STABILITY OF NUCLEOTIDE SEQUENCE OF A CHIMERIC TOBAMOVIRUS TO SERIAL PASSAGE IN TOBACCO

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LIST OF ABBREVIATIONS

A	Adenylate
BMV	Brome Mosaic Virus
C	Cytidylate
CGMMV	Cucumber Green Mottle Mosaic Virus
CP	Coat Protein
EDTA	Ethylenedinitrilo-tetraacetic acid disodium salt
G	Guanylate
kDa	kiloDalton
MOI	Multiplicity of Infection
MP	Movement Protein
MW	Molecular Weight
OAS	Origin of Assembly Site
ORF	Open Reading Frame
ORMV	Oilseed Rape Mosaic Virus
ORSV	Odontoglossum Ringspot Virus
PMMV	Pepper Mild Mottle Virus
RdRP	RNA-dependent RNA Polymerase
RMV	Ribgrass Mosaic Virus
RPA	Ribonuclease Protection Assay
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SH	Samsun High lineage
SHMV	Sunn-Hemp Mosaic Virus

SL	Samsun Low lineage
SSCP	Single-Strand Conformational Polymorphisms
T	Thymidylate
TAE	Tris-acetate EDTA
TMGMV	Tobacco Mild Green Mosaic Virus
TMV	Tobacco Mosaic Virus
TMV-OM	Tobacco Mosaic Virus strain OM
ToMV	Tomato Mosaic Virus
TVCV	Turnip Vein-Clearing Virus
TVCVrMP	Turnip Vein-Clearing Virus replaced Movement Protein

CHAPTER I

INTRODUCTION

Background

Viral genomes are in a dynamic state, with their nucleotide sequences changing as a result of accumulating mutations and recombination events. In plant viruses, such as tobamoviruses, the host plant also plays a significant role in the virus' genomic flux through positive and negative selection.

The host plant-virus relationship has been crucial in tobamovirus evolution. It is likely that the long-term survival of the tobamoviruses is dependent upon their adaptation to a particular family of plants (Lartey et al., 1996; Gibbs, 1999). Tobamovirus-host adaptation may have spanned 120-140 million years as the two may have co-evolved over this period (Gibbs, 1999). Considering that each member of the tobamovirus family has a different natural host, it is surmised that a major driving force in tobamovirus evolution is the relationship with the host plant (Fraile et al., 1995). The host plant, through the use of positive and negative selection, has kept tobamovirus genetic heterogeneity low and produced populations that are genetically very stable (Fraile et al., 1995).

However genetically stable the tobamoviruses, genetic variability occurs and variants persist. Tobamoviruses have great potential to vary through high mutation and recombination rates. Low selective constraints allow for the occurrence of heterologous recombination and the host plant is further able to select variants generated by mutations (Fraile et al., 1995). Occasionally, a genetic variant might find a niche in a host other than its original host, one that allows the association to persist. This is the case for

odontoglossum ringspot virus (ORSV), a recombinant tobamovirus, which has an anomalous host range, including orchids (Gibbs, 1999; Lartey et al., 1996).

A primary source of variability in RNA viruses is substitution mutation, as well as insertion and deletion mutations, caused by misincorporation of bases during nucleic acid replication (Fraile et al., 1995). Errors in replication result from the viral RNA-dependent RNA polymerases (RdRPs) and their intrinsic low level of fidelity, lack of proofreading activity, and lack of correction mechanisms (Steinhauer et al., 1992; Fraile et al., 1995; Lai, 1995; Ramirez et al., 1995). Limited activity of both endonucleases and 3'-->5' exonucleases associated with RdRP's has been observed by certain investigators, however. (Ishihama, 1996 and references therein). High mutation rates are a prerequisite for the survival of viruses since they constitute the basis of virus adaptation to the environment (Ramirez et al., 1995). High mutation rates produce heterogeneous viral populations termed quasi-species (Domingo et al., 1995), however, the variability generated by mutation is usually greatly decreased by selection by the host plant (Fraile et al., 1995).

The genetic errors imposed on a virus genome by the lack of proof-reading activity of the RdRPs must be corrected to ensure the long-term genetic stability of the viral RNA genome (Lai, 1995). RNA recombination could serve to correct genetic errors by generating functional RNA molecules (Lai, 1995) from among members of the quasispecies. Given the proper selection pressure provided by nature, the functional recombinant RNA molecules with the best fitness should emerge (Lai, 1995). In fact, Fernandez-Cuartero et al. (1994) showed that an RNA recombinant virus was more fit than either of the parent viruses, suggesting that RNA recombination may lead to the increased fitness of the recombinant RNA. Disruption of viral gene function due to deletions or insertions may be repaired via RNA recombination, which would account for a selective advantage for the recombinant (Lai, 1995). Thus, recombinant viruses seem to have some selective advantages under certain conditions, but it is more likely that a recombinant will be selected against

because of the potential for structural or functional incompatibility of its proteins (Lai, 1995).

RNA Virus Recombinants

While recombinants have selective disadvantages due to incompatible proteins or stable parent virus genomes, evidence confirming recombination amongst plant RNA viruses exists. Brome mosaic bromovirus (BMV) was the first plant RNA virus to demonstrate recombination (Bujarski and Kaesburg, 1986; Lai, 1995). Homologous recombination was experimentally shown to produce wild-type RNA-3 sequence from a mixture of *in vitro*-transcribed wild-type BMV RNA-1 and RNA-2 and a mutant RNA-3 (defective in the 3'-untranslated region) inoculated on barley plants (Bujarski and Kaesburg, 1986). The recombination event apparently involved recombination between the mutant RNA-3 and either RNA-1 or RNA-2 in the 3'-untranslated region common to all three RNAs (Lai, 1995). Other experiments show homologous recombination events occurring among the three RNAs of BMV's tripartite genome (Bujarski and Dzianott, 1991; Rao and Hall, 1990; Rao et al., 1990).

Among members of the tobamovirus family, at least two new virus species have been noted to result (or inferred to result) from recombination. A study by Fraile et al. (1997) involving gene sequence analysis of tobacco mosaic tobamovirus (TMV) and tobacco mild green mosaic tobamovirus (TMGMV) isolates from Australia revealed one of the isolates, H7, was a recombinant. The nucleotide sequences of two randomly chosen regions (regions I and II) in the tobamovirus genome showed that in isolate H7 region I was from TMV while region II was from TMGMV (Fraile et al, 1997). Furthermore, it was noted that H7 had a high level of fitness, exhibited by its achieving a concentration in *Nicotiana glauca* leaves greater than any other assayed isolate (Fraile et al., 1997).

A second member of the tobamovirus family has been inferred to have undergone recombination between tobamoviral genomes under natural circumstances (Lartey et al., 1996). This recombination event occurred long ago and resulted in the creation of a distinct

tobamovirus, odontoglossum ringspot virus (ORSV). Phylogenetic analysis suggests that the immediate ancestor to ORSV must have derived by recombination between a subgroup 3 tobamovirus which donated the 5' portion of the genome and a subgroup 1 tobamovirus which donated the 3' end of the genome, beginning at the movement protein (MP) gene (Lartey et al, 1996). The recombinant virus likely underwent a period in which the 5' portion of the genome and the 3' portion adapted to one another (Lartey et al, 1996). Furthermore, the recombinant tobamovirus was likely less fit than the parents due to inter-genomic and interpeptide incompatibilities until the period of adaptation was complete (Lartey et al, 1996). It has been suggested that ORSV's anomalous host range (orchids) (Gibbs, 1999) and fast rate of evolution (Lartey et al., 1996) are both effects of it being recombinant.

Moreover, homologous recombination has been observed in mutant strains of TMV (Beck and Dawson, 1990). In these mutant TMVs long repeated sequences were inserted that recombination eliminated to produce wild-type progeny (Beck and Dawson, 1990). This work suggests recombination may be an important factor in TMV evolution, especially as a repair mechanism.

RNA Recombination

Through work and study of RNA recombination in plant and animal viruses, Lai (1992) has classified RNA recombination into three types. Type I Recombination is also termed Homologous Recombination as it involves two similar or closely related RNA molecules with extensive sequence homology; crossovers occur at sites perfectly matched between the two RNAs so that the recombinant RNAs retain the exact sequence and structural organization of the parental RNA molecules (Lai, 1992). Type II or Aberrant Homologous Recombination involves two RNA molecules with similar sequences, however, crossovers occur not at homologous sites but at unrelated sites on each parental RNA molecule (Lai, 1992). As a result of type II recombination, recombinant RNAs contain sequence duplication or deletion and even insertion of nucleotides of unknown origin (Lai, 1992). This

type of recombination is unique to RNA recombination (Lai, 1992). Finally, Type III or Nonhomologous Recombination involves RNA molecules showing no sequence homology, where the crossover sites on the two RNAs may share similar secondary structure (Lai, 1992). Type III recombination may account for gene rearrangements, insertions, and deletions observed in RNA viruses (Lai, 1992).

The majority of RNA recombination events occur by a copy-choice mechanism, which involves polymerase jumping from one template to another during RNA synthesis (Lai, 1992). Several requirements are necessary for template switching to occur during RNA synthesis, including: I) RNA polymerase must pause during RNA synthesis and then dissociate from the original template; ii) another RNA template must be in close proximity for the polymerase to switch to; and iii) there must be some physical features of the new template that allow the polymerase to bind and continue RNA synthesis (Lai, 1992). The copy-choice mechanism suggests the ability of a virus to recombine probably is inversely proportional to the processivity of its own RNA polymerase (Lai, 1995). To date, no studies determining the processivity of tobamovirus RdRP have been reported.

Tobamovirus Family

The tobamovirus family of plant viruses contains at least twelve members sharing the following common features: virions are very stable, rigid rods measuring 300 X 18 nm; each virion has approximately 2000 protein subunits of a single molecule (i.e., the coat protein, MW = 17 kDa), helically arranged together with a monopartite, single-stranded, positive-sense genomic RNA (MW = 6.5 kb) (Fraile et al., 1995).

Tobacco mosaic virus (TMV) is the type member of the tobamovirus family and it has been studied more extensively than any member of the family. For this reason, the genome structure and proteins of TMV will represent the tobamovirus family. The 5' end of TMV RNA is capped and has an untranslated leader sequence of 69 nucleotides, which initiates an open reading frame (ORF) that encodes a 126 kDa protein (126 K) (Mathews, p. 208). The termination codon for the 126 K protein (UAG) is "leaky" and allows the

read-through for a second protein of 183 kDa (183 K) (Mathews, p. 208). Experimental evidence indicates both the 126 K and 183 K proteins are required for maximum efficiency of TMV replication (Buck, 1999). In addition, the read-through portion of the 183 K protein contains amino-acid motifs characteristic of RNA-dependent RNA polymerases (RdRPs) and hence the 183 K protein is likely to provide the catalytic activity for the synthesis of TMV RNA (Buck, 1999). Downstream from and adjacent to the 183 K protein is a third ORF encoding a protein of 30 kDa (30 K) (Mathews, p. 208). The 30 K protein has been shown experimentally to be required for cell-to-cell movement of TMV, thus it is termed the movement protein (MP) (Mathews, p. 211). Arce-Johnson et al. (1997) showed the MP to be involved in long distance movement as well. A fourth ORF, located closest to the 3' end, encodes a 17 kDa protein termed the coat protein (CP), which encapsidates the genomic RNA (Mathews, pp. 208, 210) and is necessary for long-distance movement of the virions (Saito et al., 1990). Members of the bromovirus and cucumovirus families require the CP for cell-to-cell and long-distance movement (Rao and Grantham, 1996; Kaplan et al., 1998). A subgenomic RNA corresponding to an ORF for a putative 54 kDa protein (54 K) in the read-through portion of the 183 K protein has been detected in RNA isolated from TMV-infected tobacco plants (Buck, 1999). The putative 54 K protein does not appear to be essential for TMV replication, but could possibly have a regulatory role (Buck, 1999). Finally, to the 3' side of the CP ORF there is an untranslated region (3' utr) 179-414 nucleotides long that can fold in the terminal region to adopt a tRNA-like structure and be amino-acylated with histidine (Mathews, p. 208; Fraile et al., 1995).

The 126 K protein contains two domains: an N-terminal and a C-terminal domain (Buck, 1999). The N-terminal domain is likely required for synthesis of the 5' genomic RNA cap structure (Buck, 1999). The C-terminal domain is helicase-like, although helicase activity has not yet been demonstrated for the 126 K protein of any tobamovirus (Buck, 1999). Helicase activity is likely necessary for TMV RNA replication for two reasons: to

unwind the duplex RNA formed during RNA synthesis and to remove second-ary structure in single-stranded RNA templates to allow replication to occur (Buck, 1999).

Tobamoviruses have historically been classified into two subgroups according to the locations of their origin of virion assembly (OAS) (Fukuda et al., 1981; Okada, 1986; Gibbs, 1986). Subgroup 1 tobamoviruses have their OAS in the MP ORF, while the OAS in subgroup 2 viruses is located within the 3' terminal ORF of the CP (Fukuda et al., 1980, 1981). Subgroup 1 viruses, which mainly infect solanaceous plants, include TMV strains vulgare and Ob, tomato mosaic virus (ToMV), pepper mild mottle virus (PMMV), ORSV, and TMGMV (Lartey et al., 1996). Subgroup 2 members include sunn-hemp mosaic virus (SHMV) and cucumber green mottle mosaic virus (CGMMV), which infect legumes and cucurbits, respectively (Lartey et al., 1996). Lartey et al. (1996) introduced a second criterion for tobamovirus classification that resulted in a third subgroup within this family. The criterion was based on the length of nucleotide overlap between the MP and CP ORFs. Subgroup 1 has a small overlap of 2 or 5 nucleotides, subgroup 2 an intermediate overlap of seven and two-thirds codons or eight and two-thirds codons, while the MP and CP ORFs in subgroup 3 overlap by 77 nucleotides (Lartey et al., 1996). The OAS of subgroup 3 members is located in the MP ORF; members include turnip vein-clearing virus (TVCV), oilseed rape mosaic virus (ORMV), and ribgrass mosaic virus (RMV), which infect cruciferous plants (Lartey et al., 1996). In subgroup 1, sequences of distinct viruses are 70-80 % identical to one another, while between subgroup 1 and 3 virus sequences are only 50-60 % identical (Zhang et al., 1999). Moreover, Lartey et al. (1996) proposed that subgroup 3 tobamoviruses evolved from a subgroup 1-like ancestor that developed the ability to infect cruciferous plants.

The members of the tobamovirus family of interest in this study, the aspects of which are discussed below, are TMV, TVCV, and the chimera TVCVrMP (Zhang et al., 1999). TMV, as mentioned above, is the type member of the tobamoviruses. As its name implies, TMV causes a mosaic pattern in infected leaves, which presents as areas of light

and dark pigmentation on a single leaf. TMV acts further as the type member of the family in its mechanism of transmission; by contact between plants, from contaminated soil or, for some virus-host combinations, through the seed, and no specific tobamovirus vectors are known (Fraile et al., 1995).

Turnip vein-clearing virus (TVCV) causes vein clearing in *Brassica rapa* cv. Just Right (turnip), a mosaic, similar to that caused by TMV, in *Nicotiana tabacum* cv. Samsun (tobacco) and severe mosaic in *N. clevelandii* (Lartey et al., 1993). TVCV contains a 6.4 kb single-stranded genomic RNA, which is structurally similar to that of TMV (Lartey et al., 1994).

TVCVrMP is a chimeric virus produced by Zhang et al. (1999), originally used to assess the ability of tobamoviruses to infect different plant families. TVCVrMP was engineered such that the MP gene of TVCV was replaced by the TMV MP gene; yet the C-terminal portion of the TVCV MP gene containing an internal ribosome entry site, the TVCV OAS and the CP subgenomic promoter were retained (Zhang et al., 1999). This chimeric virus was chosen for this study for two reasons: it was known to be infectious to *N. tabacum* cvs. Samsun and Xanthi (Zhang et al., 1999) and because it provides a good model to assess the viability and genetic adaptability of a recombinant/chimeric virus that develops in nature.

Serial Passaging

The chimera TVCVrMP was propagated in plants by a method known as serial passaging. Serial passaging involved the use of infected plant tissue as inoculum to establish infection in a second plant. In this study, ten passages were completed in each of two plant lineages. Serial passaging of TVCVrMP through the two plant lineages imposed selective pressures on the chimera. The two lineages were termed High Multiplicity of Infection (MOI) and Low MOI, where each was designed to select for or sample variants through different means. The High MOI lineage selects for a large heterogeneous population of variants. Heterogeneous populations of variants, termed quasispecies, arise in any

error-prone replication system (Novella et al., 1996), such as that of RNA viruses. Furthermore, it has been observed that repeated transmissions of large RNA virus populations from host to host in a constant environment (i.e., the High MOI lineage) leads to significant increases in mean population fitness, where fitness is defined as the overall ability of a virus to replicate progeny (Novella et al., 1995, 1996). Thus, a highly fit, heterogeneous virus population, presumably having accumulated mutations through genetic adaptation, was selected for in the High MOI lineage and the genomes of these fit variants were analyzed for the presence of mutations.

The Low MOI lineage, on the other hand, samples the population of variants differently than does the High MOI lineage. The Low MOI lineage used genetic bottlenecks to reduce the variant population to one or a few virions. Sampling of a small population of variants will usually reduce population fitness (Novella et al., 1996). Moreover, during a genetic bottleneck selection does not occur, in which case a high mutation rate can be detrimental to the fitness of RNA viruses (Duarte et al., 1994). Genetic bottlenecking and the Low MOI lineage reduced the number and the fitness of variants available to produce infection in a local-lesion host. Propagation of one or few variants in a systemic-infection host will re-establish the high fitness of the population originating from the bottlenecked variants. By propagating a large population of variants, then bottlenecking the population by sampling one or a few variants, and finally propagating the bottlenecked variants to produce a second large population, a lesser fit genome will ultimately be analyzed. It was hypothesized that the use of serial passaging in both the High MOI and Low MOI lineages would produce variants having differing genetic compositions. That is, it was proposed that by analyzing the nucleotide sequence of highly fit and lesser fit variants different patterns of mutation accumulation might be observed.

Purpose

This study utilized the chimeric tobamovirus TVCVrMP as a model to assess the extent of genetic adaptation required by a naturally occurring recombinant virus for viability

in a host plant. It was hypothesized that the chimera would accumulate mutations in order to adapt to selection pressures imposed by the host plant. Selective pressure was imposed on the chimera by serially passaging the virions in plants through two lineages: a High MOI lineage and a Low MOI lineage. Furthermore, since the MP gene of TVCVrMP is a foreign gene replacing its TVCV counterpart, it was hypothesized mutations would accumulate in order to adapt the foreign MP gene to the TVCV genome. Nucleotide sequencing was employed to determine whether mutations accumulated in the MP ORF of TVCVrMP.

CHAPTER II

MATERIALS AND METHODS

Plants and virus

This study used two separate protocols for serial passaging of the chimeric virus, TVCVrMP (Zhang et al., 1999). The first protocol exclusively utilized *N. tabacum* cv. Samsun nn (referred to simply as Samsun), which upon infection produces a systemic infection thereby yielding a large population of virus throughout the plant. The lineage arising from this first passaging protocol is termed the High Multiplicity of Infection (MOI) lineage because a large, diverse population of virus results from the infection of the plant.

The second passaging protocol of the study utilized both a systemic infection host and a localized infection host. The localized infection host was used as an intermediate for the selection of a single virion (or small number of virions) from a large, diverse population of virions; known as bottlenecking. The localized infection host was *N.tabacum* cv. Xanthi nc (or simply Xanthi) and the systemic host was again Samsun. In general, the passaging procedure of this protocol of the study proceeded as follows: a Xanthi leaf was inoculated with virus, a localized lesion developed, the lesion was harvested from the leaf and used to inoculate a Samsun leaf, which resulted in a systemic infection. Following systemic infection in Samsun, a leaf plug was harvested and used to inoculate a second Xanthi plant and the process was repeated. By using Xanthi as an infection-intermediate the virus population was bottlenecked since the large, diverse population produced during the systemic infection was in essence reduced to one or a few virions, which produced the

localized lesion on Xanthi. The lineage derived from bottlenecking the virus population was termed the Low MOI line of serial passages.

Samsun and Xanthi plants were maintained according to Lartey et al. (1993). TVCVrMP virions were directly inoculated on Samsun or Xanthi leaves having a length of 15 cm. Leaves of such length were chosen to be inoculated because they are of a good age and size, since small young leaves and old leaves are less susceptible to infection than well expanded younger leaves (Mathews, p. 451; Wang, Zhang, and Melcher unpublished observations). A volume of 20 μL of virions (isolated from turnip by a purification method developed by Sherwood and Fulton (1982) and modified by Lartey; 0.88 mg protein/mL) was rubbed gently across a Xanthi or Samsun leaf sprinkled with carborundum. Localized lesions on Xanthi developed within 3-5 days post-inoculation (dpi). A leaf plug (5 mm diameter) containing the lesion was excised from the Xanthi leaf and a homogenate was made by grinding the plug in 0.5 mL autoclaved deionized water using a small Eppendorf tube-sized pestle (Fisher Scientific, catalog number K749520-0000). Of that homogenate, 40 μL was inoculated on each of three leaves of Samsun.

At the time of the next successive inoculation (approximately 3-4 weeks post-inoculation) in either of the lines, an infected Samsun leaf sample was harvested. This harvested leaf was an upper, non-inoculated leaf of approximately 10-15 cm in length. A leaf of such size provided ample tissue for virion isolation. Following each harvest the leaves were brought from the growth chamber to the laboratory where they were weighed and stored at -20° C until needed. None of the Xanthi plants used as infection intermediates in the Low MOI passages contributed leaf samples nor were virions isolated from Xanthi leaves.

Virion Isolation

Virion isolation was performed by an adaptation of a previously established protocol designed to purify tobamoviruses from systemically infected leaf tissue (Sherwood and Fulton, 1982; modified by Lartey). The protocol was originally designed to isolate virions

from several grams of leaf tissue. In this study such large-scale isolation was not necessary since PCR was employed. The protocol was scaled down and the aver-age leaf tissue weight used was approximately 0.5 g.

The isolation of TVCVrMP virions from infected Samsun leaf tissue was accomplished by grinding the tissue in a sterile mortar in a volume of 3 mM EDTA equal in mL to twice the weight in grams of the tissue. To aid in cell disruption, an amount of alumina equal to 1/10 the leaf weight was added to the mortar. The homogenate was placed in a 1.5 mL eppendorf tube and incubated at 55° C for 10 minutes. The homogenate was then transferred to an 8.9 mL Optiseal polyallomer centrifuge tube (Beckman) and centrifuged at 10,000 rpm for 7 min at 4° C in a Beckman Type 90 Ti rotor. Following this initial centrifugation the supernatant was transferred to a new 8.9 mL Optiseal centrifuge tube, the remaining volume in the centrifuge tube was made up with 3 mM EDTA buffer, and then centrifuged at 80,000 rpm for 11.5 min at 4° C in the 90 Ti rotor. The supernatant was discarded and the pellet was suspended in 3 mM EDTA. The above centrifugation steps were repeated and the pelleted virions were suspended in 0.5 mL 3 mM EDTA.

Sequence Analysis

The objective of the study was to analyze the genomic sequence of the MP gene of TVCVrMP for changes relative to the parent virus. Sequencing is done most readily on double stranded DNA, but since TVCVrMP is a single-stranded RNA virus it was first necessary to perform reverse transcription-polymerase chain reaction (RT-PCR) to convert the virions to DNA and amplify to provide ample DNA for sequencing. The Pro-Star HF Single-Tube RT-PCR System (Stratagene, catalog number 600164) was employed for this purpose. Along with the reagents provided in the Stratagene RT-PCR kit, specific oligonucleotide primers are required in the reaction mixture. The two primers used in the RT-PCR of the sample virions are primer #1393 [5'GCCGCTCGAGGTTAA GCATTGGTAT3'; anneals to position 5666-5681 in TVCV, GenBank Accession #U03387 L22518 (Lartey et al., 1995)] and primer #362 [5'CGCCGCTCGAGATGGCTC

TAGTTGTTA AAG3'; anneals to position 4901-4921 in TMV, GenBank Accession #V01408 J02415 (Goelet et al., 1982)]. Primer #1393 anneals to a region within the TVCV coat protein ORF, being 3' of the TMV MP, whereas #362 anneals to the virion-sense complementary strand at the 5' end of the TMV MP ORF. The Stratagene RT-PCR Protocol was followed with only minor variation. That is, 2.0 µL template RNA (isolated virions) was added to the reaction mixture, which decreased the amount of RNase-free water added by 1.0 µL. The thermal cycler protocol was programmed according to the Stratgene protocol, but is listed in Table I. The RT-PCR products were visualized by agarose gel electrophoresis and ethidium bromide (EtBr) staining. The gels were 1% agarose in 1X TAE (40 mM Tris-acetate, 1 mM EDTA) running buffer, run at a constant voltage of 100 V for 1.5 hours in a model HE33 (Hoefer Scientific Instruments) gel electrophoresis apparatus.

After verifying the presence of RT-PCR product by agarose gel electrophoresis, the concentration was measured using a DNA fluorometer (Hoefer Scientific Instruments, model TKO 100). Those samples having a concentration of 10 µg/mL or greater (which 17 of 20 samples did) were filtered through PCR Select II Columns according to the manufacturer's directions (5 prime-->3 prime, Boulder, CO) as a clean-up procedure, then submitted, along with primers #362 and #363 [5'CACGCGTCGACTCCGATTCGGCGA CAGTAGCCT3'; anneals to position 5678-5699 in TMV, GenBank Accession #V01408 J02415 (Goelet et al., 1982)], to the Recombinant DNA/Protein Resource Facility (Oklahoma State University, Dept. of Biochemistry and Molecular Biology) for dye-terminator thermocycle sequencing by a Perkin Elmer-Applied Biosystems (Foster City, CA) 373 DNA Sequencer.

Along with each of the samples sequenced, two standards were sequenced in both directions as well. These standards are the plasmid form of the chimera, pTVCVrMP, and the parent virion used to start each line of serial passage, called TVCVrMP. TVCVrMP was chosen as a standard because it contains the sequence from which all other progeny virions

descended. TVCVrMP was isolated from turnip, so this virus has previously been through one round of infection. The plasmid, pTVCVrMP, was chosen as a standard because this is the "master sequence"--unaltered since being cloned. In addition, one other standard was used for sequence comparisons in the 5'-->3' direction. This standard is called TVCVrMP-0 and is the sequence of the TMV MP replacing the TVCV counterpart in the chimera. The sequence of TVCVrMP-0 was assembled according to the known sequences of TMV-OM (Goelet et al., 1982) and TVCV (Lartey et al., 1995).

TABLE I

PROTOCOL FOR RT-PCR REACTION
IN THERMAL CYCLER

Cycles	Temperature (°C)	Duration
1	37	15 min
1	95	1 min
40	95	30 sec
	60	30 sec
	68	2 min
1	68	10 min

CHAPTER III

RESULTS

Plants

Upon infection an inoculated Xanthi leaf produced small (2-4 mm diameter) circular necrotic lesions. A Samsun leaf, producing a systemic rather than localized infection, did not produce such necrotic lesions upon infection; however, symptoms were present which were indicative of infection. Healthy Samsun leaves appear robust green, while symptomatic Samsun leaves most commonly display mosaic coloration. Mosaic appears as areas of both dark green and light- or yellow-green pigmentation on the same leaf. Mosaic leaves may also have a rugose surface topography, which differs from the smooth surface of healthy leaves. In this study neither the size nor shape of the infected leaves was observed to differ from its healthy counterpart.

Virion Isolation

Once the Samsun plants (from each lineage of passaging) had been inoculated for 3-4 weeks and appeared symptomatic, infected leaf samples were harvested and virions were isolated.

Sample Nomenclature

In the High MOI lineage of passaging, ten Samsun plants were inoculated in succession, where each plant was inoculated with homogenate from the infected plant that preceded it (except, of course, the first inoculated Samsun). The naming of the samples was based on from which passage lineage the sample originated and the plant number (i.e., from number 1 to 10). For example, virion sample SH1 indicated this was the first sample recovered from Samsun in the High MOI lineage. Homogenate SH1 was used to inoculate a Samsun, which upon infection harbored SH2 virions. A similar system of nomenclature

was used to name the virion samples produced in the Low MOI lineage, where SL is the prefix.

Amplification and Detection

Fortunately for the sake of simplicity and time, the RT-PCR kit and accompanying protocol provided optimal results without the need for optimization or modification. That is, of the 20 virion samples [10 samples from both lineages (SL1 through SL10 and SH1 through SH10)] 17 were amplified by RT-PCR (85%). Although amplification by RT-PCR occurred for samples SL1, SH1, and SL2, further amplification by an additional PCR step was performed to ensure ample product for sequencing since a minimum concentration is required.

However, in the early stages of the project several RT-PCR attempts failed (producing either no product or artifactual smearing) due to the addition of too much TaqPlus Precision DNA polymerase (Stratagene) because of pipetting error. By dialing the pipettor to the desired volume (0.5 μL) excess DNA polymerase solution was drawn up due to the nature of glycerol. Decreasing the amount of DNA polymerase produced positive results in all subsequent RT-PCR amplifications.

RT-PCR-amplified MP ORF fragments were detected by agarose gel electrophoresis. DNA bands corresponding to the correct sized fragment of ca. 1100 bp were observed (Figure 1). Those virion samples sequenced showed no other unexpected bands or smearing of bands on the gels. The presence of only the correct sized band indicated no large insertions or deletions and no RNase digestion occurred prior to the RT-PCR reaction.

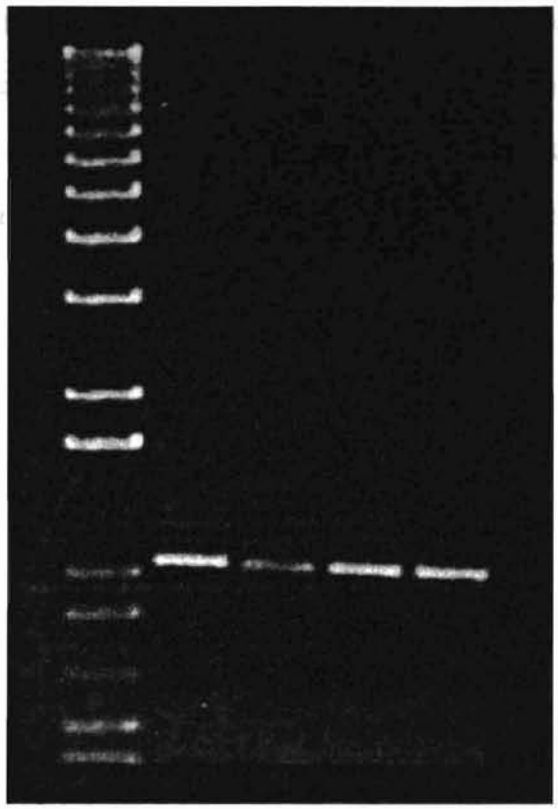

FIGURE 1. Representative Gel Displaying RT-PCR Amplification Products From TVCVrMP Samples.

From left to right, lanes 1 through 5 contain the following: 1) Molecular Weight Marker: 1 Kb Plus DNA Ladder (GibcoBRL); 2) Sample SL3; 3) Sample SL4; 4) Sample SH3; 5) Sample SH4. Each sample electrophoresed to a position corresponding to ca. 1100 bp, which was the expected length of the amplified fragment (i.e., the MP ORF).

Sequence Analysis

This study determined by nucleotide sequencing whether mutations occurred in the TVCVrMP MP ORF during passaging. Since mutations can be as subtle as a single nucleotide change (e.g., point mutation, insertion, or deletion), sequence analyses provide the highest possible resolution. The software program AssemblyLign (Oxford Molecular Group PLC) was utilized for aligning related sequences and controls in the form of contigs. Sequence alignments made it possible to directly compare complementary sequences from a single sample and were very effective in determining whether mutations occurred.

Serially passaged samples which were successfully amplified by RT-PCR were submitted for Automated DNA Sequencing. Those samples submitted for sequencing came from both High and Low MOI lineages and are listed in Table II.

TABLE II

A LIST OF ALL THE SERIAL PASSAGE SAMPLES FROM BOTH HIGH MOI AND LOW MOI LINEAGES THAT WERE SUCCESSFULLY AMPLIFIED AND SEQUENCED

	Lineage	
	High	Low
Passage	SH1	SL1
	SH3	SL2
	SH4	SL3
	SH5	SL4
	SH6	SL5
	SH8	SL6
	SH9	SL7
	SH10	SL9
		SL10

Thus, 17 of 20 samples were amplified by RT-PCR and sequenced. Two controls were sequenced as well; these were the plasmid form of the chimera, termed pTVCVrMP, and the original virions used to start the infections in both lineages, termed TVCVrMP. All samples, including the two controls, were sequenced in both the 5'-->3' (using primer #362) and 3'-->5' (using primer #363) directions. Having two complementary sequences available for each sample provided the means to confidently determine the correct nucleotide at a position when the identity was ambiguous.

The primer set used for sequencing (#362 and #363) differs from the primer set used in the RT-PCR reactions, in that primers #362 and #1393 were used for RT-PCR. Sequence reactions containing primer #1393 produced no "readable" sequences. In 10 of the samples sequenced 5'-->3' by primer #362, a 32 bp region corresponding to bases 5081-5113 was observed to be particularly ambiguous and appeared as though two (or more) sequences were present. In addition to many double peaks and ambiguous base calling in this region, the amplitude of the peaks on the electropherogram noticeably decreased relative to the region directly upstream. Furthermore, the region upstream from the 32 bp region contained many double peaks, but base calling was not ambiguous, whereas very few double peaks were present downstream of this region and base calling also was not ambiguous. The analogous 32 bp region sequenced 3'-->5' was not ambiguous and no double peaks were present. A comparison of the 5'-->3' and 3'-->5' sequences of this 32 bp region revealed that the virion sequence was homogeneous, and also confirmed that two (or more) sequences were present in this region when sequenced 5'-->3'. Since at least two sequences were present in the 32 bp region, of which one sequence was known to be correct, a "subtractive" sequence was determined by assigning nucleotides to the corresponding peaks. Since one means of obtaining two overlapping sequences on an electropherogram is through a mispriming event, attention was turned to the primers used for both RT-PCR and sequencing. The subtractive sequence determined from the 32 bp region and the sequence of primer #1393 were compared and the subtractive

sequence contained the #1393 sequence. Thus, during the RT-PCR reactions of these 10 samples primer #1393 annealed to a false priming site on the virion template RNA. The sequence data of the 10 samples determined to have been false primed by primer #1393 were analyzed no further. The false priming by primer #1393 affected only sequence data determined in the 5'-->3' direction (i.e., sequences primed by #362) and the 3'-->5' sequences (i.e., sequences primed by #363) were not affected. That is, base calling in the sequences primed by #363 were not affected because only those RT-PCR products not false primed were available for sequencing by #363. By disregarding the 10 false primed sequences, seven samples from both passage lineages remained for sequence analysis (Table III).

TABLE III

A LIST OF PASSAGED SAMPLES WHOSE SEQUENCES WERE ANALYZED FOR THE PRESENCE OF MUTATIONS

Lineage	Sample
High	SH3
	SH4
	SH6
	SH8
Low	SL3
	SL5
	SL6

The distance between primers #362 and #363 is approximately 800 nts; none of the samples in this study was completely sequenced. Typically, 500-650 nts of accurate, readable sequence was obtained for each of the samples (Table IV).

TABLE IV

A LIST OF THE PASSAGED SAMPLES FOR BOTH LINEAGES THAT WERE SEQUENCED, THE DIRECTION IN WHICH EACH WAS SEQUENCED, AND THE NUMBER OF UNAMBIGUOUS NUCLEOTIDES ANALYZED

Lineage	Sample	Sense (+/-)*	#nts Sequenced
High	SH3	+	485
		-	605
	SH4	+	516
		-	638
	SH6	+	591
		-	646
	SH8	+	476
		-	646
Low	SL3	+	636
		-	567
	SL5	+	379
		-	606
	SL6	+	629
		-	639

*Note: Sense (+/-) refers to the direction in which the sample was sequenced relative to virion RNA, where + is 5'-->3' and - is 3'-->5'.

Many more nucleotides appeared on the automated sequencing results (called an electropherogram) than could be accurately determined by analysis. These extraneous nucleotides appeared at both the far 5' and 3' ends and were deleted from the sequence text files using the editing capabilities of AssemblyLign, which allows the user to correct insertions, deletions, and incorrect nucleotide substitutions. Deleting the extraneous nucleotides from the sequences allowed the analysis to be concentrated on the portion of the sequence considered to be accurate.

Determination of the correct, complete nucleotide sequence of the TVCVrMP MP ORF for each of the seven samples required the two complementary sequences. Occasionally the sequence and the corresponding electropherogram did not agree. For example, a peak appeared in the electropherogram while the corresponding nucleotide assigned to that peak was not present in the sequence. Comparison of the complementary sequences for a sample eliminated such ambiguities in nearly all occasions when a manual sequence change (i.e., editing) was performed. In only two samples was editing required in which neither of the two complementary sequences contained the nucleotide corresponding to the peak in the electropherogram. In sample SH8, 15 of 16 manual sequence changes were made following confirmation by one of the two complementary sequences; the one sequence change which could not be confirmed by either electropherogram was the addition of an A to the sequences at position 5053. In sample SH4 24 of 25 manual sequence changes were confirmed; in this sample the one sequence change not confirmed by either electropherogram was a T at position 5034. For the remaining five samples a combined 81 manual sequence changes were made where each change made was confirmed through comparison of the two complementary sequences.

Once the sample sequences were analyzed and the editing was completed, each of the sequences and the two controls, pTVCVrMP and TVCVrMP-0 (i.e., the computer-assembled sequence of TVCVrMP) were aligned as contigs using AssemblyLign. The AssemblyLign program simplified the analysis of the alignments by making each sequence

the same sense (i.e., each sample is aligned in the 3'-->5' direction). Thus, even sequences which were originally complementary to one another were converted to the same sense in the alignment.

The result of the alignments of the seven samples (i.e., the number of passaged samples that were not false primed during either RT-PCR or automated sequencing) showed that no mutations or other sequence changes were introduced into the MP ORF of TVCVrMP. In each alignment of the seven samples one nucleotide near the 3' end of the MP ORF at position 5566 was flagged by AssemblyLign as differing (i.e., an A-->G change, resulting in an Asn-->Ser coding change) from the TVCVrMP-0 sequence. In fact, this same nucleotide was flagged in the pTVCVrMP sequence as well. As stated above, the TVCVrMP-0 sequence was computer assembled according to the known sequences of TMV and TVCV. Thus, TVCVrMP-0 was considered the "master" sequence to which all other sample and control sequences were compared. Furthermore, pTVCVrMP was produced by both assembling a series of cDNA clones and utilizing TMV MP sequence information obtained from GenBank (Zhang et al., 1999). Since pTVCVrMP was produced in the laboratory according to the "master" sequence, TVCVrMP-0, the A-->G change in pTVCVrMP was erroneously introduced by being present in the GenBank TMV MP sequence. This erroneous nucleotide substitution was further propagated by the infectious virions used in this study. Therefore, the only nucleotide flagged in the alignments was not the result of a natural mutation but one introduced in the production of pTVCVrMP.

The absence of mutations in the sample sequences relative to the controls indicated that the predominant sequences isolated following passaging were the same as the sequence in the original inoculum (i.e., TVCVrMP virions). To determine whether any additional sequences were present in a level detectable by nucleotide sequencing, sample electropherograms were analyzed by eye for the presence of nucleotides occupying the same position (i.e., double peaks at a location on the electropherogram). Double peaks in the samples were compared with the analogous location on the control pTVCVrMP electro-

pherogram. This comparative analysis did not identify any positions which possibly contained two sequences.

CHAPTER IV

DISCUSSION

This study hypothesized the genome of the chimeric virus, TVCVrMP, would undergo genetic adaptation of the TMV MP ORF to the TVCV genome into which it was inserted. It was further hypothesized that genetic adaptability might occur through point mutations (i.e., insertions, deletions, or substitutions) causing amino acid coding changes which might reduce or eliminate peptide incompatibilities resulting from TVCVrMP being recombinant. In addition, it was hypothesized that any mutation introduced into the genome would also be an attempt by the chimeric virus to adapt to the host plant. Thorough analysis of the samples' sequences revealed that no mutations occurred in the 650 bp region of the TVCVrMP MP ORF. The TVCVrMP MP ORF is actually 807 bp, but as stated above, many of the nucleotides at both the 5' and 3' ends were unreadable and were deleted. Editing each of the sequences left an approximately 650 bp region of se-quence to be analyzed.

Following virion isolation from infected plant tissue, each sample was amplified by RT-PCR. The RT-PCR fragments were visualized by agarose gel electrophoresis, which demonstrated only the correct sized bands of ca. 1100 bp. The presence of only the correct sized band indicated no large insertions or deletions occurred at any step. Deletions were possible due to the duplicated OASs in TVCVrMP, in which one OAS might have been eliminated as a result of viral genome size constraints imposed by the virus.

The samples sequenced from the High MOI lineage spanned a large portion of the samples in the lineage. That is, of the ten total samples in the High MOI lineage, four samples representing the beginning (SH3), the middle (SH4 and SH6), and the end (SH8)

of the lineage were sequenced and analyzed. Since no mutations occurred in any of these four samples it was surmised that the probability of mutations having occurred in the TVCVrMP MP ORF of the other samples from the High MOI lineage (especially samples SH9 and SH10) was low. Such an assumption was more difficult to make for the samples from the Low MOI lineage. Three samples from the Low MOI lineage representing the beginning (SL3) and middle (SL5 and SL6) were sequenced and analyzed. The possibility exists that mutations may have been introduced in the last four samples of the lineage (i.e., SL7-SL10).

Each of the sample sequences was determined to contain only a single sequence through analysis comparing the samples to the controls. There are several proposed reasons no mutations were observed in these sequenced samples. First, the chimera is a recombinant between members of the same virus family (i.e., the tobamovirus family). The relationship between the MP gene (and gene product) of TVCV and TMV may be so close that the exchange of one MP ORF for the other (as occurs in this chimera) had little effect on the viability of the recombinant virus. In other words, the replacement of the foreign but related TMV MP ORF in the TVCV genome did not cause the need for adaptation because the replaced gene and its product were structurally and functionally compatible with TVCV.

A second reason no mutations were observed in the TVCVrMP MP ORF may be because the recombination involved the replacement of the complete MP ORF. Assuming the copy-choice mechanism of RNA recombination to be correct, recombination events would most likely not occur precisely at ORF junctions. Perhaps mutations would accumulate in a chimeric virus in which the recombination event occurred in a "random" region of the genome. Such a random recombination event would likely produce structural and functional incompatibilities requiring adaptation. Chimeras created by Zhang et al. (1999) which had intergenic junctions were not infectious, presumably because their chimeric protein products were unable to function.

A less theoretical possibility for the lack of observed mutations in the sequenced samples involves the region of TVCVrMP sequenced. That is, the sequencing efforts and analysis was focused on the MP ORF. Perhaps no mutations were observed in this region of the genome because no adaptation was necessary for the proper functioning of the MP gene product. Analysis of the regions upstream and downstream of the replacement MP ORF might reveal mutations introduced for adaptation purposes.

A fourth reason for the absence of mutations in the sequenced region of TVCVrMP involves selection pressures imposed by the host plant. As noted by Fraile et al. (1995), the host plant, through the use of positive and negative selection, has kept tobamovirus genetic heterogeneity low and produced populations that are genetically very stable. Kearney et al. (1993) demonstrated the stability of the TMV genome by determining that mutations accumulated at a low rate, implying that sequence drift has less effect on TMV evolution than was supposed.

Yet another possible reason no mutations were observed is because the length of the time course of the experiment was not long enough for mutations to accumulate. Based on previous passaging studies involving mutant generation (Kurath and Palukaitis, 1990; Palukaitis and Roossinck, 1996), it was proposed that ten serial passages each in two lineages should establish whether the chimeric virus genome was genetically stable or required adaptation. A representative sample from each lineage was sequenced and analyzed, which revealed no mutations had occurred, suggesting the chimera's genome was stable. Further passaging of the chimera might either further confirm or refute the above results.

Finally, although nucleotide sequencing is an efficient and effective method for determining whether mutations have occurred in a genome, it is not the only method designed for such analysis. Methods such as single-strand conformational polymorphisms (SSCP) analysis, heteroduplex analysis, and ribonuclease protection assay (RPA) could be used in addition to nucleotide sequencing to provide a comprehensive analysis of the TVCVrMP genome.

CHAPTER V

CONCLUSIONS

The focus of this study was to determine whether mutations occurred in the MP ORF of the chimeric virus TVCVrMP following serial passaging in two separate lineages in tobacco. Nucleotide sequencing was used as the method of analysis to determine the presence or absence of mutations. The study was performed as a model to assess what kind of genetic adaptability might occur in a naturally occurring recombinant virus.

The 650 bp region of the TVCVrMP MP ORF that was sequenced contained no sequence mutations. The lack of mutations might be due to several reasons, including: i) the parent viruses are both members of the tobamovirus family, ii) the chimeric virus was recombined precisely at the MP ORF, iii) sequence analysis was performed only within the recombined MP ORF, iv) selection pressure from the host plant may have eliminated variants, v) the time course of the experiment was not long enough for variants to accumulate, and vi) methods in addition to nucleotide sequencing might be performed to obtain a comprehensive analysis of the chimera's genome.

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