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Molecular Analysis of Replication and Packaging Mechanism of Satellite Tobacco Mosaic Virus

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Genetics, Genomics and Bioinformatics

by

Venkatesh Sivanandam

March 2014

Dissertation Committee:
Dr. A.L.N. Rao, Chairperson
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Dr. Shou Wei Ding

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The Dissertation of Venkatesh Sivanandam is approved:

Committee Chairperson

University of California, Riverside

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Life is not about being successful or being famous, according to me life is about getting inspiration from others and inspiring other people especially our brothers, sisters and students of younger generations. I learned that during my years of doctorate study in the United States at the University of California, Riverside.

I came to the US with a common ‘Indian attitude’ of getting to be well known among my friends and family in India as I have stepped my foot in the US and did my PhD in one of the best schools in the US. Recently my parents, especially my mom started looking for a wealthy bride to get me married. But my six years of study in a place inebriated by inspiring people (UCR) made me think and act differently. I would like to mention few of the many people who inspired me to be a happy human.

I would like to thank my co-professor Dr. Deborah M. Mathews (Dr.Deb) without whom my PhD would have been the abbreviation for ‘permanent head damage’. She is an easy going person but very astute when it comes to work. I always loved to work in her lab. She taught me everything, those tricks when handling equipment, centrifuges etc. I was humbled by her way of looking at life which is about helping people not caring for the credits. She even gave me tips in

handling the girl whom I met at UCR. She was always supporting my ideas and views on doing my research on STMV. I had more failures than success with my research, additionally I get tired after a long hours of teaching as a teaching assistant. Whenever I am not happy or frustrated with my research I go to her lab and talk with her and her lab mates Greg, Neeraj and Sohrab. They encouraged me a lot. I really wish that I will be happy and caring like Dr.Sohrab and Dr.Deb.

I am the eldest son in my family but I sometimes wish I had an elder brother and I found Dr. Soon Ho Choi (Dr. ALN Rao's lab). He was a brother to not only me but also to my other labmates Dr.Devinka and Sonali. Dr. Soon accompanied me when I was working late night in the lab we talked a lot about viruses, movies, philosophy and electronic gadgets.

In addition to Dr. Soon, I received humbleness from my former lab mate Dr. Sun Jung Kwon. She is a great researcher. She taught me the tricks in cloning/subcloning especially involving blunt-end ligation. She was the only person in the lab who would ask about the progress of my project everyday. She was like a sister to me. I was a lazy person coming late to lab and chatting with my friends on facebook, but her hardwork and dedication to science changed me. Nowadays I don't come to lab too late. I miss her and her family a lot.

Being a teaching assistant especially for introductory biology (Bio 5A) discussion was considered a difficult task until I had Dr. Richard Cardullo and Dr. Anandashankar Ray as my Instructors. To be honest, I learned a lot from their lectures even though I am their teaching assistant. I was not good at giving a talk at seminars, I learned a lot from Dr. Anand Ray's lectures. I am also happy that I rotated in his lab. I don't know how to thank Dr. Richard Cardullo. He inspired me to be a better TA and he was the only person who saw my passion to teach. I will never forget his encouragement. Thank you sir for everything. It was an honor to teach by having you as my instructor.

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I would like to thank my beloved mom, dad and my brother. I love them a lot. Finally I would like to thank the love of my life Ms. Youngmi Park. It is difficult for me to thank her simply, I can write pages and pages of how she inspired me and loved me. Instead of thanking her I would like to say I love you.

I am what I am now because of you guys. There are more people in my memory than in this acknowledgement page and I will be thanking them now and forever.

I would like to end my acknowledgement by quoting this sentence: ‘Life is not measured by the number of breaths you take, it is measured by the moments that take your breath away’.

Venkatesh S

ABSTRACT OF THE DISSERTATION

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of Satellite Tobacco Mosaic Virus

by

Venkatesh Sivanandam

Doctor of Philosophy, Graduate Program in
Genetics, Genomics and Bioinformatics
University of California, Riverside, March 2014
Dr. A.L.N. Rao, Chairperson

Satellite tobacco mosaic virus (STMV) as the name implies is the satellite virus of the well characterized helper *Tobacco mosaic virus* (TMV). This satellite virus is 18nm in diameter having a T1 symmetry encapsidating a positive sense genomic RNA of 1058nt in size whose 3' 150nt shares 65% homology with that of its Helper virus. Extensive Structural studies on STMV suggested that its coat protein interacts extensively with the stem-loops of the packaged genomic RNA and hence would be unstable to form virions without interacting with its genomic RNA internally. However *invivo* experiments to prove the same were not performed. Host range effects and the role of the positively charged amino acids at the N-terminal of its capsid protein on replication and packaging have not been studied yet on this Satellite virus (or in any other satellite virus). Chapter 1 of this

dissertation describes T-DNA based agrotransformants of STMV to synthesize its genomic RNA in either (+) or (-) orientation when expressed ectopically in *Nicotiana benthamiana* plants. The biological activity of ectopically expressed agrotransformants of STMV (+) or (-) transcripts was confirmed when co-expressed with TMV. Furthermore, progeny virions of STMV recovered from agroinfiltrations were indistinguishable from those recovered from mechanically inoculated plants. However, STMV virions assembled in *N. benthamiana*, but not in tobacco, efficiently packaged a variant form of the genomic RNA. Chapter 2 describes Individual substitution of the positively charged amino acids at the N-terminal region of its Coat protein to a neutrally charged amino acid Alanine affecting dramatically the replication and packaging of these mutant viruses. Chapter 3 describes Agrobacterium mediated transient expression of the three genomic RNA of *Tomato Aspermy virus* in *N.benthamiana* and by utilizing this system we demonstrate that expressed genomic RNA were biologically active and also induced symptoms in *N.benthamiana*.

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GENERAL INTRODUCTION

Satellite RNAs & Viruses:

Plant and animal RNA viruses are commonly associated with subviral agents. There are basically two types of subviral agents: self replicating and non self-replicating. Self replicating agents are plant viroids. Viroids are infectious single-stranded closed circular RNA molecules existing as highly base-paired rod-like structures. The non-self replicating agents are usually parasites of viruses. They depend on a host virus for replication and sometimes encapsidation. These type of subviral agents include satellite RNAs, satellite viruses, virusoid, defective interfering (DI) RNA, and hepatitis delta virus. DI RNAs are mutated sub-viral RNAs spontaneously produced by error-prone viral replicase during viral replication. DIs are commonly associated with animal viruses. Satellite RNAs are linear or circular RNAs that require a helper virus to supply proteins for replication, but share little or no sequence relatedness with the helper virus. Satellite RNAs that encode their own coat protein (CP) are termed as Satellite viruses. Both satellite RNAs and satellite viruses require their helper and a susceptible plant host for replication of their genome. Satellite RNAs require their helper coat protein for encapsidation of their genomic RNA while the satellite viruses do not require the helper for encapsidation as they encode their own capsid protein. The coat proteins of all known satellite viruses are

antigenically different from the coat protein of their helper virus and they are always icosahedral in shape.

There are several known Satellite RNAs, the notable ones are *Cucumber mosaic virus* (CMV) satellite RNA (Avila-Rincon, et al. 1986), Peanut stunt virus (PSV) satellite RNA (Collmer, et al. 1985), *Pea enation virus* (PEV) satellite RNA (Demler, et al. 1989), *Turnip crinkle virus* (TCV) Satellite RNA (Altenbach, et al. 1981). There are five known satellite viruses: *satellite maize white line mosaic virus* (SMWLMV, Zhang et al. 1991), *satellite tobacco necrosis virus* (STNV, Kassanis 1962; Ysabert et al 1980), *satellite panicum mosaic virus* (SPMV, Buzen et al 1984), *satellite St. Augustine decline virus* (Berger et al. 1994) and *satellite tobacco mosaic virus* (STMV, Valverde et al. 1987; Dodds 1991). Satellite RNAs frequently alter the symptoms induced by their helper viruses (Roosinck et al 1992), sometimes leading to a severe disease on their host plants (Kaper and Waterworth 1977). Satellite viruses generally do not modify symptomology. These five plant satellite viruses along with their respective helper viruses are depicted in Table 1.

Satellite Virus		Helper Virus
Satellite maize white line mosaic virus (Icosahedral:30-35 nm)		Maize white line mosaic virus (Icosahedral: 30-35nm)
Satellite panicum mosaic virus. (Icosahedral: 16-17nm)		Panicum mosaic virus. (Icosahedral: 29-30nm)
Satellite St.Augustine decline virus. (Icosahedral: 15-18nm)		St.Augustine decline virus (Icosahedral: 15-18nm)
Satellite tobacco mosaic virus (Icosahedral: 17nm)		Tobacco mosaic virus. (Rod shaped: 300nm by 18nm)
Satellite tobacco necrosis virus (Isocahedral: 16-20 nm)		Tobacco necrosis virus. (Icosahedral: 29-32nm)

Table 1. Comparision of structures of Plant satellite viruses with their Helper viruses

Notably, all the satellite viruses and their helper are icosahedral in structure except *tobacco mosaic virus*, a rod-shaped virus which acts as the helper for satellite TMV, a spherical virus.

The requirement of satellite RNAs and satellite viruses of their cognate helper viruses was demonstrated by co-inoculating host plants with the satellites and their helper virus. Only upon co-inoculation was there accumulation of these

sub viral pathogens. The requirement for helper virus replicase has been shown for STNV (Andreissen et al. 1995), by demonstrating replication of its genomic RNA in the presence of the TNV replicase only instead of the whole TNV virus. The requirement for Helper virus replicase for Satellite RNA replication has been shown for CMV satellite RNA *in vitro* by expressing CMV replicase (Hayes et al. 1992; Wu et al. 1993). Recently it has been discovered that CMV satellite RNA can still replicate in the absence of its helper virus or helper replicase by using Host Pol II in the nucleus.(Choi et al 2012). Similar mechanism of replication for subviral RNAs was found in viroids. Like a virus, the viroid invades a cell and hijacks its reproductive mechanisms. It forces the cell to duplicate the viroid's RNA instead of its own.

Construction and Mutagenesis of Viable cDNA clones of Satellite Viruses

Infectious cDNA clones of RNA viruses have proven invaluable for elucidating the roles that viral genes and nucleic acid sequence play in cell to cell and long distance movement of viruses, their replication and transmission to new hosts (Donson et al. 1991). Cloning of RNA virus genomes of as cDNA was made possible during the late 1970’s and the 1980’s. Construction of viable clones of satellite viruses was accomplished during the 80’s, e.g. STNV (Van Emmelo et al. 1987) and STMV (Mirkov et al. 1989).

Relatively few molecular analyses have been performed on the genome of satellite viruses. The only two satellite viral genomes that underwent mutagenesis studies were STNV and STMV. Mutagenesis analysis was performed on the 5' and 3' UTRs (untranslated regions) of the STNV genome (single-stranded positive sense RNA of 1239 nucleotides). The effects of these mutations on translational efficiency were measured in an *in vitro* translation system. Removal of the first 12 nucleotides or 10 changes in the nucleotide sequence of the 5' UTR reduced translational efficiency of its coat protein approximately 3-fold. Truncation of the 3' UTR to nucleotide 627 or 700, or deletion of nucleotides 627-737, reduced translational efficiency more than 20-fold. Chimeric mRNAs were constructed which contained the coding region of rabbit alpha-globin mRNA and either the 5' UTR, 3' UTR, or both the 5' and 3' UTRs of STNV RNA. Both the 5' and 3' UTRs of STNV RNA were necessary to obtain cap-independent translation. These findings indicate that interaction between the 5' UTR and the region between nucleotides 627 and 737 in the 3' UTR are required for cap-independent translation. The translation mechanisms of STMV or other satellite virus genomes have not been investigated yet. Current mutagenesis studies on STMV will be described below.

Satellite Tobacco Mosaic Virus:

Satellite tobacco mosaic virus (STMV) was discovered in Riverside, California by researchers in the laboratory of Dr.J.A.Dodds. It was discovered

while doing a survey on RNA viruses present in *Nicotiana glauca* plants found locally(Valverde et al 1986, Valverde at al 1987). Upon Transmission electron microscopy (TEM) icosahedral particles of 18nm in size were seen. Further research showed this small spherical was a satellite associated with tobacco mild green mosaic virus (TMGMV or TMV U5) infections of tree tobacco (*Nicotiana glauca*) in southern California. It is the only satellite virus that has rod shaped viruses (tobamoviruses) as a helper. It is capable of encoding its own capsid protein and thus encapsidates its own genomic RNA which is 1058 nt in length. The presence of unique STMV related inclusion bodies in leaf hair cells of N.Tabacum has been reported. These granular inclusion bodies occurred only in cells in which TMV was accumulating (Kim et al. 1989). The helper TMGMV is also common in N. glauca in Spain, Israel, Australia, and other countries with Mediterranean climates, but there have been no additional reports of STMV. STMV has a wide host range which includes tobacco, pepper, and tomato, and infects several agronomic hosts experimentally using a range of tobamoviruses like *Tomato mosaic virus* (ToMV), *Green tomato atypical mosaic virus* (GTAMV) and *Odontoglossum ringspot* ORSV as alternate helper viruses, but STMV has yet to be reported from a crop plant. It was proposed that STMV requires the TMV RdRp for replication. STMV alone can be purified from TMV/STMV infected tissue by precipitating an n-butanol clarified leaf extract with 4% polyethylene glycol (PEG) and 1 M sodium chloride to collect the TMV, followed by 8% PEG precipitation of the aqueous phase to collect the STMV. Electron microscopy of

STMV extracted by this method and purified by sucrose density gradient purification method is shown in Figure 1.

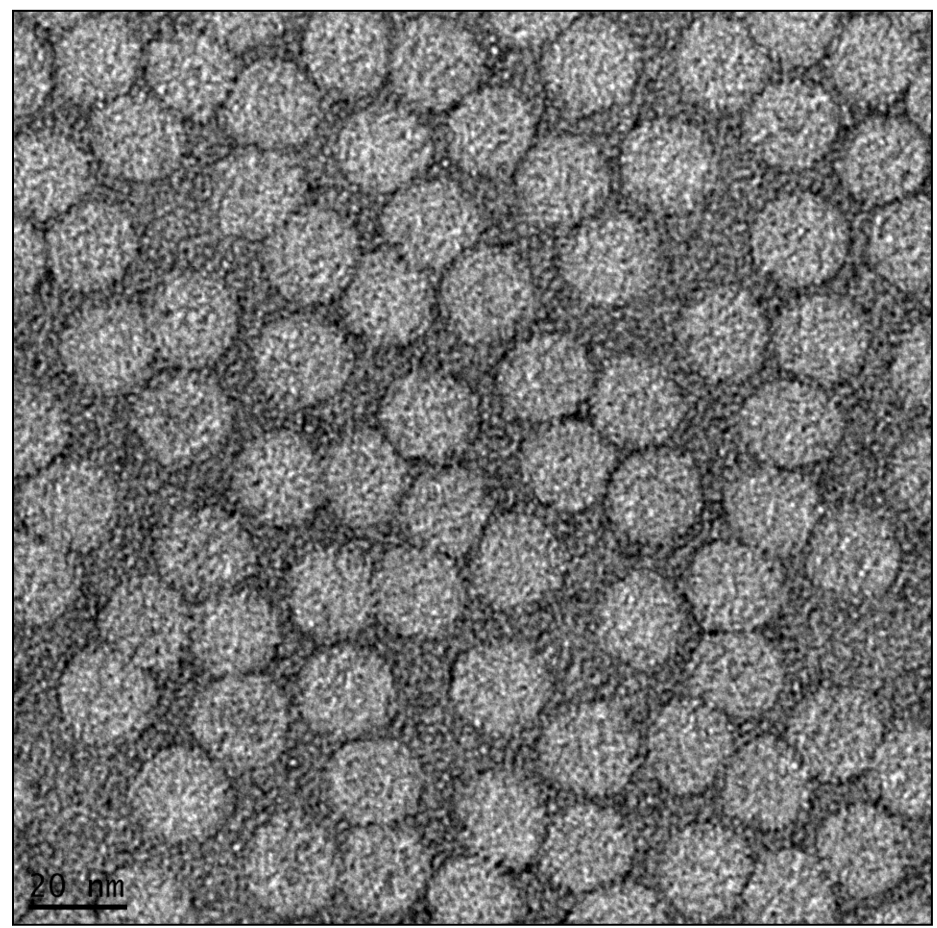


Figure 1.Transmission Electron microscopy of *Satellite tobacco mosaic virus*

The genome of STMV has two open reading frames (Fig 2). The first open reading frame (ORF) starts from base 52 to 228 encodes a 6800 Da protein that is translated only *in vitro*. A second ORF, starts from 162 to 641, encodes the

capsid protein of 17500 Da. Virions of STMV are assembled from 60 identical subunits of CP exhibiting T=1 symmetry.

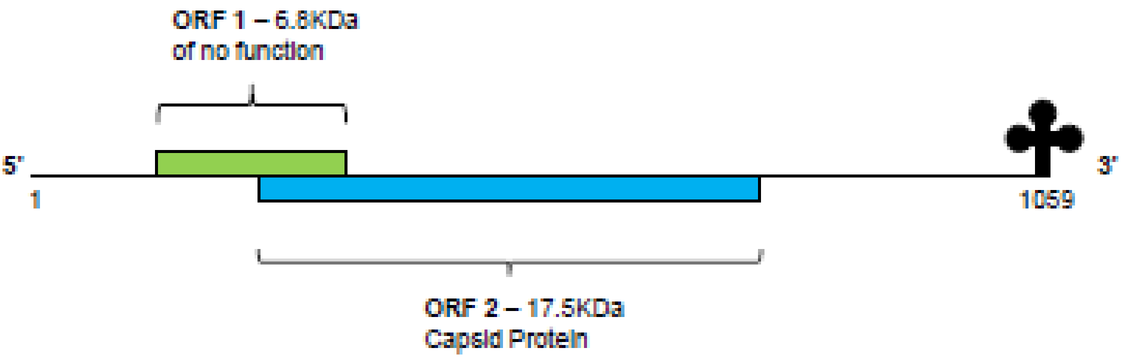


Figure 2. Genome Organization of *Satellite Tobacco Mosaic Virus*

Research on Satellite Tobacco Mosaic Virus:

Since STMV tended to crystallize spontaneously upon purification, it was adopted by the laboratory of Dr. Alexander McPherson for crystallographic studies and its crystal structure revealed a great deal of information on the assembled capsid structure and its RNA content (Kozalek et al. 1989; Larson, et al. 1993a; Larson et al 1993b). STMV genomic RNA is highly structured and it interacts with the positively charged N-terminal of the capsid protein to enable assembly into highly stable virions. The 3' 150 nucleotides of the STMV genome have 65% homology with that of TMV (TMV U1 strain) with two domains of high

sequence homology. These two domains encompass the 3’ T-RNA like structure and the proximal pseudoknot wherein the first domain encompass nucleotides from 899 to 949 of STMV corresponding with nucleotides 6259 to 6300 of TMV U1 and the second domain encompasses nucleotides 1009 to 1059 of STMV that are homologous with nucleotides 6343 to 6395 of TMV U1. The 3’ t-RNA like structure and 3’ proximal pseudoknot of STMV are believed to play an important role in translation due to its homology with the 3’ end of its helper TMV as it was found that the 3’ UTR of the TMV genomic RNA plays an important role in its own translation. (Gultyaev et al., 1994).

In cross protection assays, it is been found that the STMV type strain (original strain) successfully cross-protected against the other four types (not widely prevalent) of STMV when tobacco plants were inoculated 10 days apart (Mathews et al. 1998). STMV does not elicit any symptoms in their plant hosts except Pimenta dioica (Jalapeno pepper plant) which is characterized by necrotic lesions on the leaves few days after STMV infection on this plant that had already been infected with TMV (Rodriguez-Alvarado et al. 1994). A chimeric STMV clone containing the 3' terminus of TMV (TMV-U1) RNA was infectious in coinfections with TMGMV, but it did not replicate with TMV-U1 revealing that the RNA sequences involved in helper specific replication reside at the 5’ end of the molecule and not the 3’ terminus (Kurath et al. 1993). Populations of STMV adapted to replication with four alternative helper tobamoviruses which were generated by serial passage in tobacco. RNase protection analyses of these

RNA populations showed that in all cases there had been a genetic change approximately 50 to 60 bases from the 5' terminus of the STMV genome (Kurath et al. 1993). Similar changes were detected in several progenies of STMV clones replicated with TMV-U1, indicating that change at this site was essential for replication with a helper virus other than TMGMV. Sequence analyses of the changes at this helper adaptation domain consistently showed the deletion of a single G from five consecutive Gs at bases 61 to 65. On changing the helper from TMV U5 to TMV U1 and on serial passaging, STMV still replicated its genomic RNA and formed virions like that with TMV U5 but on sequence analysis it was found that the STMV genomic RNA lost its terminal 5'end nucleotide namely the 'adenine'. This adenine devoid STMV RNA could replicate and form virions when co-infected with TMV U5.

A series of frame shift and deletion mutations was created in the genome of STMV by modifying full-length cDNA clones of the type strain, from which biologically active transcripts could be synthesized in vitro. Deletions and frameshift mutations in the 5' open reading frame had no effect, compared to wild-type STMV, on RNA accumulation, systemic movement, or the symptoms induced by STMV in *N. tabacum* (Turkish) co-inoculated with tobacco mild green mosaic tobamovirus TMGMV (or TMV U5). This implies that the protein encoded by this reading frame is not necessary for biological activity. Deletions and frameshift mutations in the coat protein open reading frame resulted in decreased accumulation of STMV RNA in *N. tabacum*, although these mutants

were still capable of systemic movement, presumably in a nonencapsidated or free RNA form. Furthermore, the mild symptoms induced in tobacco by co-inoculations of wild-type STMV/TMGMV or infection with TMGMV alone were altered to severe systemic necrosis when plants were co-inoculated with these STMV coat protein mutants and TMGMV. Mutants within the 3' untranslated region were much less able to accumulate in TMGMV-infected plants than was wild-type STMV.

While the encapsidated form of the RNA has been extensively studied, less is known about the structure of the free RNA, aside from a purported tRNA-like structure at the 3' end. Selective 2′-hydroxyl acylation *analyzed* by primer extension (SHAPE) analysis to reveal the exact secondary structure of STMV genomic RNA was consistent with the previously predicted tRNA-like fold at the 3' end of the molecule and the first set of three pseudoknots but it failed to agree with the second set of pseudoknots that was predicted before (Athavale, et al. 2013).

Studies on replication independent translation and encapsidation and the effect of amino-acid substitution on the N-terminal positively charged amino acids of the coat protein of STMV or other satellite viruses have not been performed yet and are addressed in this thesis.

Chapter 1 of this dissertation analyzes replication and packaging of STMV when it is delivered by an agrobacterium-mediated transient gene expression system that allows delivery of satellite viral RNA in both polarities

(positive and negative sense) *in vivo* namely *Nicotiana benthamiana*. This chapter also analyzes the translation, assembly and packaging mechanism of STMV coat protein when it is delivered without its helper virus (HV) but with a suppressor of RNA silencing, namely P19 by an agrobacterium-mediated expression system.

Chapter 2 describes the replication, translation and packaging mechanism of STMV when the N-terminal positively charged aminoacids of its coat protein, namely arginine and lysine are substituted individually by a neutrally charged amino-acid: alanine.

Chapter 3 describes about the biological activity of *Tomato Aspermy virus* when its three genomic RNA is delivered to the leaves of *Nicotiana benthamiana* through agroinfiltration.

References:

Ahlquist P, French R, Janda M, Loesch-Fries LS. Multicomponent RNA plant virus infection derived from cloned viral cDNA. Proc Natl Acad Sci U S A. 1984 Nov;81(22):7066–7070.

Andriessen, M., Meulewaeter, F. & Cornelissen, M. (1995). Expression of tobacco necrosis virus open reading frame 1 and 2 is sufficient for the replication of satellite tobacco necrosis virus. Virology 212, 222-224.

Berger PH, Shiel PJ, Gunasinghe U. The nucleotide sequence of satellite St. Augustine decline virus. Mol Plant Microbe Interact. 1994 Mar-Apr;7(2):313-6.

Buzayan JM, Gerlach WL, Bruening G. Non-enzymatic cleavage and ligation of RNA complementary to a plant virus satellite RNA. Nature 1986; 323:349–353.

Buzen F. G., Niblett C. L., Hooper G. R., Hubbard J., Newman M. A. (1984) Further characterization of panicum mosaic virus and its associated satellite virus. Phytopathology 74:313–318

Collmer, C. W., Kaper J. M; Infectious RNA transcripts from cloned cDNAs of cucumber mosaic viral satellites. Biochem. Biophys. Res. Commun., 135 (1986), pp. 290–296

Culver JN, Dawson WO. Tobacco mosaic virus coat protein: an elicitor of the hypersensitive reaction but not required for the development of mosaic symptoms in Nicotiana sylvestris.Virology. 1989 Dec;173(2):755–758.

Culver, J. N., and Dawson, W. O. 1991. Tobacco mosaic virus elicitor coat protein genes produce a hypersensitive phenotype in transgenic Nicotiana sylvestris plants. Molecular Plant-Microbe Interactions 4, 458-463

Culver, J. N., Lehto, K., Close, S. M., Hilf, M. E. & Dawson, W. O. Genomic position affects the expression of tobacco mosaic virus movement and coat protein genes. Proc Natl Acad Sci USA 90, 2055–2059 (1993).

Culver, J. N. Dawson, W. O., Plonk, K., and Stubbs, G. (1995). Site-directed mutagenesis confirms the involvement of carboxylate groups in the disassembly of tobacco mosaic virus. Virology 206, 724-730

Davies, C. Haseloff, J. and Symons, R. H. (1990) Structure, self-cleavage, and replication of two viroid-like satellite RNAs (virusoids) of subterranean clover mottle virus. Virology 177, 216-224

Dawson, W. O. Beck, d., I., Knorr, D. A, and Grandthani, G. L. (1986) cDNA cloning of the complete genome of tobacco mosaic virus and production of infectious transcripts. Proc. Nat'l Acad. Sci. USA 86, 3509-3513

Demier, S. A., Rucker, D. G., Nooruddin, L., and Dezocten, G. A. (1994) Replication of the satellite RNA of pea enation mosaic virus is encoded by RNA 2-encoded functions. J gen. Vir. 75, 997-1007

Dejong, W., Chu, A., and Ahiquist, P. (1995). Coding changes in the 3A cell-to-cell movement gene can extend the host range of brome mosaic virus systemic infection. Virology 214, 464-474.

Danthinne, X., Seurinck, J., Meulewaeter, F., Vanmontagu, M., and Cornelisson (1993) The 3' untranslated region of satellite tobacco necrosis virus RNA stimulates translation in vitro. Mol. And Cell Biol. 13, 3340-3349.

Diener, T. O. (1991) Subviral pathogens of plants – viroids and viroid-like satellite RNAs. FASEB Journal 5, 2808-2813

Dinant, S., Janda, M., Kroner, P. A., and Ahlquist, P. (1993) Bromovirus RNA replication and transcription require compatibility between the polymerase- and helicase- like viral RNA synthesis J. Vir. 67, 7181-7189

Dodds, J. A. (1991) Structure and function of the genome of satellite tobacco mosaic virus. Canodian Journal of Plant Pathology 13, 192-195

Donson, J., Kearney, C. M., Hilf, M. E., and Dawson, W. O. (1991) Systemic expression of a bacterial gene by a tobacco mosaic virus-based vector. Proc. Natl. Acad. Sci. (USA) 88, 7204-7208.

Duran-Vila, N., Roistacher, C. N. Rivera-Bustamante, R., and Semancik, J. S., (1988) A definition of citrus viroid groups and their relationship to the execortis disease. J. gen Vir. 69, 3069-3080.

Dzianott, A., Flasinski, S and Bujerski, J. J. (1995) Foreign complementary sequences facilitate genetic recombination in brome mosaic virus. Virology 208, 370-375

Eggen, R., Verver, J., Wellink, J., Dejong, A., Goldbach, R., and Van Kammen, A. (1989) Improvements in the infectivity of in viro transcripts from cloned cowpea-mosaic virus cDNA: impact of terminal nucleotide sequences. Virology 173, 447-455

Felden, B., Florentz, C., McPherson, A., and Giege, R. (1994). A histidine accepting tRNA-like fold at the 3' and of satellite tobacco mosaic virus RNA. Nucleic Acids Res 22, 2882-2886

Flasinaski, S., Dzianott, A., Pratt, S., and Bujarski, J. J. (1995). Mutational analysis of the coat protein gene of brome mosaic virus – effects of replication and movement in barley and in chenopodium hybridum. Mol. Plant-Microb. Interact 8, 23-31.

Forster, A., C. and Symons, R. H. (1987) Self cleavage of plus and minus RNAs of a virusoid and a structural model for the active sites. Cell 49, 211-220

Gerlach, W. L., Buzzyan, J., Schneider, I. R., and Bruening, G. (1986) Satellite tobacco ringspot virus RNA: biological activity of cDNA clones and their in vitro transcripts. Virology 151, 172-185.

Gerlach, W. L., Liewellyn, D., and Hasseloff, J. (1987) Construction of a plant disease resistance gene from the satellite RNA of tobacco ringspot virus. Nature (London) 328, 802-805.

Grief, C., Hemmer, O., Demangeat, G., and Fritsch, C. (1990) In vitro synthesis of biologically active transcripts of tomato black ring virus satellite RNA. J. gen. Vir. 71, 907-915.

Gultyaev, A. P., Van Batenburg, E., and Pleij, C. W. A. (1994) Similatities between the secondary structure of satellite tobacco mosaic virus and tobamovirus RNAs. J. gen. Vir. 75, 2851-2856.

Hamamoto, H., Sugiyama Y., Nakagawa N., Hashida, E., Matsunaga, Y., Takemoto, S., Watanabe, Y., and Okada, Y. (1993) A new tobacco mosaic virus vector and its use for the systemic production of angiotensis-I-converting enzyme inhibition in transgenic tobacco and tomato. Bio-Technology 11, 930-932.

Hanold, D and Randles, J. W. (1991) Coconut cadang-cadang and its viroid agent. Plant Disease 75, 330-335.

Hans, F., Fuchs, M., and Pinck, L. (1992). Replication of grapevine fanleaf virus satellite RNA transcripts in Chenopodium-quinoa protoplasts. J. gen. Vir. 73, 2517-2523

Hayes, R. J, Tousch, D., Jacqemond, M., Pereita, V. C., Buck, K. W. and Tepfer, M. (1992) Complete replication of a satellite RNA in vitro by a purified RNA-dependent RNA polymerase. J. gen. Vir. 73, 1597-1600.

Heaton, L. A., Carrington, J. C., Morris, T. J. (1989) Turnip crinkle virus infection from RNA synthesized in vitro. Virology 170, 214-218.

Hemmer, O., Oncino, C., and Fritsch, C. (1993) Efficient replication of the in vitro transcripts from cloned cDNA of tomato black ring virus satellite RNA requires the 48K satellite RNA-encoded protein. Virology, 194, 800-806.

Hillman, B. I., Carrington, J. C., and Morris, T. J. (1987) A defective interfering RNA that contains a mosaic of a plant virus genome. Cell (Cambridge Mass.) 51, 427-433.

Ikeda, R., Watanabe, E., Watanabe, Y., and Okada, Y. (1993) Nucleotide sequence of tobamovirus OB which can spread systemically in N-gene tobacco J. gen. Vir. 74, 1939-1944.

Ismail, I. D., and Milner, I. J. (1988) Isolation of defective interfering particles of Sonchus yellow net virus from chronically infected plants. J. gen. Vir. 69, 9999-1006.

Janda, M., French, R., and Ahlquist, P. (1987) High efficiency T7 polymerase synthesis of infectious RNA from cloned brome mosaic virus cDNA and effects of 5’ extensions of transcript activity. Virology 158, 259-262.

Kaper, J. M. and Waterworth, H. E., (1977) Cucumber mosaic virus associated RNA5. Causal agent for tomato necrosis. Science 196, 429-431.

Kassanis, B. (1962) Properties and behavior of a virus depending for its multiplication on another. J. ge. Microbiol. 27, 477-488.

Kassanis, B. (1981) Portraits of viruses: Tobacco necrosis virus and its satellite virus Intervirology 15, 57-70.

Kim, K. S., Valverde, R. A., and Dodds, J. A. (1989) Cytopathology of satellite tobacco mosaic virus and its helper virus in tobacco. J. Ultrastruct and Mol. Struct Res. 102 196-204.

Keszelak, S., Dodds, J. A., and McPherson, and McPherson, A. (1989). Preliminary analysis of crystals of satellite tobacco mosaic virus. J. Mol. Biol. 209, 323-325.

Kroner, P., Richards, D., Traynor, P., and Ahlquist, P. (1989) Defined mutations in a small region of the brome mosaic virus-2A gene cause diverse temperature-sensitive RNA replication phenotypes. J. Vir. 63, 5302-5309.

Kroner, P. A., Young, B. M., and Ahlquist, P. (1990). Analysis of the role of brome mosaic virus 1A-Protein domains in RNA replication using linker insertion mutagenesis. J. Vir. 64, 6110-6120.

Kurath, G. and Palukaitis, P. (1987) Biological activity of T7 transcripts of a prototype clone and a sequence variant clone of a satellite RNA of cucumber mosaic virus. Virology 159, 199-208.

Kurath, G., Rey, M., E., C and Dodds, J. A. (1992) Analysis genetic heterogeneity within the type strain of satellite tobacco mosaic virus reveals several variants and a strong bias for g to a substitution mutations. Virology 189, 233-244.

Kurath, G., Heick, and J. A., Dodds, (1993a) Rnase protection analyses show high genetic diversity among field isolates of satellite tobacco mosaic virus. Virology 194, 414-418.

Kurath, G., Rey, M. E. C., and Dodds, J. A. (1993b) Tobamovirus helper specificity of satellite tobacco mosaic virus involves a domain near the 5' end of the satellite genome. J. gen. Vir. 74, 1233-1243.

Kurath, G., and Dodds, J. A. (1994) Satellite tobacco mosaic virus sequence variants with only five nucleotide differences can interfere with each other in a cross protection-like phenomenon in plants. Virology 202, 1065-1069.

Kurath, G., and Dodds, J. A. (1995) Mutation analysis of molecularly cloned satellite tobacco mosaic virus during serial passage in plants – evidence for

hotspots of genetic change. RNA-A Publication of the RNA Society 1, 491-500.

Larson, S. B., Koszelak, S., Day, J. Greenwood, A., Dodds, J. A., and McPherson, A., (1993) 3-Dimensional structure of satellite tobacco mosaic virus at 2 9 Angstrom resolution. J. Mol. Biol. 231, 375-391.

Larson, S. B., Koszelak, S., Day, J., Greenwood, A., Dodds, J. A., and McPherson, A. (1993) Double-helical RNA in satellite tobacco mosaic virus. Nature 361, 179-182.

Mirkov, T. E., Kurath, G., Mathews, D. M., Elliott, K., Dodds, J. A., and Fitzmaurice, L. (1990) Factors affecting efficient infection of tobacco with in vitro RNA transcripts from cloned cDNAs of satellite tobacco mosaic virus. Virology 179, 395-402.

Ogawa, T., Watanabe, Y., and Okada, Y. (1992) Cis-acting elements for in-trans complementation of replication-defective mutant of tobacco mosaic virus. Virology 191, 454-458.

Nagy, P. D., Daianott, A., Ahlquist, P., and Bujarski, J. J. (1995) Mutations in the helicase-like domain of protein 1A alter the sites of RNA-RNA recombination in brome mosaic virus. J. Vir. 69, 131-140.

Nagy, P. D., and Bujarski, J. J. (1996) Homologous RNA recombination in brome mosaic virus-AU-rich sequences decrease the accuracy of crossovers. J. Vir. 70, 415-426.

Padgett H. S., and Beachy, R. N., (1993) Analysis of a tobacco mosaic virus strain capable of overcoming N gene mediated resistance. Plant Cell 5, 577-586.

Passmore, B. K., Sager, M., Chin., L. S., Falk, B. W., and Bruening, G. (1993) Beet western yellows virus-associated RNA – an independently replicating RNA that stiulates virus accumulation. Proc. Natl Acad. Sci. 90, 10168-10172.

Pogue, G. P., Marsh, L. E., and Hall, T. C. (1990) Point mutations in the ICR2 motif of brome mosaic virus RNAs debilitate (+)-strand replication. Virology 178, 152-160.

Pogue, G. P., Marsh, L. E., Connel, J. P., and Hall, T. C. (1992a) Requirement for ICR-like sequences in the replication of brome mosaic virus genomic RNA. Virology 188, 742-753.

Pogue, G. P., and Hall, T. C. (1992b) The requirement for a 5 stem-loop structure in brome mosaic virus replication supports a new model for viral positive-strand RNA initiation. J. Vir. 66, 674-684.

Racienello, V. R., and Baltimore, D. (1981) Cloned poliovirus DNA is infectious in mammalian cells. Science 214, 916-919.

Rao, A. L. N., Dreher, T. W., Marsh, I. E., and Hall, T. C. (1989) Telomeric function of the transfer RNA-like structure of brome mosaic virus RNA. Proc. Natl Acad. Sci. (USA) 86, 5335-5339.

CHAPTER 1

Molecular Studies on Satellite Tobacco Mosaic Virus: Host passage effects and helper-independent assembly

Abstract

The genome of a *Satellite tobacco mosaic virus* (STMV) is 1058 nt long and encodes a single capsid protein of 17.5 kDa assembling into a18 nm icosahedral virus whose replication is dependent on the rod shaped *Tobacco mosaic virus* (TMV). Although STMV was structurally well characterized previously, the mechanism of genome packaging in STMV is not known. In this study we assembled T-DNA based agrotransformants of STMV to synthesize its genomic RNA in either (+) or (-) orientation when expressed ectopically in *Nicotiana benthamiana* plants. The biological activity of ectopically expressed agrotransformants of STMV (+) or (-) transcripts was confirmed when co-expressed with TMV. Furthermore, progeny virions of STMV recovered from agroinfiltrations were indistinguishable from those recovered from mechanically inoculated plants. However, STMV virions assembled in *N. benthamiana*, but not in tobacco, efficiently packaged a variant form lacking the 3' 150 nt (STMVΔ150). Further characterization of STMVΔ150 revealed it was defective in HV-dependent replication.

Introduction

Satellite RNAs and satellite viruses each require their associated helper virus for replication of their genome. Satellite viruses encode their own coat protein while satellite RNAs require the helper virus coat protein for encapsidating their genome (Hu et al., 2009). *Satellite tobacco mosaic virus* (STMV) was first isolated from *Nicotiana glauca* in Southern California in 1998 (Dodds, 1998). STMV is a small icosahedral virus 17 nm diameter (Valverde and Dodds, 1986, 1987). The particles of STMV have T=1 symmetry (Larson et al., 1993a) assembled from 60 capsid protein subunits of 17,500 Daltons and encapsidate a single strand, positive sense RNA of 1,059 nucleotides (nt) (Mirkov et al., 1989). The RNA has ORFs for two proteins of 6.8 kDa and 17.5 kDA. The function of the former protein is unknown and has only been detected using *in vitro* translation, while that of latter is the capsid protein. The 3' proximal 150 nt of STMV exhibit sequence similarity to the 3' tRNA-like structure (TLS) of tobacco mosaic virus (TMV) (Mirkov et al., 1989). By definition, a satellite virus or satellite RNA cannot replicate without a helper virus (HV) (Hu et al., 2009). Consequently TMV is the HV for STMV that can replicate in a wide range of host plants with six different tobamoviruses tested (Dodds, 1998). Structurally STMV is well characterized (Larson et al., 1993a) and sequences required for efficient replication and systemic movement were mapped (Routh et al., 1995). The simple genome organization of STMV and the fact that STMV is the only known icosahedral satellite virus whose replication is dependent on a well-characterized

rod shaped TMV (Valverde and Dodds, 1986, 1987), offers a unique system for studying genome packaging. Since the genome packaging in RNA viruses is functionally coupled to replication (Annamalai and Rao, 2006b; Annamalai et al., 2008; Nugent et al., 1999b), it is important to dissect the replication of STMV from the packaging. Therefore, in this study we established an *Agrobacterium*-mediated transient expression system for STMV to study replication independent expression of STMV CP followed by virion assembly.

Materials and methods

Agro-constructs and Agroinfiltration

To construct T-DNA based plasmids of STMV, a full-length cDNA of STMV was amplified from pSTMV6 (Mirkov et al., 1990) by PCR using a 5' forward primer (5' TGA ATA <u>TAC GTA</u> AAC TTA CCA ATC AAA AGA CC 3'; *Sna*BI Site is underlined) and the 3' reverse primer (5' AAA AAA <u>TAC GTA</u> TGG GCC GCT TAC CCG CGG 3'; *Sna*BI Site is underlined). The resulting PCR product was digested with *Sna*BI and ligated into a *Stu*I digested binary vector pCASS RZ (Annamalai and Rao, 2005). The resulting plasmids of pSTMV(+) or (-) (Fig. 3) contain in sequential order: double 35 S promoters, cDNA complementary to full length STMV in either (+) or (-) orientation, Rz sequence and a *Nos* terminator. An agroconstruct, referred to as Δ35S-pSTMV(-) (Fig. 3), was constructed by deleting the sequence encompassing the 35S promoter region as described previously (Kwon and Rao, 2012). A variant of STMVΔ150 was

constructed by PCR using a forward primer (5'TGAATATACGTAAACTTACCAATCAAAAGACC3'; *Sna*BI site is underlined) and a reverse primer (5'GGGAGGACACAGCCAACATACGTA 3'; *Sna*BI site is underlined). The resulting PCR product was digested with *Sna*BI and ligated into the binary vector pCASSRz as described above. An agroconstruct, referred to as pJL 63 (Fig. 3) containing a full-length cDNA of *Tobacco mosaic virus* (TMV) was obtained from Dr. John Lindbo's lab (Ohio State University). A variant agroconstruct of TMV, referred to as pRP (Fig. 3), designed to express a functional viral RNA-dependent RNA polymerase (RdRp) gene was obtained from Dr. Barbara Baker's lab (UC Berkeley). Growth conditions of agrotransformants and the agroinfiltration procedure was as described previously (Annamalai and Rao, 2006a) except that each agroculture was adjusted to 0.5 OD at 600nm. The final inoculum was prepared by mixing equal volumes of each culture and infiltrating it into the abaxial surface of fully expanded *N. benthamiana* leaves using a 1 ml syringe without a needle.

Progeny analysis

Total RNA from agroinfiltrated and mechanically inoculated leaves was extracted using the TRIZOL method (Ambion) and the RNA pellet was suspended in RNAse-free water. Virions were purified from these leaves as described previously (Mirkov et al., 1987). Total and virion RNA was subjected to Northern blot analysis as described previously (Annamalai and Rao, 2005). To

prepare strand specific riboprobes, a sequence located between nucleotides 601 to 789 of the STMV genome was generated by digesting the cDNA with *Sal*I and *Bgl*II and sub-cloning it into a pT3/T7 vector digested with *Sal*I and *Bam*HI. STMV CP samples were analyzed using Western blots as described previously (Osman et al., 1997).

Virion fractionation and Electron microscopy

For electron microscopy analysis, STMV virions were purified from *N.benthamiana* as described (Dodds et al 1987), subjected to 10% - 40% sucrose gradient centrifugation and fractions of 500ul were collected after centrifugation using an ISCO UA5 Gradient Fractionator while recording the UV absorbance along the length of the gradient. Ten microliters of these fractions were subjected to western blot analysis to determine the fractions harboring the virions, and each fraction was then subjected to virion RNA extraction (Dodds 1987) for agarose gel electrophoresis and Northern blot analysis. The virus harboring fractions were pooled together and concentrated by high-speed centrifugation at 30,000 RPM for 4 hours and the pellets were re-suspended in water. Approximately 10µl (100ng/µl) of these highly purified virions were spread on glow-discharged carbon coated grids and negatively stained with 1% uranyl acetate prior to examining with a FEI Tecnai12 transmission electron microscope (Annamalai and Rao, 2005) and images were recorded digitally.

Results

Characteristic features and biological activity of an agrotransformant of pSTMV(+)

STMV agrotransformants, pSTMV(+) and pSTMV (-) (Fig. 3), were engineered such that, following agroinfiltration *in planta*, the transiently expressed STMV (+) and (-)-strand RNA transcripts would have the following characteristics. Plus-strand RNA transcripts would have an authentic 5' end due to precise initiation of transcription by the 35S promoter (Fig. 3) while the 3' end would have an additional 22-nt extension beyond the natural 3' CCA due to the presence of the self-cleaving TBSV ribozyme (Fig. 3). Similarly, the (-)-strand STMV RNA transcripts would be the exact complementary sequences to the corresponding (+)-strand, terminating with +22-nt non-viral extensions at the 5'end (Fig. 3).

To test the biological activity of transiently expressed STMV (+)-strand transcripts, the following two experiments were performed. In the first experiment, *N. benthamiana* plants were agroinfiltrated with a mixture of agrotransformants containing pSTMV (+) and either pJL 36 or pRP. Agrotransformants pJL 36 (Lindbo, 2007) and pRP (Erickson et al., 1999) were engineered respectively to express full-length TMV or only the TMV replicase gene . In the second experiment, following agroinfiltration of *N. benthamiana* with pSTMV, at 4 dpi, infiltrated leaves were mechanically inoculated with TMV. *N. benthamiana* and *N. tabacum* plants mechanically inoculated with wild type

STMV and TMV served as controls. Total RNAs recovered from experimental and control plants were subjected to Northern blot hybridization and results are shown in Fig. 4A. As expected, in the absence of HV, transcripts of STMV RNA were not detectable (Fig. 4A, lane 1). However, co-expression with either pJL 36 or pRP resulted in efficient expression and accumulation of STMV RNA (Fig. 4A, lanes 2 and 3) similar to control plants (Fig. 4A, lanes 5 and 6). Likewise, detectable levels of STMV RNA accumulated in leaves agroinfiltrated with STMV followed by mechanical inoculation with TMV (Fig. 4A, lane 5). Western blot analysis with anti-STMV CP antibody (Fig. 4B) and electron microscopic examination of purified virions from each case (Fig. 4C), confirmed that transiently expressed RNA transcripts of pSTMV (+) are biologically active and the progeny were indistinguishable from control plants.

Ectopically expressed STMV (-)- transcripts are biologically active

We recently demonstrated that ectopically expressed (-)-strand RNA transcripts of *Brome mosaic virus* (BMV) RNA3 or satellite RNA of *Cucumber mosaic* virus (CMV) are competent to serve as templates to initiate replication by their respective viral replicases (Kwon and Rao, 2012; Seo et al., 2013). To verify whether (-)-strand transcripts of STMV would be biologically active when complemented with its HV replicase, pSTMV(-) was co-expressed in *N. benthamiana* plants with pRP. Plants infiltrated only with either pSTMV(+) or pSTMV (-) in the presence and absence of an RNA silencing suppressor P19

(Lakatos et al., 2004) served as controls. Results of Northern blot (plus and minus-strands), Western blot and EM analysis are shown in Fig. 5 (A-D). In the absence of P19, only transcripts of (-)-strand RNA but not those of (+)-strand were detectable (compare lanes 1 and 2 in Fig. 5A, B). By contrast, co-expression of P19 resulted in detectable levels of both (+) and (-)-transcripts (Fig. 5A, lanes 3 and 4). It is interesting to note that although no complementary (-)-strands were detected in leaves co-expressing P19 and pSTMV (+) (compare lane 3 in Fig. 5A, B), a low but weakly visible level of (+)-strand RNAs in leaves co-expressing P19 and pSTMV (-) were detected (compare lane 4 in Fig. 5A, B). Western blot analysis confirmed that the STMV (+)-strand transcripts derived from ectopically expressed STMV (-)-strands are competent for CP translation (compare lanes 3 and 4 in Fig. 5C). When plants were co-infiltrated with pSTMV(-) and pRP, detectable levels of progeny STMV (+)-strand transcripts were accumulated (Fig. 5A, lane 5), although this level of (+)-strand synthesis was prominently less than that of progeny derived from transiently expressed pSTMV (+) and pRP (compare lanes 5 and 6 in Fig. 5A). In addition, EM analysis of virion preparations recovered from *N. benthamiana* leaves coinfiltrated with pRP and either pSTMV(+) or pSTMV(-) were of 18 nm and indistinguishable from native STMV virions (Fig. 5D). Collectively the data suggested that ectopically expressed STMV (-)-strand transcripts are biologically active (see Discussion).

A remote possibility exists that initiation of STMV (+)-strands from ectopically expressed STMV (-)-strands could be due to the action of an

inconspicuous cryptic promoter presented in the pSTMV (-) agrotransformant. To verify this possibility, a region encompassing the double 35S promoter was deleted from pSTMV (-) agroconstruct, resulting in Δ35SpSTMV (-) (Fig. 3). Agrocultures of Δ35SpSTMV (-) and pRP were coinfiltrated into *N. benthamiana* leaves and progeny was subjected to northern blot hybridization at 4dpi. Absence of any detectable STMV RNA (Fig. 5 A, lane 7) confirmed that initiation of STMV(+)-strand synthesis on ectopically expressed STMV(-)-strands by HV RdRp is authentic and is not mediated by the action of any cryptic promoter.

Transiently expressed STMV CP is competent to form virions

Results shown in Fig. 5C demonstrate that STMV transcripts expressed in the absence of HV are competent to translate. To verify whether the STMV CP translated in the absence of its HV could form virions we performed the following experiment. Following agroinfiltration of *N. benthamiana* leaves with pSTMV+P19 (P19 is the suppressor of RNA silencing of *Cymbidium ringspot virus* (CymRSV)) virions were purified and subjected to density gradient centrifugation followed by fractionation and EM analysis of pooled fractions as described under Materials and Methods. Sucrose density gradient fractionation of virions purified from plants infiltrated with wt pSTMV+pRP served as a control. Results are shown in Fig. 6.

Sucrose density gradient sedimentation of virions purified from *N. benthamiana* leaves infiltrated with pSTMV+pRP revealed a single sedimenting

major peak approximately 3 cm from the meniscus (Fig. 6A) characteristic of wt STMV virions purified from *N. tabacum* plants (Valverde and Dodds, 1986, 1987). Gradient fractions encompassing the single major peak contained STMV CP as evidenced by Western blot analysis (Fig. 6B) and the detection of icosahedral virions 18 nm in diameter (Fig. 6C, panel ii). By contrast, virions purified from *N. benthamiana* leaves infiltrated with pSTMV+P19 revealed two sedimenting peaks approximately 3 cm and 5 cm from the meniscus (Fig. 6B). When fractions encompassing the entire gradient were subjected to Western blot hybridization (Fig. 6E) and EM analysis (Fig. 6F), STMV CP was found to be associated only with fractions from near the top of the gradient and not within the UV absorbing peaks below.

To analyze the nucleic acid composition of each gradient, samples encompassing the 28 fractions shown in Fig. 6A and D, were pooled pair-wise (resulting in a total of 14 pooled fractions), then RNA was extracted and subjected to Northern blot hybridization. Results are shown in Fig. 7 (A-F). As expected the STMV RNA profile was identical to that shown in Fig. 6A and was associated with the single peak recovered from the gradient with pSTMV+pRP virions (Fig. 7B). By contrast, STMV RNA was not detected in any of the fractions recovered from the gradient containing pSTMV+P19 (Fig. 7D). However, ethidium bromide stained agarose gel analysis revealed the presence of a group of nucleic acids characteristic of a cellular RNA profile in faster migrating fractions (Fig. 7E). RNase treatment confirmed that the detected nucleic acids

are single strand RNAs (Fig. 7F). Collectively these observations suggest that STMV virions associated with the slower sedimenting fractions of pSTMV+P19 are empty (see discussion).

STMV virions from N. benthamiana package a truncated species

When RNA was isolated from STMV virions shown in Fig. 5D and was subjected to Northern blot hybridization an unexpected truncated RNA that migrated faster than wild type (wt) was seen (Fig. 8A, lanes 1 and 2). This truncated RNA was not seen in virions recovered from mechanically inoculated plants *N. tabacum* (Fig. 8A, lane 3). To confirm that production of this truncated RNA species is not due to agroinfiltration, *N. benthamiana* plants were mechanically inoculated with STMV+TMV. *N. tabacum* plants mechanically inoculated with the same inoculum served as a control. Northern blot hybridization and PCR results of virion RNA recovered from agroinfiltration and mechanical inoculation shown in Fig. 8B confirmed that production of truncated STMV RNA species is not an artifact of agroinfiltration but is specifically generated in *N. benthamiana,* but not in *N. tabacum.*

The truncated PCR product of STMV was sequenced and found to lack the terminal 3’ 150 nt, as schematically shown in Fig. 8C (referred to as STMVΔ150). To further characterize the nature of this truncated species of STMV, an agroconstruct referred to pSTMVΔ150, as schematically shown in Fig.

8D was made. To test the biological activity, pSTMVΔ150 was agroinfiltrated to *N. benthamiana* in the presence and absence of P19. Plants infiltrated with wt pSTMV served as controls. Total RNA was subjected to duplicated Northern blots and hybridized with STMV (+) and (-) riboprobes. Results are shown in Fig. 8E. Like wt STMV, although transcripts of STMVΔ150 were accumulated to detectable levels (compare lanes 2 and 4 in Fig. 8E), the absence of progeny (-)-strands confirmed that it did not replicate when co-expressed with pRP (compare lanes 6 and 8 in Fig. 8E).

Discussion

This study was initiated to understand the mechanism regulating genome packaging in STMV. We hypothesized that packaging STMV could be functionally coupled to replication as observed in other positive strand RNA viruses such as BMV, polio, flock house and kunjin viruses (Annamalai and Rao, 2005; Khromykh et al., 2001; Nugent et al., 1999a; Venter et al., 2005.)To verify this hypothesis, we need assemble an *Agrobacterium*-mediated transient expression system, similar to that of BMV (Annamalai and Rao, 2006b) that is amenable for dissecting replication from packaging. To this end, we assembled a set of agrobacterium transformants of STMV competent to synthesize either (+) or (-)-strand transcripts when expressed *in planta*. Results showed that (i) co-expression with helper virus TMV, the transiently expressed (+)-strand STMV transcripts are biologically active; (ii) transiently expressed (-)-strand STMV

transcripts also served as templates to initiate (+)-strand synthesis by HV replicase; (iii) most importantly, either mechanical or agroinfiltration of STMV in *Nicotiana benthamiana*, but not in *N. tabacum*, generated a truncated STMV lacking the 3' 150 nt and this truncated form efficiently encapsidated by STMV CP and finally (iv) HV-independent expression of STMV CP was competent to assemble into empty capsids. The significance of these results is briefly discussed below.

Agrobacterium-derived STMV mRNAs having extended 3' non-viral bases are biologically functional

In positive strand RNA viruses, initiation of negative strand synthesis by viral Replicase occurs at the 3' terminus and therefore authentic 3' termini are considered to be crucial for efficient initiation of viral replication. However, this was not case in many RNA viruses. For example, (Dreher et al., 1989)showed that inoculation of whole plants with in vitro transcripts derived from BMV cDNAs having + 11-nt 3' extensions induced infectivity that was comparable to that of wt BMV RNAs. Studies involving DNA-based expression of BMV RNAs in yeast system revealed that initiation of BMV RNA replication from DNAs transformed into yeast cells is highly sensitive to 3' extensions (Ishikawa et al., 1997). These authors observed that in vivo transcripts terminating with either authentic 3' ends (+ 0) or + 3-nt extensions, but not + 19-nt extensions, were found to be efficient templates for negative strand synthesis (Ishikawa et al., 1997). However, as observed with BMV(Annamalai and Rao, 2005) *Agrobacterium* generated STMV

RNAs terminating with + 22-nt 3’ extensions appear to have no effect on initiating negative strand synthesis (Fig. 3B). Taken together, the data suggested that the 3’ extensions required for initiation of RNA synthesis are more relaxed in plant system.

In vivo initiation of STMV replication on an ectopically expressed (-)-strand template.

In (+)-strand RNA viruses, undoubtedly (-)-strand RNAs are the most efficient templates for (+)-strand synthesis, since each (-) strand serves as a template for 100-fold excess of (+) strands (Marsh., et al. 1991; Nassuth., et al. 1983) However, Only in a few cases has either replication (Bal, L.A., 1994; Shaklee et al., 1990; Song, C. & Simon, A. E., 1994) or infectivity (Tousch. D, Jacquemond. M and Tepfe.r M., 1994) been reported to occur from (-)-strand templates. Despite these few reports, for unknown reasons, no further advancement in using (-) strands as templates toward elucidation of viral RNA replication have been made. We provide the following explanations for unsuccessful attempts in previous studies to initiate replication on in vitro-synthesized (-)-strand templates. First, the lack of biological activity of (-)-strand templates may be attributed to extraneous non-viral nucleotides at the termini of (-)-strands (Miller, A and Bujarski, JJ., 1986). Second, some inherent requirements of replication-derived RNA molecules may preclude replication, as in the case of poliovirus (Richards, O.C., Ehrenfeld, E., 1990). Third, procedures

used to deliver (-)-strand RNA templates, either by mechanical inoculation (as in the case of plant viruses) or by transfections, result in uncontrolled cellular partitioning and may either affect the stability (degraded by either RNases or the RNAi pathway) or prevent access to interactions with viral replicase. The precise reasons for successful initiation of replication by STMV replicase from (-)-strand templates delivered by agroinfiltration are currently obscure. It is likely that, compared to mechanical inoculation, agroinfiltration results in the accumulation of much higher concentrations of RNA transcripts of (-)-strand polarity. This would increase the chances for (-) strands to be “seen” and used by HV RdRp. Alternatively, synthesis of RNA transcripts of (-) polarity by the 35S promoter is an exclusively host-controlled process that directs RNA templates to subcellular compartments of the cytoplasm, providing access for viral RdRp to initiate replication. Regarding the delayed onset of replication with STMV (-)-strand templates (Fig. 3B), we provide the following explanation. STMV replication occurs in the cytoplasm. Since agroinfiltration results in continuous synthesis of RNA transcripts up to 7 days, it likely that excessive (-)-strands inhibit (+)-strand synthesis. More experiments are required to substantiate this hypothesis.

Host passage effects of STMV

Prior to this study, STMV inoculations were performed in several *Nicotiana* spp including *N. benthaminana* but no work on the effects of host passage has been studied yet (Rodrigo A.Valverde, 1990). A comparative analysis of STMV

replication in *N. tabcum vs N. benthamiana* revealed that in the later host a truncated form of STMV is consistently generated. Since this truncated form could also be seen in mechanically inoculated plants, we argue that its generation is not an artifact of agroinfiltration. Sequence analysis showed that the truncated STMV lacked 3' 150 nt (STMVΔ150) region encompassing the (-)-strand promoter. Consequently, transient expression of STMVΔ150 in the presence of its HV failed to replicate. However, an interesting outcome is the packaging efficiency of STMVΔ150. For example, although the accumulation level of STMVΔ150 in total RNA was significantly lower than that of in packaged virions (Fig 5A). This suggested that STMV CP has high affinity to package STMVΔ150. Furthermore these results also suggest that packaging signal of STMV is not localized in the 3' 150 nt region. Additional studies are required identify the precise packaging signal(s) distributed on the STMV genomic RNA.

HV-independent expression of STMV CP

STMV was successfully crystallized and its structure was determined at 2.9Å and 1.8Å (Larson et al 1993, 1998). Based on these structural studies it was hypothesized that assembly of STMV virions require nucleic acid (Larson et al, 1998) However, HV-independent expression of STMV in this study proved otherwise. For example, when wild type STMV was expressed in the absence of its HV and virions were fractionated on sucrose density gradient, we observed the presence of STMV virions in the lighter most fractions (Fig 6E). Additional

experiments performed to analyze the contents revealed that virions associated with lighter most fractions are empty. These observations suggested that STMV CP is competent to form empty virions.

In conclusion, this chapter summarizes some of the interesting features associated with STMV that have not been observed before. We attribute this advancement is mainly due to the development of *Agrobacterium*-mediated transient expression system. Chapter 2 represents an extension of this system for analyzing the role of N-proximal region of STMV CP revealed another novel aspect associated with STMV replication.

Figures:

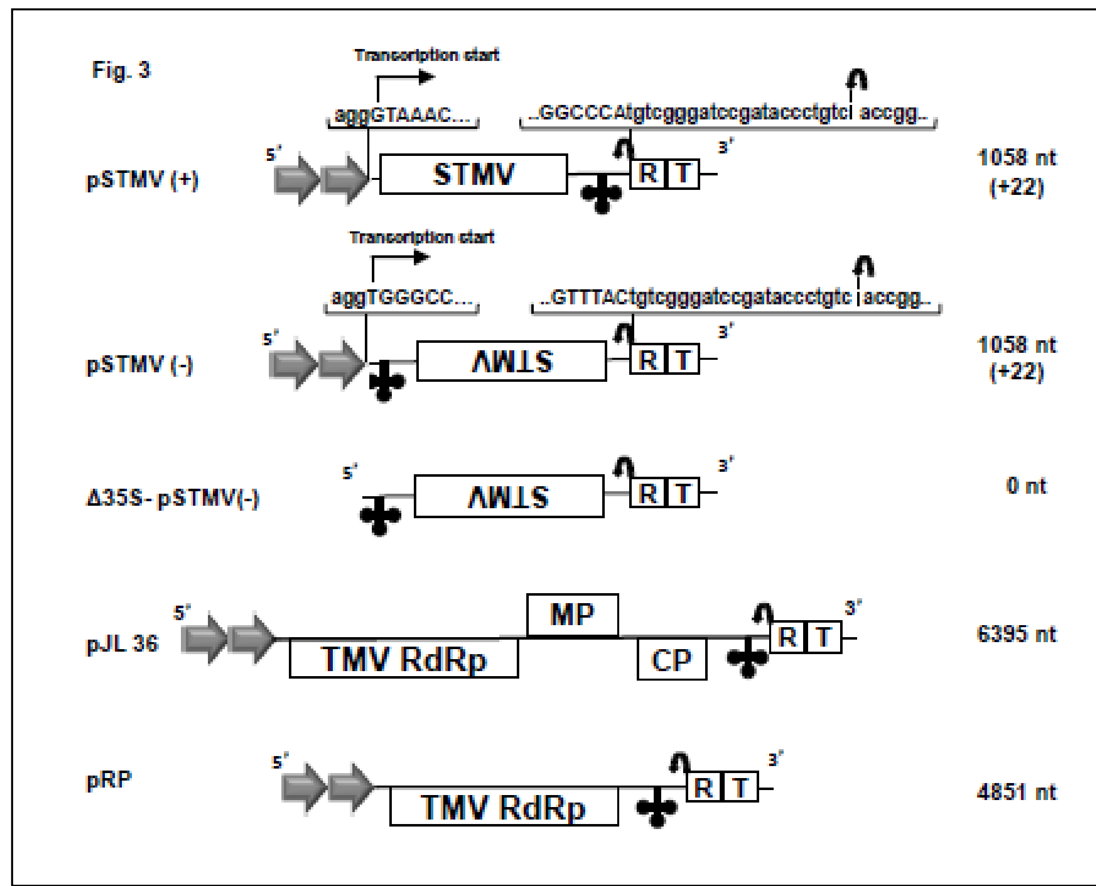


Figure 3: Agroconstructs of STMV and its helper TMV.

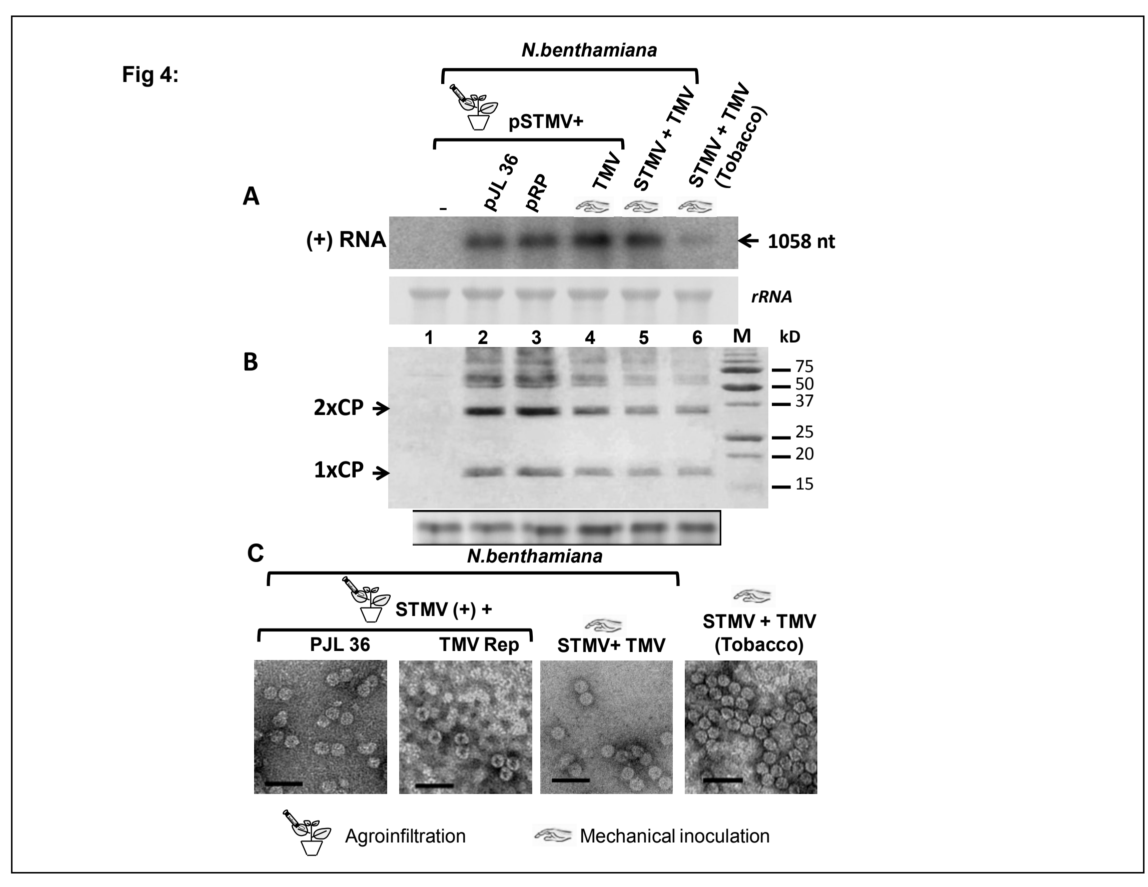


Figure 4: Panel A: Northern blot analysis of agroinfiltrated or mechanically inoculated (hand logo) STMV clones with various helper combinations; Panel B: Western blot analysis of infections established in Panel A using an STMV coat protein specific antibody; Panel C: TEM of virions purified from noted infections.

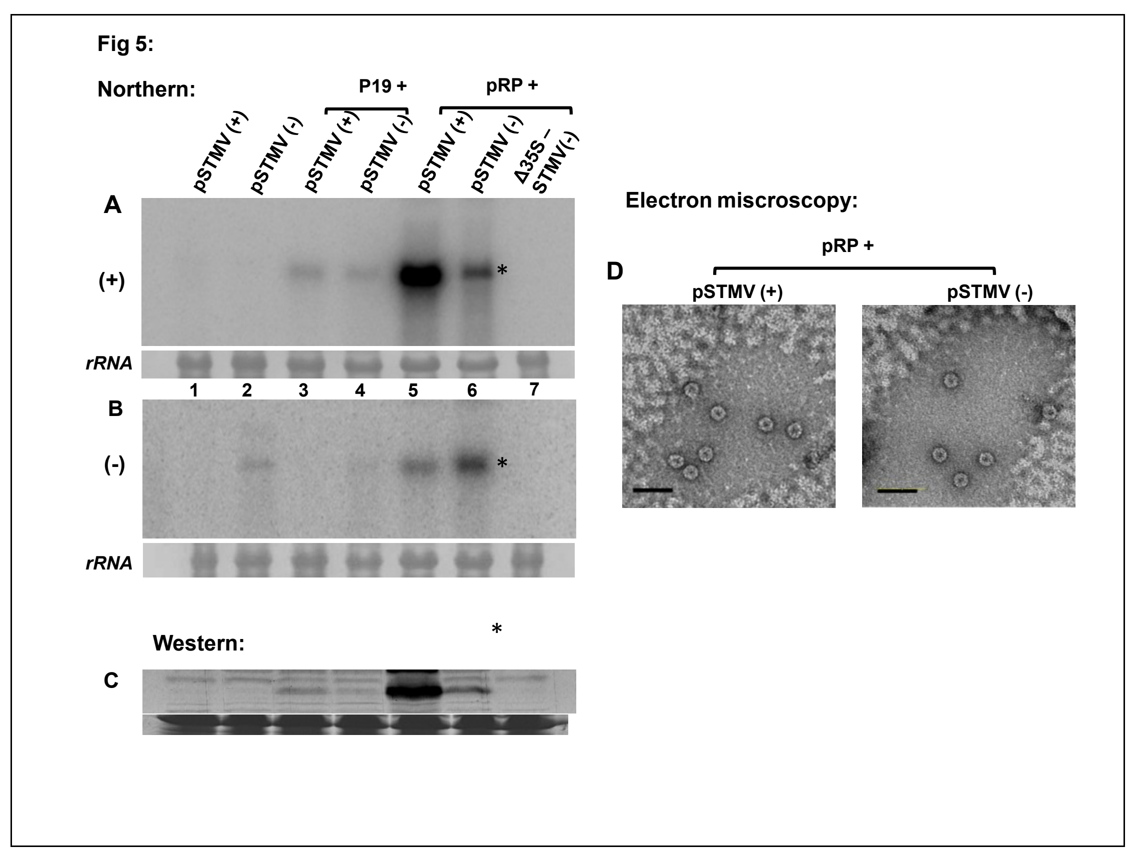


Figure 5: Northern blot analysis using a plus-sense (A) or minus-sense (B) probe for detection of STMV RNA when inoculated with the silencing suppressor p19 or TMV replicase alone; Western blot analysis (C) using an STMV coat protein antibody on the same plants in Panels A and B; Electron microscopy of STMV virions formed when the STMV agroconstructs were inoculated with TMV replicase.

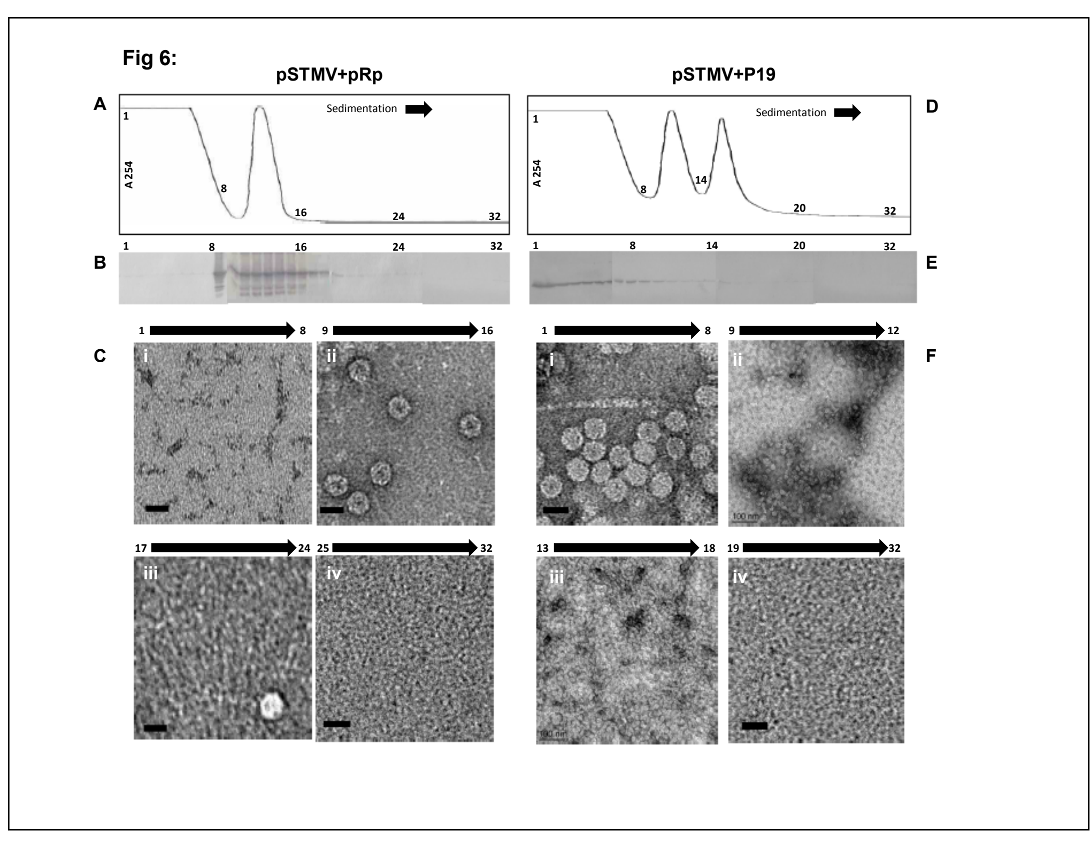


Figure 6: Panel A: UV absorption scan of purified virions showing relative positions of fractions collected; Panel B: Western blot analysis of each fraction from panel A using an STMV coat protein specific antibody; Panel C: TEM analysis of virions found in various fraction pools from panel A.

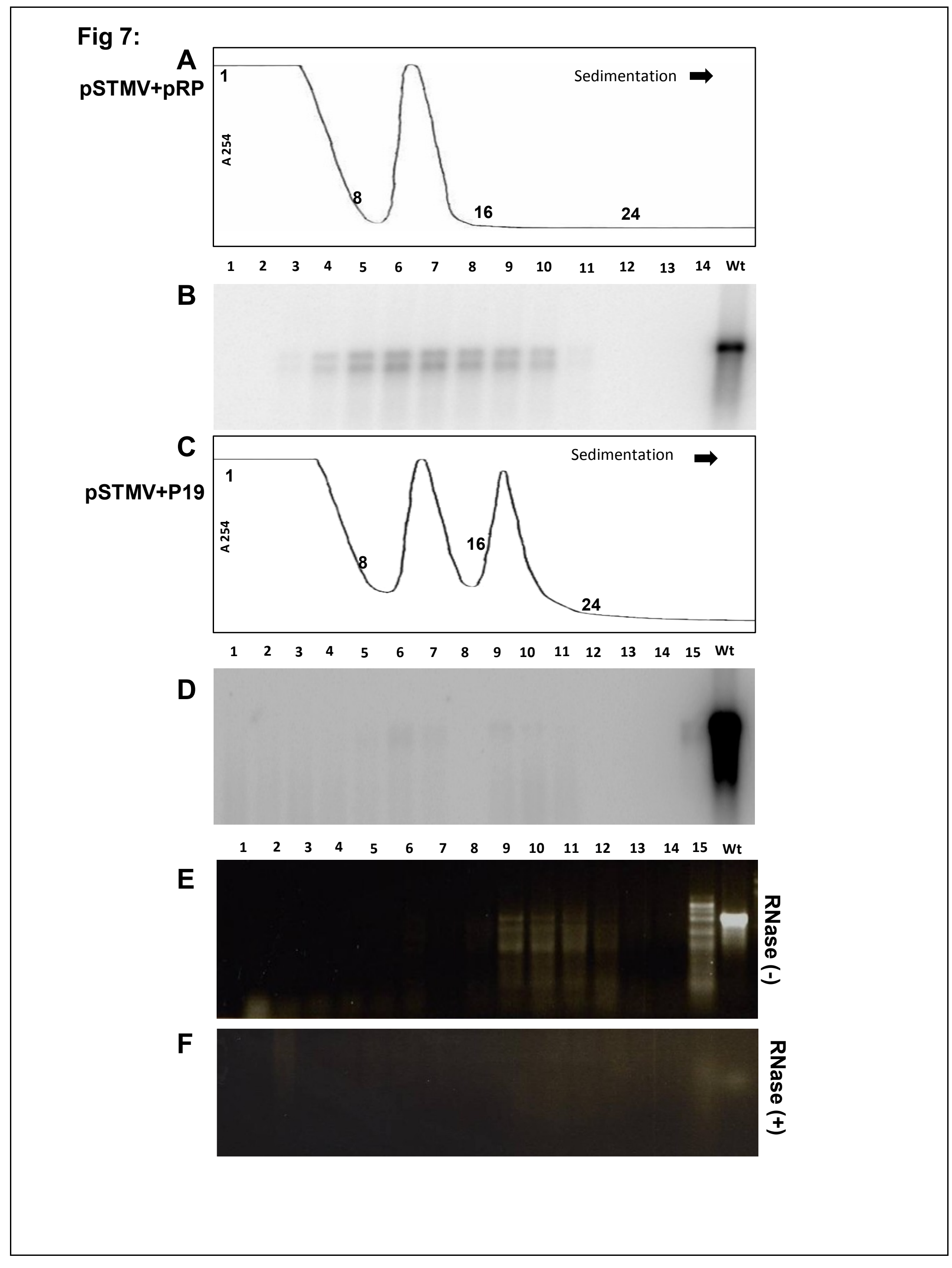


Figure 7: Sucrose gradient sedimentation profiles and fractions collected of virions purified from pSTMV + pRP or pSTMV + p19 infections (A and C). Fractions were pooled pairwise (1+2, 3+4, etc), RNA was extracted from virions and analyzed using Northern blot analysis (B and D) or agarose gel electrophoresis without RNase treatment (E) or with RNase A treatment (F).

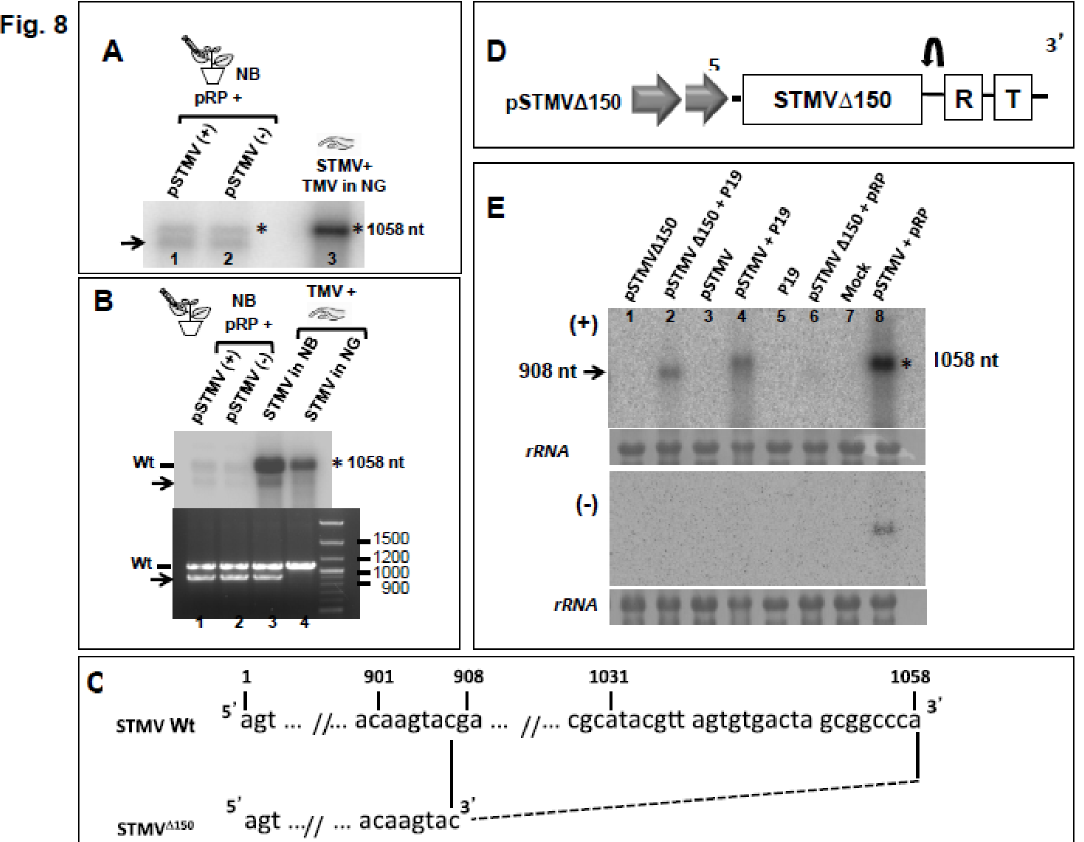


Figure 8: Panel A: Northern blot analysis of RNA purified from pSTMV + TMV-RP infections in *N. benthamiana* (NB) or *N. tabacum* (NT); Panel B: Northern blot and agarose gel electrophoresis of RNAs purified from agroinfiltrations of pSTMV + TMV-RP in NB or STMV virions mechanically inoculated (hand symbol) with TMV in NB or NT; Panel C: comparison of sequences from wild type STMV vs the truncated STMVΔ150; Panel D: agroconstruct of pSTMVΔ150; Panel E: Northern blot analysis using plus-sense or minus-sense probes on various RNAs purified from infections of STMV or STMVΔ150 with or without p19 or TMV-RP.

References:

Annamalai, P., Rao, A.L., 2005. Replication-independent expression of genome components and capsid protein of brome mosaic virus in planta: a functional role for viral replicase in RNA packaging. Virology 338, 96-111.

Annamalai, P., Rao, A.L., 2006a. Delivery and Expression of functional viral RNA genomes in planta by agroinfiltration, in: Downey, T. (Ed.), Current Protocols in MIcrobiology. John Wiley & Sons, New York, pp. p2.1-2.15.

Annamalai, P., Rao, A.L., 2006b. Packaging of brome mosaic virus subgenomic RNA is functionally coupled to replication-dependent transcription and translation of coat protein. Journal of virology 80, 10096-10108.

Annamalai, P., Rofail, F., Demason, D.A., Rao, A.L., 2008. Replication-coupled packaging mechanism in positive-strand RNA viruses: synchronized coexpression of functional multigenome RNA components of an animal and a plant virus in Nicotiana benthamiana cells by agroinfiltration. J Virol 82, 1484-1495.

Ball, L.A., 1994. Replication of the genomic RNA of a positive-strand RNA animal virus from negative-sense transcripts+Proc Natl Acad Sci USA 91:12443–12447+Dodds, J.A., 1998. Satellite tobacco mosaic virus. Annual review of phytopathology 36, 295-310.

Dreher, T.W., Rao, A.L., Hall, T.C., 1989. Replication in vivo of mutant brome mosaic virus RNAs defective in aminoacylation. Journal of molecular biology 206, 425-438.

Erickson, F.L., Holzberg, S., Calderon-Urrea, A., Handley, V., Axtell, M., Corr, C., Baker, B., 1999. The helicase domain of the TMV replicase proteins induces the N-mediated defence response in tobacco. The Plant journal : for cell and molecular biology 18, 67-75.

Hu, C.-C., Hsu, Y.-H., Lin, N.-S., 2009. Satellite RNAs and Satellite Viruses of Plants. Viruses 1, 1325-1350.

Ishikawa, M., M. Janda, M. A. Krol, and P. Ahlquist. 1997. In vivo DNA expression of functional brome mosaic virus RNA replicons in *Saccharomyces cerevisiae*. J. Virol. 717781-7790.

Khromykh, A.A., Varnavski, A.N., Sedlak, P.L., Westaway, E.G., 2001. Coupling between Replication and Packaging of Flavivirus RNA: Evidence Derived from the Use of DNA-Based Full-Length cDNA Clones of Kunjin Virus. Journal of virology 75, 4633-4640.

Kwon, S.J., Rao, A.L., 2012. Emergence of distinct brome mosaic virus recombinants is determined by the polarity of the inoculum RNA. Journal of Virology 86, 5204-5220.

Lakatos, L., Szittya, G., Silhavy, D., Burgyan, J., 2004. Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses. The EMBO journal 23, 876-884.

Larson SB, Day J, Greenwood A, McPherson A.J Mol Biol. 1998 Mar 20; 277(1):37-59.Refined structure of satellite tobacco mosaic virus at 1.8 A resolution.

Larson, S.B., Koszelak, S., Day, J., Greenwood, A., Dodds, J.A., McPherson, A., 1993a. Three-dimensional structure of satellite tobacco mosaic virus at 2.9 A resolution. Journal of molecular biology 231, 375-391.

Lindbo, J.A., 2007. TRBO: a high-efficiency tobacco mosaic virus RNA-based overexpression vector. Plant physiology 145, 1232-1240.

Marsh LE, Huntley CC, Pogue GP, Connell JP, Hall TC. Regulation of (+):(-)-strand asymmetry in replication of brome mosaic virus RNA. Virology. 1991 May;182(1):76–83.

Miller WA, Bujarski JJ, Dreher TW, Hall TC. Minus-strand initiation by brome mosaic virus replicase within the 3' tRNA-like structure of native and modified RNA templates. *J Mol Biol.*1986 Feb 20;187(4):537–546.

Mirkov, T.E., Kurath, G., Mathews, D.M., Elliott, K., Dodds, J.A., Fitzmaurice, L., 1990. Factors affecting efficient infection of tobacco with in vitro RNA transcripts from cloned cDNAs of satellite tobacco mosaic virus. Virology 179, 395-402.

Mirkov, T.E., Mathews, D.M., Du Plessis, D.H., Dodds, J.A., 1989. Nucleotide sequence and translation of satellite tobacco mosaic virus RNA. Virology 170, 139-146.

Nassuth, A., & Bol, J. F., (1983). Altered balance of the synthesis of plus- and minus-strand RNAs induced by RNAs

1 and 2 of alfalfa mosaic virus in the absence of RNA 3. Virology 124, 75-85.Nugent, C.I., Johnson, K.L., Sarnow, P., Kirkegaard, K., 1999a. Functional Coupling between Replication and Packaging of Poliovirus Replicon RNA. Journal of virology 73, 427-435.

Nugent, C.I., Johnson, K.L., Sarnow, P., Kirkegaard, K., 1999b. Functional coupling between replication and packaging of poliovirus replicon RNA. J Virol 73, 427-435.

Osman, F., Grantham, G.L., Rao, A.L., 1997. Molecular studies on bromovirus capsid protein. IV. Coat protein exchanges between brome mosaic and cowpea chlorotic mottle viruses exhibit neutral effects in heterologous hosts. Virology 238, 452-459.

Richards, O.C., Ehrenfeld, E., 1990. Poliovirus RNA Replication, in: Racaniello, V. (Ed.), Picornaviruses. Springer Berlin Heidelberg, pp. 89-119.

Rodrigo A.Valverde, J.A.H.a.J.A.D., 1990. Interactions Between Satellite Tobacco Mosaic Virus, Helper Tobamoviruses, and Their Hosts. Phytopathology, 99-104.

Routh, G., Dodds, J.A., Fitzmaurice, L., Mirkov, T.E., 1995. Characterization of deletion and frameshift mutants of satellite tobacco mosaic virus. Virology 212, 121-127.

Seo, J.K., Kwon, S.J., Chaturvedi, S., Ho Choi, S., Rao, A.L., 2013. Functional significance of a hepta nucleotide motif present at the junction of Cucumber mosaic virus satellite RNA multimers in helper-virus dependent replication. Virology 435, 214-219.

Shaklee PN. Negative-strand RNA replication by Q beta and MS2 positive-strand RNA phage replicases. Virology. 1990 Sep;**178**(1):340–343.

Song, C. & Simon, A. E. (**1994**). RNA-dependent RNA polymerase from plants infected with turnip crinkle virus can transcribe (+)- and (−)-strands of virus-associated RNAs. *Proc Natl Acad Sci U S A* **91**, 8792–8796

Tousch, D., Jacquemond, M. & Tepfer, M. (1994). Replication of cucumber mosaic virus satellite RNA from negative-sense transcripts produced eitherin vitro or in transgenic plants. Journal of General Virology 75, 1009±1014

Valverde, R.A., Dodds, J.A., 1986. Evidence for a satellite RNA associated naturally with the U5 strain and experimentally with the U1 strain of tobacco mosaic virus. Journal of virology 67, 1875-1884.

Valverde, R.A., Dodds, J.A., 1987. Some properties of isometric virus particles which contain the satellite RNA of tobacco mosaic virus. Jornal of General Virology 68, 965-972.

Venter, P. A., N. K. Krishna, and A. Schneemann. 2005. Capsid protein synthesis from replicating RNA directs specific packaging of the genome of a multipartite, positive-strand RNA virus. J. Virol.796239-6248

Chapter 2

Mutations in the N-terminus of the STMV coat protein affect RNA replication and genome packaging

Abstract

STMV infects *N.benthamiana* both by mechanical inoculation and agroinfiltration but interestingly the STMV progeny virions packaged a truncated STMV genomic RNA (STMVΔ150) along with its wt 1058nt genomic RNA (Chapter 1). Like many RNA viruses, the first 13 amino acids of N-terminal region of the STMV coat protein was found to have five positively charged amino-acids at positions 3,5,7,10 & 11 which were thought to interact with highly secondary structured areas of the genomic RNA (Day J Fau - Kuznetsov et al.). Individual substitution of these positively charged amino acids to a neutrally charged amino acid alanine dramatically affected the replication and packaging of these mutant viruses. Substitution at position 3 (from the N-terminal) reduced replication but changes 5,7,10 or 11 increased replication and virions formed by mutants 5A, 7A, 10A and 11A all packaged STMVΔ150 exclusively.

Introduction

Satellite tobacco mosaic virus (STMV) was originally found in association with the t*obacco mild green mosaic virus* (TMGMV or TMV strain U5) in natural infections of tree tobacco (*Nicotiana glauca*) (Valverde and Dodds, 1986). Thus, TMGMV is the designated helper virus (HV) for STMV. However, other tobamoviruses such as TMV U1 and U2 have been shown to also support the replication of STMV (Valverde and Dodds, 1986). The genome of STMV is composed of a single-stranded, positive-sense RNA of 1059 nucleotides (nt) (Mirkov et al., 1989; Valverde and Dodds, 1987). STMV RNA does not show appreciable sequence homology with helper TMV RNA, except for the 3'-terminal 150nt that exhibit 65% homology with the corresponding 3' end of TMV RNA (Mirkov et al., 1989). Given the partial homology between the 3' ends of STMV and TMV RNA, a tRNA-like region of secondary folding capable of accepting a histidine residue was identified (Felden et al., 1994). The genome of STMV is encapsidated into icosahedral virions of 17 nm in diameter and are assembled from 60 identical capsid proteins of 17.5 kDa that encapsidate the genome (Mirkov et al., 1989). STMV virions display a T=1 symmetry (Larson et al., 1993). The three-dimensional structure of the STMV has been determined at 1.8 Å (Larson and McPherson, 2001). A unique feature of the STMV capsid is the presence of 30 RNA double helices, each consisting of 9 base pairs and an unpaired nucleotide at the 3' end of each strand (Larson et al., 1993). Closer

analysis revealed that only 57% of the STMV genome is visible in the crystal structure (Larson et al., 1993).

Although the capsid structure of STMV has been characterized in detail (Larson et al., 1998; Zeng et al., 2012), (Day J Fau - Kuznetsov et al.) the mechanism regulating the genome packaging in STMV, specifically the CP domains involved therein has not been investigated. Capsid proteins of many plant viruses have highly basic (positively charged) N-terminal regions that are thought to interact with viral RNA either during encapsidation or inside the capsid (Rao, 2006). Interestingly, the N-proximal 13 amino acid region of STMV CP is enriched with positively charged residues whose function in STMV packaging has not been determined. It was also predicted that the genomic RNA associates with N-terminal amino acids 2 - 25 (Day, J.F and Kuznetsov et al.) and additionally deletion of CP amino acids except the first 13, allowed the genomic RNA to replicate to wild-type level in *N.benthamiana* protoplasts (Routh et al., 1997). Thus, in this study, we have engineered a series of mutations affecting the positively charged amino acids located within the N-proximal 13 amino acid region, and their effect on replication and *in vivo* packaging was evaluated.

Materials and Methods

Agro-constructs and Agro-infiltration

Agro-constructs of STMV cDNA driven by CaMV 35S promoters (pSTMV), from which biologically active STMV can be transcribed, have been constructed

using the type strain pSTMV6 construct (Mirkov et al., 1990;Kurath et al., 1992) (Chapter 1 of this thesis). All coat protein mutants constructed in this study were incorporated into the genetic background of this plasmid using a mega PCR approach. Briefly, to construct the N-terminal coat protein mutants 3(A), 5(A), 7(A),10(A),11(A), 5,7(A) the CP gene harboring nucleotides (nt)159 to 614 was first amplified with each forward primer (5' TACGTAAACTTACCAATCAAAAG 3') having the single mutation and a commonly shared reverse primer (5' GGCGACTTGTCGACAGTTGC 3'; *Sal* I site is underlined). The resulting PCR products were gel purified and used as megaprimers in a PCR reaction. A region spanning the sequence of pSTMV from 768-614 nt was amplified using a forward primer (5'CGCCAAGCTTGCATGCCTGCAGG3'; *Hind* III site is underlined) and each megaprimer for the respective mutant prepared above. The resulting PCR products were digested with *Hind* III and *Sal* I and subcloned into similarly treated pSTMV. The nature of all recombinant clones was verified by DNA sequencing. As a result of these cloning manipulations, each plasmid contains, in sequential order, a double 35S promoter, cDNA complementary to respective full-length STMV RNAs, a ribozyme sequence of *satellite tobacco ring spot virus* (Rz) and a 35S-terminator (T) (Fig 9B). *Agrobacterium* strain GV3101 containing the desired transformant was initially streaked on an LB plate containing antibiotics (kanamycin 50 mg/ml, rifampicin 10 mg/ml) and incubated at 28 °C. A single colony was inoculated into 2ml LB medium with the above antibiotics, and grown at 28 °C for 48 h with vigorous shaking. One ml of the culture was transferred to

50 ml LB medium containing the above antibiotics, 10 mM MES (pH 5.6) and 40 μM acetosyringone. After incubation at 28 °C for 16 h with vigorous shaking, the OD_{600} of the culture was allowed to reach 1.0. The bacteria were spun down at 2000×g for 10 min, the pellet was resuspended in 50 ml 10 mM MgCl2, and then 125 ml 100 mM acetosyringone was added. To maintain a uniform concentration of *Agrobacterium* in co-infiltration experiments involving two or more *Agrobacterium* cultures, the OD_{600} of each culture was adjusted to 0.5. The final inoculum was prepared by mixing equal volumes of each culture. The bacteria were kept at room temperature for at least 3 h without shaking. These cultures were then infiltrated into abaxial surface of the fully expanded *N. benthamiana* leaves using a $1cm^3$ syringe without a needle, as described previously (Annamalai and Rao, 2006).

STMV virion purification

For virion purification, wild-type and mutant agroconstructs of STMV were co-infiltrated with pRP agroconstruct (TMV Replicase only, see chapter 1) to *N.benthamiana* plants and purified 6 days post infiltration as described previously (Valverde and Dodds, 1987). Approximately, 20 g fresh leaf tissue was ground in a buffer containing 0.2M potassium phosphate buffer, pH 7.2 and 0.4 ml of 2-mercaptoethanol using pestle and mortar and acid washed sand to facilitate maceration of leaf tissue. The extract was then strained through Miracloth, centrifuged at 8000 g for 15 mins, the supernatant is removed carefully and

stirred with 8% (v/v) butanol and centrifuged at 8000 g for 15 min using a JA 20 rotor. The supernatant was adjusted to 8.0% polyethylene glycol (PEG) in 0.1M NaCl and STMV virions were allowed to precipitate overnight at 4ºC. The precipitate was centrifuged at 8000 g for 15 min and the pellet was resuspended in 0.2 ml distilled water. The suspension was centrifuged at 10,000 g for 10 mins to remove undesired plant material. The supernatant containing STMV virions was further purified by rate zonal sucrose density gradients (10-40% sucrose prepared in water). Absorbance profiles were recorded at 254nm and the peak corresponding to the STMV virions was collected, diluted with water and concentrated by centrifuging at 90,000 rpm for 3 hours. The final pellet was resuspended in water and virion concentration was determined using 6 as the extinction coefficient of STMV (J. A. Dodds, unpublished results).

Progeny analysis and packaging assays

Total RNA from agroinfiltrated leaves was extracted using the TRIZOL method and the RNA pellet was suspended in RNAse-free water. Virions were purified from agroinfiltrated leaves as described previously (Valverde and Dodds, 1987). For Northern blot analysis (Annamalai and Rao, 2005) total RNA (5 µg) or virion RNA (0.5 µg) recovered from either leaf discs (20mm diameter) or infiltrated leaves was dried in a microfuge and suspended in 10 µl of sample buffer (10× MOPS buffer/formamide/formaldehyde/H2O in a ratio of 1:1.8:5:2.2, respectively). The reaction mixture was heated at 65 °C for 10 min and subjected

to electrophoresis in a 1.5% agarose–formaldehyde gel (Sambrook and Russel, 2001). Following a 3 hr electrophoresis, fractionated RNA was transferred to a nylon membrane with a VacuGene XL blotting unit (Pharmacia Biotech). The blot was then processed for prehybridization and hybridization using ^{32}P-labeled riboprobes corresponding to STMV CP ORF and the 3′ conserved tRNA-like structure (TLS) of BMV (Rao et al., 1989). STMV CP samples were analyzed by Western blot analysis as described previously (Osman et al., 1997). For electron microscopy analysis, purified virions are further subjected to 10%-40% sucrose gradient centrifugation and spread on glow-discharged grids followed by negative staining with 1% uranyl acetate prior to examining with a FEI Tecnai12 transmission electron microscope (Annamalai and Rao, 2005). Images were recorded digitally.

RT-PCR

Virion RNA from wild type and CP mutants were subjected to Poly–A tailing using *E.coli* poly A polymerase according to the manufacturer's protocol (NEB). Following inactivation of poly A polymerase by heat (95ºC for 2 to 3 minutes), first strand cDNA was synthesized using a reverse primer (5' GGGAGGACACAGCCAACA<u>TACGTA</u>TTTTTTTTTTTTTTTTTTTT 3'; *Sna*B I site is underlined) and M-MulV reverse transcriptase (New England Biolabs). The resulting product was subjected to PCR using a forward primer (5' TACGTAAACTTACCAATCAAAAG 3') and a reverse primer

(5'GGGAGGACACAGCCAACATACGTA3'; *Sna*B I site is underlined). PCR products were finally analyzed by agarose gel (1%) electrophoresis and visualized and photographed under UV light following ethidium bromide staining.

Biological activity of STMV mutant virions

The biological activity of purified STMV wild type and mutant virions was tested by mechanically inoculating their Virion RNA to the leaves of *N.benthamiana* along with TMV U5 (HV) RNA. Total RNA was isolated from-inoculated leaves at 7 dpi and subjected to Northern blot analysis as described above.

Results

Replication and encapsidation of CP N-terminal mutants

To ascertain the importance of positively charged 6 amino acids located within the N-proximal 13 amino acid region (Fig. 9A), each of the two arginine (at positions 3 and 10) and three lysine (at positions 5, 7 and 10) residues was substituted with an alanine residue (Fig. 9B) into the genetic background of pSTMV, a biologically active agrotransformant of STMV (Chapter 1). Each mutant agrotransformant was co-infiltrated with pRP (an agrotransformant engineered to provide TMV RdRp; see Chapter 1) in *N. benthamiana* leaves and progeny RNA was subjected to Northern blot hybridization with riboprobes specific for STMV (+) or (-)-strands. Results are shown in Fig. 10. As expected, compared to

healthy (Fig. 10A, lane 1) or empty vector (Fig. 10A, lane 7), control infiltrations performed with wild type pSTMV resulted in the accumulation of progeny (+) and (-)-strands (Fig. 10A and B, lane 8). Interestingly, contrasting RNA profiles resulting from both down and up regulation of progeny was seen for each mutant. For example, mutation of arginine at the 3rd position down regulated progeny accumulation of STMV (+)-strand synthesis by 69% (Fig. 10A, lane 2) although no significant reduction in its corresponding (-)-strand was observed (Fig. 10B, lane 2). Whereas, for the remaining set of four mutants (5A, 7A, 10A and 11A), depending on the location of the engineered mutation, a 2-4 fold up regulation in the accumulation of (+)-strand was observed (Fig. 10A, compare lanes 3-6). Similar up-regulation in (-)-strand synthesis for each of these four mutants was observed (Fig. 10B, compare lanes 3-6). These observations suggest that engineered mutations specifically affected (+)-strand synthesis (see Discussion).

A western blot assay using anti-STMV CP antibody was performed to monitor the accumulation levels of CP in leaves infiltrated with mutant inocula. A correlation between (+)-strand accumulations for each mutant and the corresponding CP synthesis was observed (Fig. 11A). For example, compared to wt (Fig. 11A, lane 8), up-regulation of CP synthesis was observed for mutants 5A, 7A, 10A and 11A (Fig. 11A, lanes 3-6) while no CP was detected for the 3A mutant (Fig. 11A, lane 2). Consistent with an increase in CP synthesis, virion yield for mutants 5A, 7A, 10A and 11A was higher than wt (Fig. 11B).

Assembled virions exhibit unusual packaging profile

Next, to verify the virion formation in each case, partially purified virions were subjected to EM analysis. Results are shown in Fig. 11C. Absence of CP correlated with failure to recover any virions for mutant A (Fig. 11A). The CP of the remaining four mutants is competent to assemble into virions that are indistinguishable from that of the wt control (Fig. 11C). Finally, to verify the nucleic acid composition of the assembled virions, RNA was isolated and subjected to Northern blot hybridization using riboprobes specific for STMV (+)-strands. Results are shown in Fig. 11D. As shown under Chapter 1, virions purified from wild type control infiltrations contained full length as well as truncated RNA species referred to STMVΔ150 (Fig. 11D, lane 6). Despite efficient synthesis of full length STMV RNA (Fig. 10A), virions of 5A, 7A, 10A and 11A mutants packaged exclusively STMVΔ150 (Fig. 11D, lanes 2- 6). To confirm the absence of full length STMV RNA, two additional experiments were performed. In the first experiment, virion RNA of wt and each mutant was mixed with HV RNA and mechanically co-inoculated to *N. benthamiana* plants and at 7 dpi total RNA was subjected to Northern blot hybridization. Results are shown in Fig. 12A. As demonstrated previously (Chapter 1), only full length STMV RNA was capable of replicating in the presence of its helper replicase, hence viron RNA from wt were able to replicate and make progeny RNA (Fig. 12A, lane 6) while such progeny STMV RNA were not detected in leaves infiltrated with five CP mutant virion RNA (Fig. 12A, lanes 1-5). These results confirmed that none of mutant virions

packaged infectious full length STMV RNA. In the second assay, virion RNA from each sample shown in Fig. 11D was subjected to poly (A) tailing followed by RT-PCR as described under Materials and Methods. Results shown in Fig. 12B, confirmed that virions of mutants 5A, 7A, 10A and 11A packaged only the truncated STMVΔ150 (Fig. 12B, compare lanes 2 to 5). Taken together these results demonstrate that the mutant CPs examined here are competent to package truncated STMVΔ150 but not full length STMV RNA.

Replication and packaging phenotype of variant 5/7A

Results shown in Fig. 10 demonstrate that mutation of either 5K➔5A or 7K➔5A up-regulated STMV replication. To verify the concurrent effect of these two mutations, another variant with changes at both positions referred to as 5/7A (Fig. 9B) was created and co-expressed *N. benthamiana* with pRP. Results of Northern blot hybridization are shown in Fig. 13A. Unlike individual mutations, concurrent mutation of the 5K and 7K to alanine residues (i.e. 5/7A) down regulated STMV replication by 40% of wt (Fig. 13A). Western blot hybridization showed that variant 5/7A is competent to translate STMV CP (Fig. 13C). To verify the packaging phenotype for variant 5/7A, RNA was isolated from purified virions and subjected to Northern blot hybridization. The packaging phenotype for variant 5/7A was indistinguishable from that of either 5A or 7A i.e. only the

truncated STMV Δ150 was packaged (Fig. 13D). RT-PCR assays further confirmed that like 5A or 7A, 57A was defective in packaging STMV RNA (Fig. 13E).

Discussion:

In this study we analyzed the role of six positively charged amino acids located within the N-proximal region of STMV CP in packaging. The data revealed the following: (i) an arginine residue at the third position is critical since 3R➔3A substitution down regulated STMV replication; (ii) whereas 5K➔5A, 7K➔7A, 10R➔10A or 11K➔11A up regulated STMV replication and (iii) each CP mutant exclusively packaged the truncated form of STMV (i.e. STMVΔ150). The significance of these observations is discussed below.

Role of CP in STMV replication

In all positive-strand RNA viruses the main function of the CP is to protect the replicated progeny from cellular nucleases by encapsidation into stable virions. Nevertheless, viral CPs have been found to be multifunctional by regulating various aspects of the virus life cycle including strand asymmetry, symptom expression, cell-to-cell and long distance spread. With regards to encapsidation, N-proximal positively charged amino acids have been shown to affect genome packaging by interacting with negatively charged RNA. Surprisingly, CP mutants examined in this study revealed an unexpected facet

associated with STMV. For example, a 3R➔3A substitution in mutant 3A severely down regulated STMV replication (Fig.10A, B). This cannot be attributed to the RNA stability since detectable levels of progeny (-)-strand RNA were accumulated (Fig 10B). Likewise, compared to wild type, (+)-and (-)-strand synthesis was significantly up regulated by CP mutants 5A, 7A, 10A and 11A (Fig.10A, B). This up-regulation was reversed by the double mutant 5/7A (Fig 13A, B). Collectively these data suggest that CP plays an important role in STMV replication. Although the mechanism of CP-mediated STMV replication is currently obscure, it is likely that STMV CP plays an integral role in the ability of HV replicase to replicate STMV RNA.

Packaging of STMV

In eukaryotic RNA viruses, the most thoroughly characterized packaging signal is that of *tobacco mosaic virus*, a helical RNA plant virus in which the specific interaction between the CP and a 69-nucleotide (nt) region internal to the RNA genome leads to the specificity of virion assembly. Among viruses with icosahedral symmetry, RNA elements that act as specific packaging signals have been characterized for enveloped

animal viruses such as coronaviruses, and alphaviruses, human and murine retroviruses and for the nonenveloped plant viruses *Turnip crinkle* and *Brome mosaic viruses*. Although STMV is structurally well characterized, sequences functioning as packaging signals have not been identified. It is interesting to note

that CP mutants examined in this study have a propensity to preferentially package STMVΔ150 but not the wild type. As hypothesized under Chapter 1, if the 3' 150 nt sequences are not required by STMV, then why was the wild type genomic RNA was not packaged by each of the mutant CPs? RNA features that specifically interact with CP to mediate the assembly of icosahedral virions have been described for retroviruses, *Sindbis virus* and *turnip crinkle virus* (Berkowitz, 1996; Wei and Morris., 1991; Wei, N., 1990; Weiss, B., 1994). Such specific interactions are envisioned to play a significant role in selectively removing viral RNAs from the pool of heterologous host RNAs present in the cytoplasm. Keeping these results in perspective, we offer the following likely scenarios for the packaging phenotypes exhibited by STMV CP mutants. *First*, it is likely that mutant STMV RNA and its CP could have been compartmentalized differently than wild type. *Second*, the structure of the mutant CP could be different from that of wild type, exhibiting high affinity to mutant RNA. *Third*, if packaging of STMV is structure based rather than sequence specific packaging signals, the structures of mutant sequences are favorable for packaging over wild type. Additional studies are required to substantiate these predicted scenarios.

Figures:

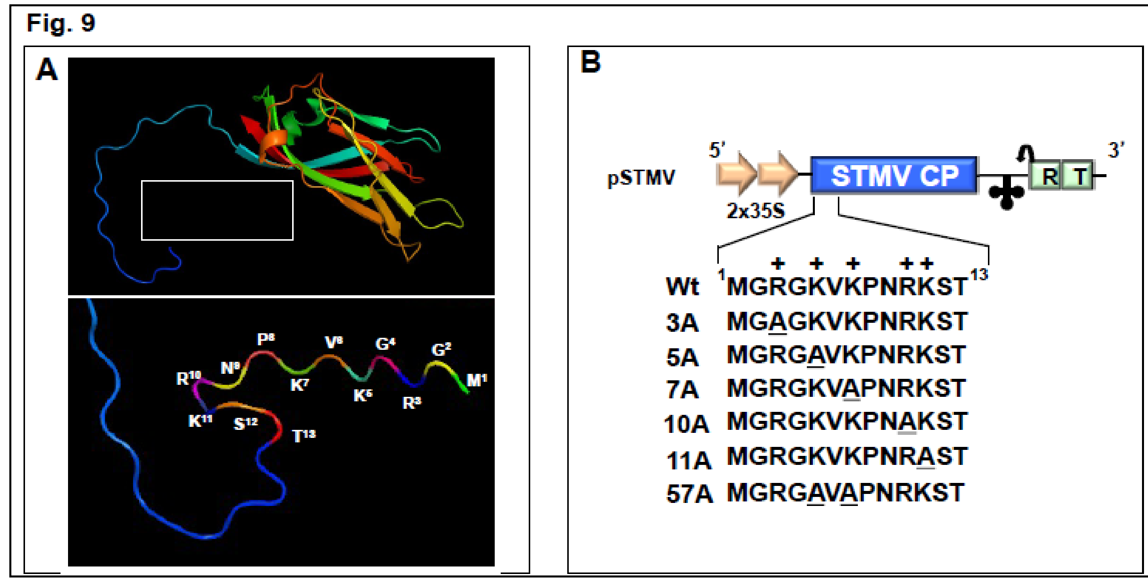


Figure 9: Proposed protein folding model (A) with the area of interest for mutation highlighted. Positions of mutations for positively charged amino acids (+) and their resulting clones changed to the neutral amino acid alanine (underlined, B)

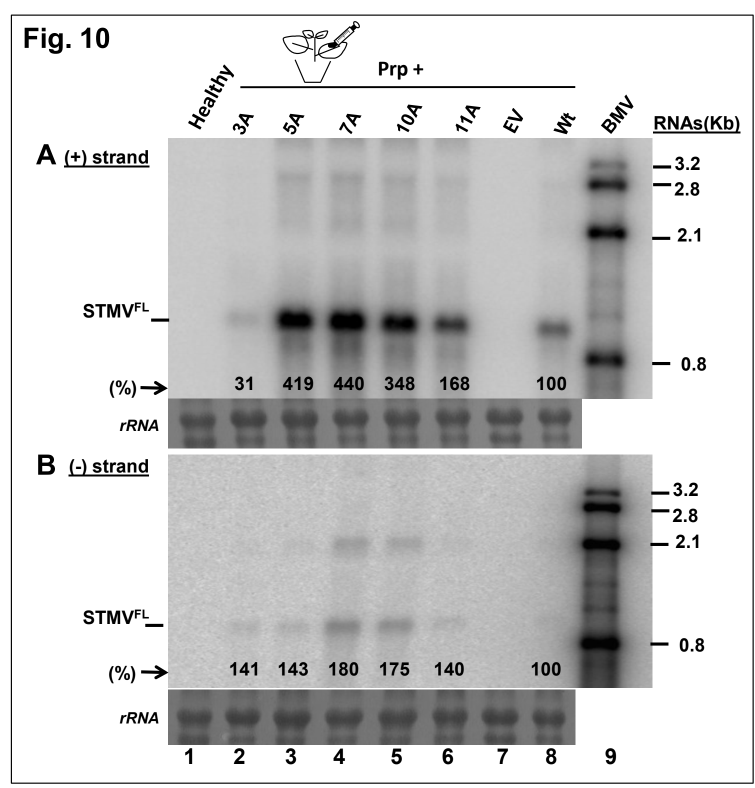


Figure 10: Northern blot analysis (using + or - sense probes) of total RNA purified from agroinfiltrated infections of each amino acid mutant (3A-11A) with TMV-RP compared to infections of pSTMV (Wt). BMV

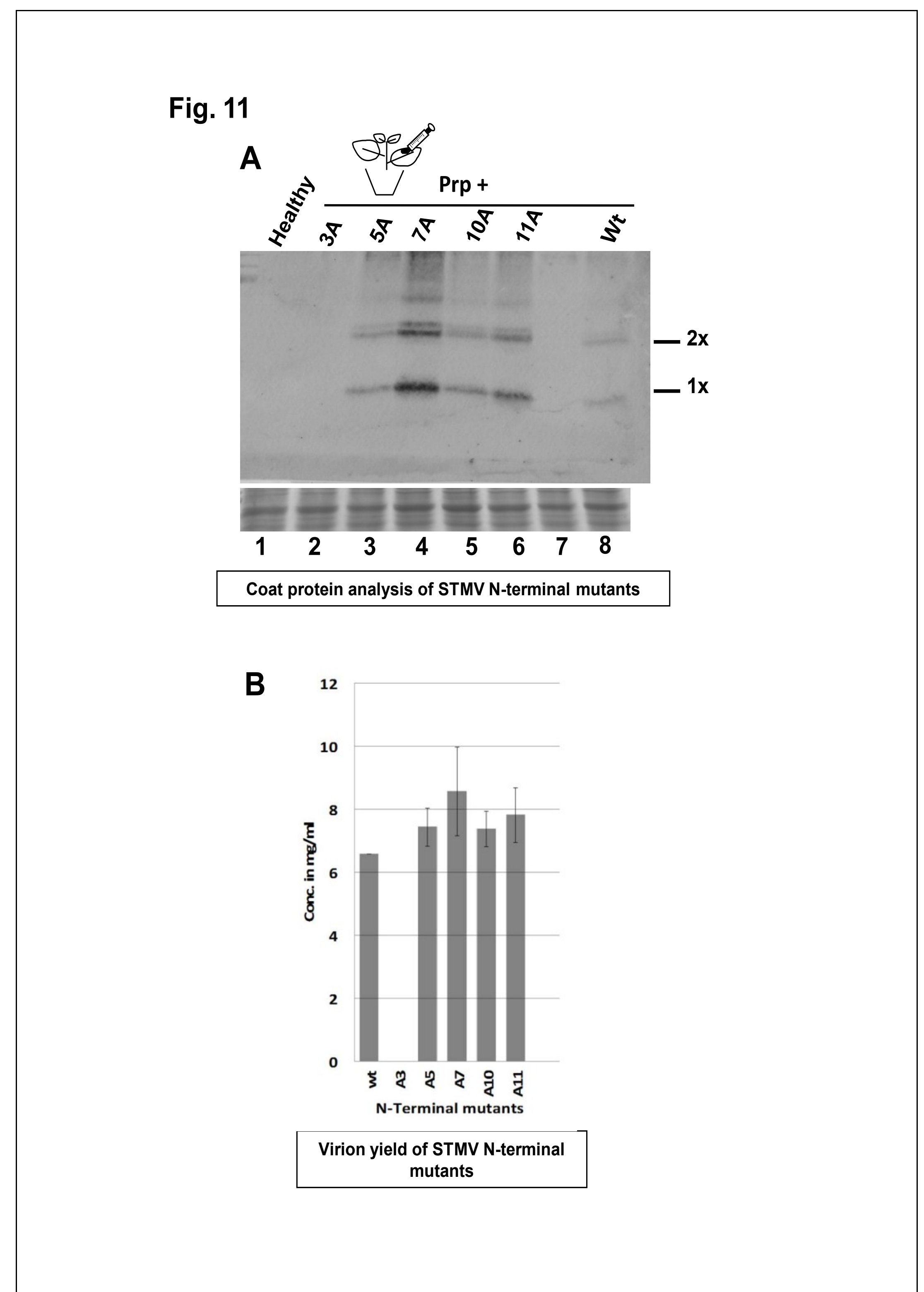
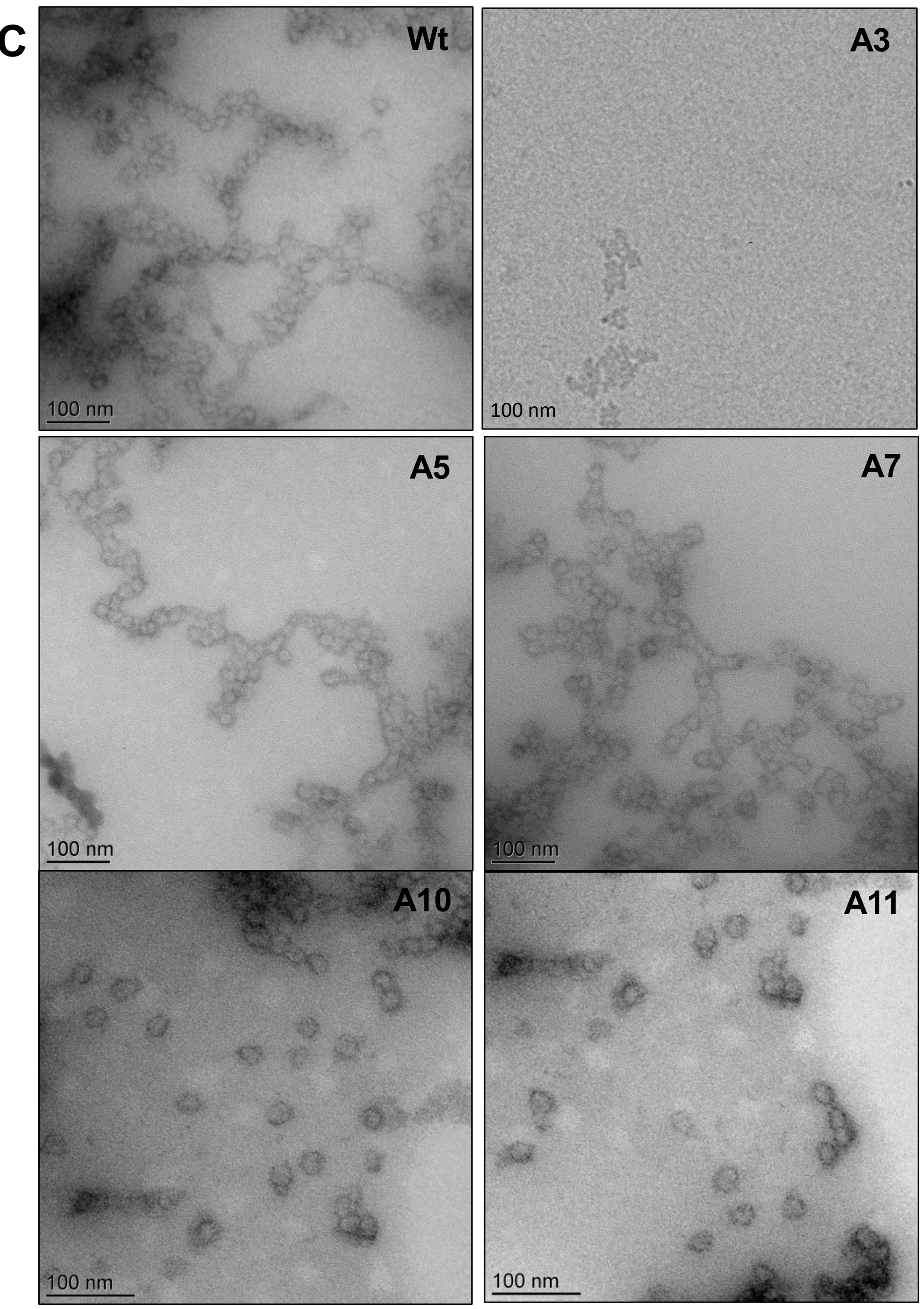
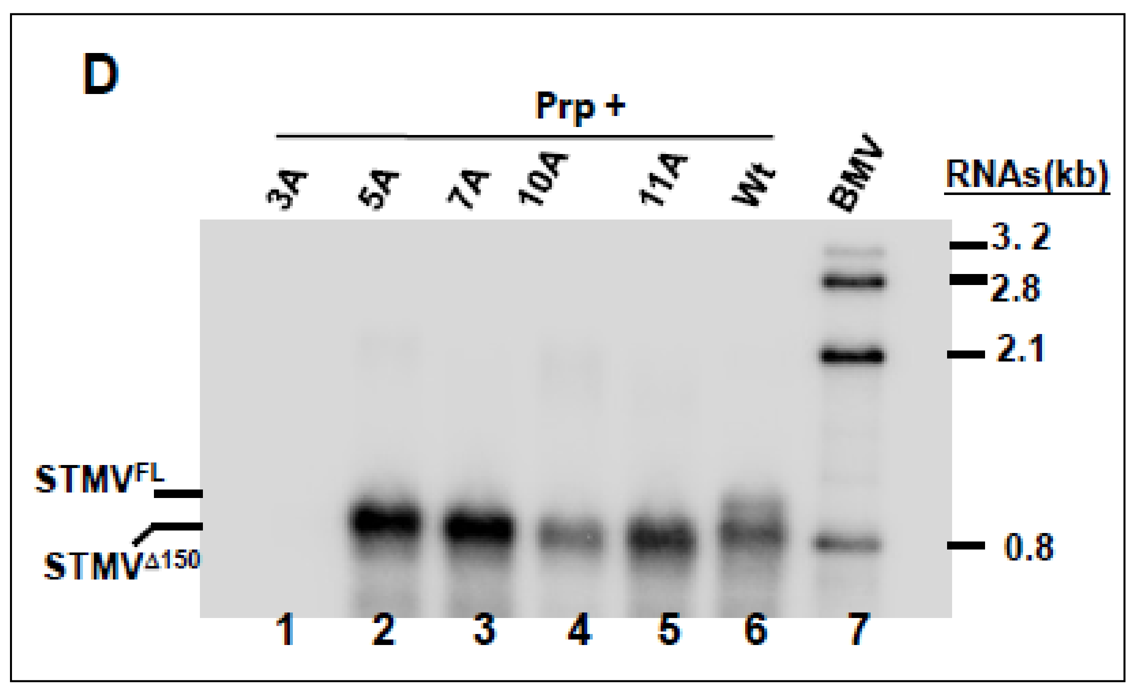


Fig. 11
A
Healthy
Prp +
3A
5A
7A
10A
11A
Wt
2x
1x
1
2
3
4
5
6
7
8
Coat protein analysis of STMV N-terminal mutants
B
12
10
8
6
4
2
0
Conc. in mg/ml
wt
A3
A5
A7
A10
A11
N-Terminal mutants
Virion yield of STMV N-terminal mutants



Electron microscopy of STMV N-terminal mutants



Virion RNA analysis of STMV N-terminal mutants

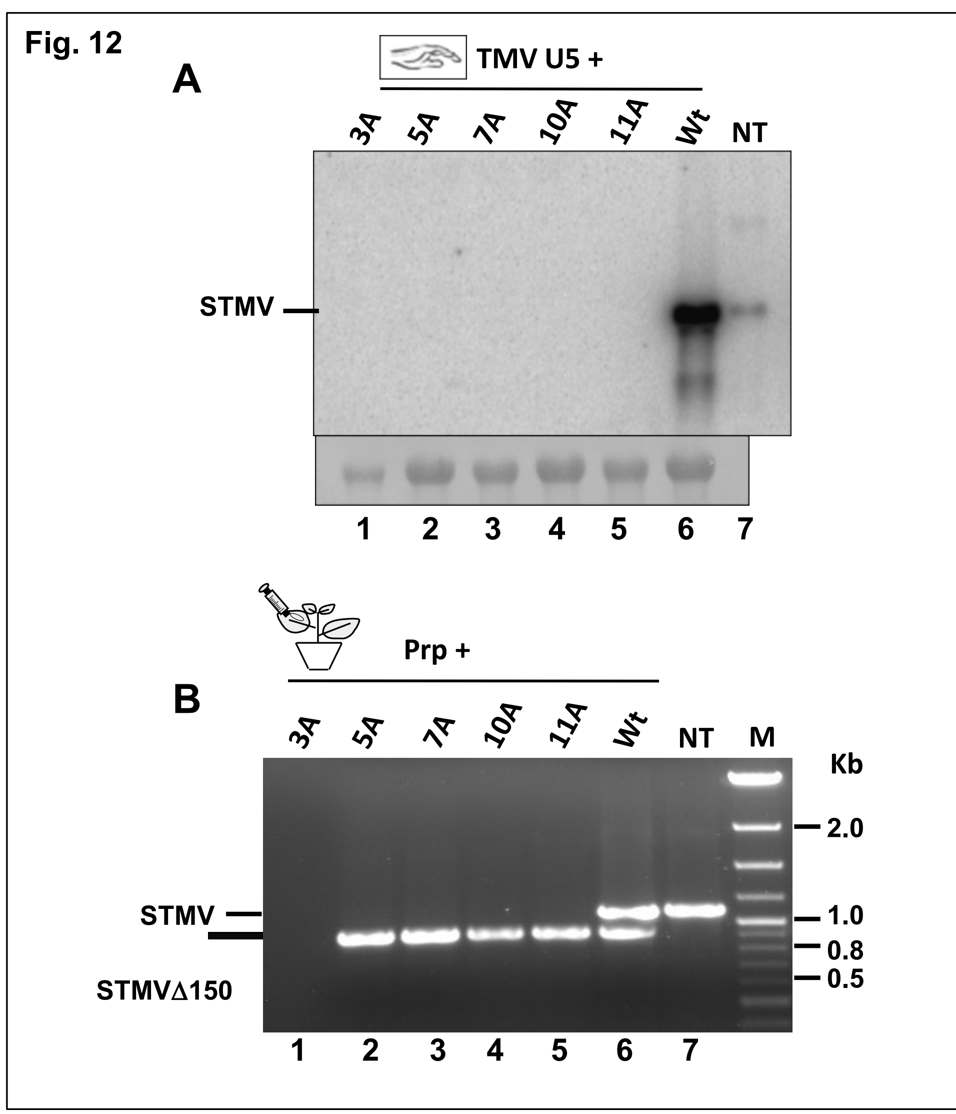


Figure 12: Northern blot (A) and RT-PCR analysis (B) of RNAs purified from mutant and wild type STMV + TMV-RP agroinfiltrated virions in *N. benthamiana* compared to a wild type infection in *N. tabacum* (NT)

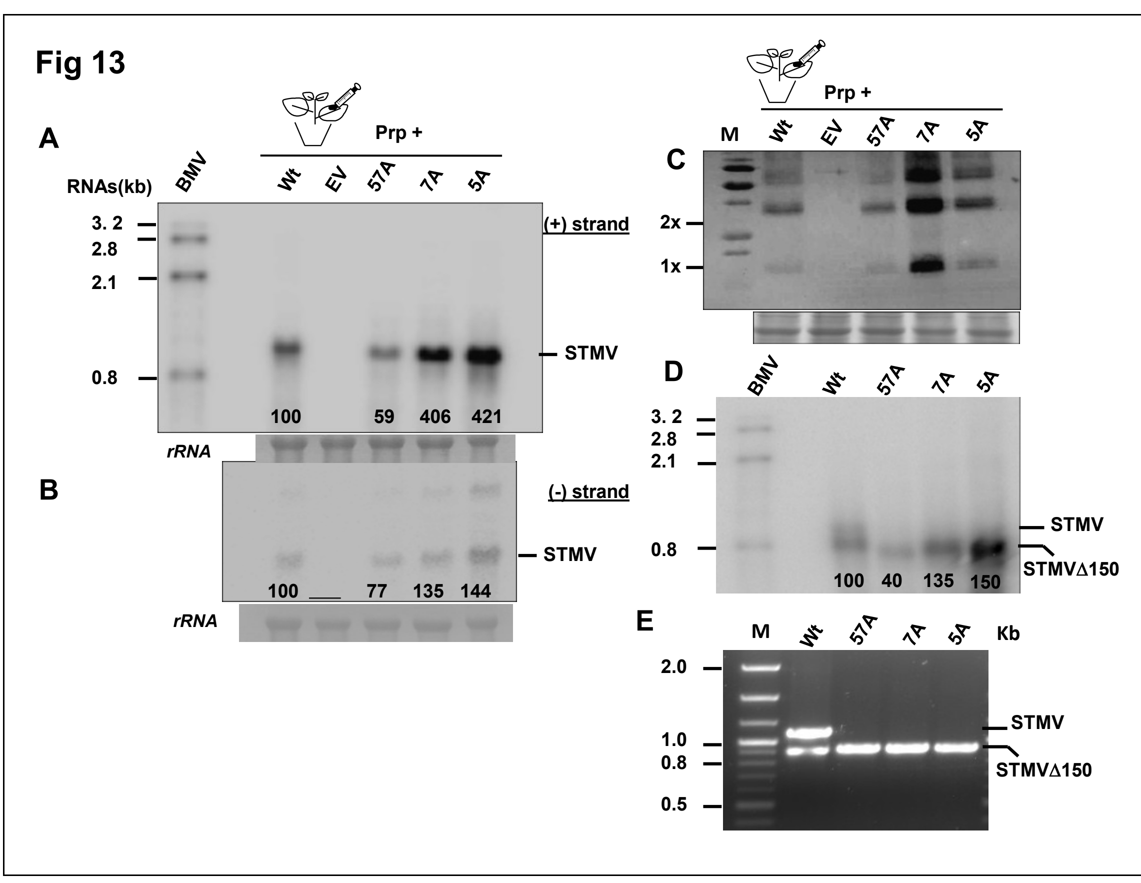


Figure 13: **Relative accumulations of STMV RNA (A, B and D) and CP (C) from agroinfections of mutants 5A, 7A or 5/7A (both mutations in the same clone) compared to wild type STMV. WT STMV was set at 100% and numbers on lanes reflect a relative increase or decrease of that value. Panel E is an ethidium bromide stained agarose gel of each RNA sample.**

References

Annamalai, P., Rao, A.L., 2005. Replication-independent expression of genome components and capsid protein of brome mosaic virus in planta: a functional role for viral replicase in RNA packaging. Virology 338, 96-111.

Annamalai, P., Rao, A.L., 2006. Packaging of brome mosaic virus subgenomic RNA is functionally coupled to replication-dependent transcription and translation of coat protein. Journal of virology 80, 10096-10108.

Berkowitz, R., J. Fisher, and S. P. Goff., 1996. RNA packaging. Curr. Top. Microbiol. Immunol. 214, 177–218.

Day J., Kuznetsov, Y.G., Kuznetsov Yg Fau - Larson, S.B., Larson Sb Fau - Greenwood, A., Greenwood A - McPherson, A., Biophysical studies on the RNA cores of satellite tobacco mosaic virus.

Felden, B., Florentz, C., McPherson, A., Giege, R., 1994. A histidine accepting tRNA-like fold at the 3'-end of satellite tobacco mosaic virus RNA. Nucleic acids research 22, 2882-2886.

Kurath, G., Rey, M.E., Dodds, J.A., 1992. Analysis of genetic heterogeneity within the type strain of satellite tobacco mosaic virus reveals variants and a strong bias for G to A substitution mutations. Virology 189, 233-244.

Larson, S.B., Day, J., Greenwood, A., McPherson, A., 1998. Refined structure of satellite tobacco mosaic virus at 1.8 A resolution. Journal of molecular biology 277, 37-59.

Larson, S.B., Koszelak, S., Day, J., Greenwood, A., Dodds, J.A., McPherson, A., 1993. Double-helical RNA in satellite tobacco mosaic virus. Nature 361, 179-182.

Larson, S.B., McPherson, A., 2001. Satellite tobacco mosaic virus RNA: structure and implications for assembly. Curr Opin Struct Biol 11, 59-65.

Mirkov, T.E., Kurath, G., Mathews, D.M., Elliott, K., Dodds, J.A., Fitzmaurice, L., 1990. Factors affecting efficient infection of tobacco with in vitro RNA transcripts from cloned cDNAs of satellite tobacco mosaic virus. Virology 179, 395-402.

Mirkov, T.E., Mathews, D.M., Du Plessis, D.H., Dodds, J.A., 1989. Nucleotide sequence and translation of satellite tobacco mosaic virus RNA. Virology 170, 139-146.

Osman, F., Grantham, G.L., Rao, A.L., 1997. Molecular studies on bromovirus capsid protein. IV. Coat protein exchanges between brome mosaic and cowpea chlorotic mottle viruses exhibit neutral effects in heterologous hosts. Virology 238, 452-459.

Rao, A.L., 2006. Genome packaging by spherical plant RNA viruses. Annu Rev Phytopathol 44, 61-87.

Rao, A.L., Dreher, T.W., Marsh, L.E., Hall, T.C., 1989. Telomeric function of the tRNA-like structure of brome mosaic virus RNA. Proceedings of the National Academy of Sciences 86, 5335-5339.

Routh, G., Yassi, M.N., Rao, A.L., Mirkov, T.E., Dodds, J.A., 1997. Replication of wild-type and mutant clones of satellite tobacco mosaic virus in Nicotiana benthamiana protoplasts. The Journal of general virology 78 (Pt 6), 1271-1275.

Valverde, R.A., Dodds, J.A., 1986. Evidence for a satellite RNA associated naturally with the U5 strain and experimentally with the U1 strain of tobacco mosaic virus. Journal of virology 67, 1875-1884.

Valverde, R.A., Dodds, J.A., 1987. Some properties of isometric virus particles which contain the satellite RNA of tobacco mosaic virus. Jornal of General Virology 68, 965-972.

Wei N, Heaton LA, Morris TJ, Harrison SC. Structure and assembly of turnip crinkle virus. VI. Identification of coat protein binding sites on the RNA. J Mol Biol. 1990 Jul 5;214(1):85–95.

Wei, N., Morris, T.J., 1991. Interactions between viral coat protein and a specific binding region on turnip crinkle virus RNA. Journal of molecular biology 222, 437-443.

Weiss, B., Geigenmuller-Gnirke, U., and Schlesinger, S. 1994. Interactions between Sindbis virus RNAs and a 68 amino acid derivative of the viral capsid protein further defines the capsid binding site. Nucleic Acids Res. 22: 780–786.

Zeng, Y., Larson, S.B., Heitsch, C.E., McPherson, A., Harvey, S.C., 2012. A model for the structure of satellite tobacco mosaic virus. J Struct Biol 180, 110-116.

Chapter 3

Studies on *Tomato aspermy virus* in *Nicotiana benthamiana* by *in vivo* expression of their genomes by Agro-infiltration.

Abstract

Studies on *Tomato aspermy virus* were done by mechanically inoculating either the RNA transcripts or cDNAs of the three genomic segments on plants, and for synchronous delivery of all three genomes to plants, agroconstructs of the V strain of TAV (V-TAV) were made. Results obtained after agroinfiltration of these agroconstructs to leaves of *Nicotiana benthamiana* were the same as previously reported. However we are additionally reporting symptom modification on the systemic leaves of *N.benthamiana* and a reduction of TAV replication in the presence of the satellite RNA of Q strain of Cucumber mosaic virus.

Introduction:

Tomato aspermy virus (TAV) which was first reported in 1946 by Caldwell in infected chrysanthemum stocks belongs to the family *Bromoviridae* and is similar to *Cucumber mosaic virus* (CMV) in host range and symptom expression, vector and physical properties (Stace-Smith and Tremaine, 1973). It is a tripartite virus (3 particle) that has three single stranded positive sense genomic RNAs

namely RNA1 (TAV1) of 3410nt in one separate virion particle, RNA2 (TAV2) of 3074nt in another separate virion particle and RNA3 (TAV3) of 2386nt and subgenomic RNA4 (sgTAV4) in a third particle. The 3' terminal 200nt of TAV RNA 1, 2 and 3 are homologous . These three particles are identical in size and density.

Viral RNA replication is dependent on efficient interaction between two non-structural proteins, 1a and 2a, encoded by monocistronic RNAs1 and 2, respectively (Bernal et al., 1991; Moriones E Fau - Roossinck et al.). Non-structural protein 1a (111.9 kDa) contains a methyltransferase/guanylyltransferase domain in its N-terminal half and a helicase domain in its C-terminal half while non-structural protein 2a (93.4 kDa) contains central polymerase-like domain (Hayes Rj Fau - Buck and Buck; Nitta et al., 1988; Rezaian Ma Fau - Williams et al.; Rezaian Ma Fau - Williams et al.). The RNA 2 of TAV encodes an overlapping gene (2b) of 11.2 kDa in addition to the 2a gene(Shi Bj Fau - Ding et al.). These replication proteins share amino acid sequence similarity with proteins encoded by many morphologically and genetically diverse (+) stranded RNA viruses of plants (O'Reilly D Fau - Thomas et al.). Genomic RNA3 encodes a non-structural movement protein 3a (MP) of 30.4 kDa and a capsid protein (CP) of 24 kDa (Salanki K Fau - Balazs et al.; Schwinghamer Mw Fau - Symons and Symons). Only the 5' proximal MP gene, but not the 3' proximal CP gene, is translated directly from the genomic TAV3. Instead, the CP is translated from a subgenomic mRNA transcribed from

genomic RNA 3. Apart from sub-genomic RNA 4, two novel subgenomic RNAs namely 3B and RNA 5 are also transcribed from RNA 3. On testing the compatibility of the TAV genomic RNA with that of CMV, it was found that RNA 3 of these two cucumoviruses are interchangeable, thus TAV RNA 3 can replicate in the presence of CMV RNA 1 and 2 (Rao and Francki, 1981). TAV subgenomic RNAs 3B and 5 were detected in the infection of a mixture TAV RNA 3 with RNAs 1 and 2 of TAV and also with RNA 1 and 2 of CMV (Shi et al., 1997).

This virus causes serious losses in chrysanthemum crops and the symptoms include severe “flower break” or “color-break” in flowers, distortion of flowers and dwarfing of the chrysanthemum plant. Symptoms may not be expressed in the first year of infection. TAV is also transferred from plant to plant by aphids (Blencowe and Caldwell., 1949). TAV causes severe systemic leaf mottle, enation, dwarfing and seedlessness (aspermy) in infected tomato plants (Hollings and Stone 1971). Infected *Chenopodium amaranticolor* and *C. quinoa* shows *n*umerous chlorotic or necrotic local lesions but not systemic infections. Additionally, a strain of TAV called V-TAV was found to replicate the satellite RNA of Q-CMV, the Q-SatRNA (Q-Sat RNA is a 5′-capped, noncoding, single-stranded RNA genomes of 330 to 405 nucleotides nt long) but these satellite RNA were not encapsidated by TAV(Garcia-Arenal F Fau - Palukaitis and Palukaitis; Moriones et al., 1992; Simon Ae Fau - Roossinck et al.). Another strain called TAV-B also replicates the satellite RNA of CMV D and K and interestingly they encapsidate these satellite RNAs. These two satellite RNAs attenuate TAV

symptoms in infected tobacco correlated with a reduction of virus content in the plant. The CMV satellite RNAs also caused lethal necrosis in TAV-infected tomato as when CMV acts as the helper (Lee, 1986). Infectious cDNA clones of TAV were constructed using a T7 promoter. *In-vitro* RNA transcripts, which were produced using T7 RNA polymerase from full-length cDNAs, could be mechanically inoculated to *Nicotiana tabacum*, Xanti-nc plant. (Choi Sk Fau - Choi et al.). cDNA clones of TAV with a 35S promoter were also made (pCass TAV1,2 & 3) wherein the inserts were removed along with the 35S promoter using restriction enzymes and equal concentration of the three genomic cDNAs (10ug in water) were inoculated on *N. glutinosa* plants which had been in the dark for 24 h. However, mechanical has one major setback; not all the plant cells will receive all three genomic RNAs/cDNAs and since these inoculated RNAs reach the cytoplasm first, they will mostly be degraded by endonucleases found in the cytoplasm. These limitations can be circumvented by the application of *Agrobacterium*-mediated transient expression (agroinfiltration) (Annamalai and Rao, 2005). Agroinfiltration of viral components offers the following benefits: (i) synchronized delivery of multiple plasmids to the same cell (Annamalai and Rao, 2005); (ii) A steady-state supply of ectopically expressed RNA transcripts for up to 4 days (Annamalai and Rao, 2005; Annamalai et al., 2008).

Materials and Methods:

To construct T-DNA based plasmids of TAV, full-length cDNAs of TAV were amplified from pCass TAV1,2& 3 (Shi Bj Fau - Ding et al.) by PCR using a common forward primer (5' GTT TGT CTA TCA AGA GCG TAC GGT TCA ACC 3') and a 3' reverse primer (5' TGG GAC CCC TAG GGG GAA CC 3'; for TAV 1 and 2 and a unique forward primer (5' GTT TAC CAA CCA ACC AAC CAC TAC TAT CTA TCT ATC TG 3') and a reverse primer (5' TGG GAC CCC TAG GGG GAC C 3') for TAV 3. The primers were kinased at their 5' end and 3' end by Polynucleotide Kinase at 37 C for 30 minutes prior to the PCR cycle. The resultant PCR product is ligated into a *Stu*I digested binary vector pCASS RZ (Annamalai and Rao, 2005). The resulting plasmids of TAV (Fig. 14) contain in sequential order: double 35 S promoters, cDNA complementary to full length TAV, Rz sequence and a terminator. Growth conditions of agrotransformants and the agroinfiltration procedure was as described previously (Annamalai and Rao, 2006) except that each agroculture was adjusted to 0.5 OD at 600nm. The final inoculum was prepared by mixing equal volumes of each culture and infiltrating it into the abaxial surface of fully expanded *N. benthamiana* leaves using a 1 ml syringe without a needle. Agro constructs of the Q strain of CMV, its satellite RNA (Q-Sat) and its mutants namely Q-SatΔ3, Q-SatΔ7, Q-SatΔ9, Q-SatΔ13, Q-SatΔ17, Q-SatΔ23 (courtesy of Dr. Sun Jung Kwon) were also used in some the experiments.

Progeny analysis

Total RNA from agroinfiltrated and mechanically inoculated *N.benthamiana* leaves was extracted using the TRIZOL method (Ambion) and the RNA pellet was suspended in RNAse-free water. Virions were purified from these leaves fifteen days post agro-infiltration or mechanical inoculation as described previously (Peden Kw Fau - Symons and Symons) but EDTA was not added to the extraction buffer as it disrupts the TAV, but not the CMV during extraction. TAV Virion RNA was isolated by treating the purified Virions with a Phenol-chloroform (1:1 v/v) solution by gently vortexing them together for three minutes and centrifuging the mixture at 6000 rpm for 5 minutes and carefully removing the aqueous phase and precipitating the virion RNA overnight using 10% ammonium acetate and two volumes of 100% ethanol. The precipitated Virion RNA was pelleted by centrifuging at 12000 RPM for 30 minutes at 4ºC and the resuspended in RNAse-free water. Total and virion RNA was subjected to Northern blot analysis as described previously (Annamalai and Rao, 2005). To prepare strand specific riboprobes, a sequence located between nucleotides 2855 to 3061 of the TAV 2 genome was generated by inserting two restriction sites for Hind III and EcoR1 at the 5'and the 3' end respectively by using Forward primers 5' ATCAAGCTTACGGCTAAAATGG 3' (Hind III site is underlined) and Reverse Primer 5' CCGGAATTCGGACCCCTAGGGGGAACC 3' (EcoR1 site is underlined) and digesting the PCR product with *Hind III* and *EcoR1,* then sub-cloning it into a pT3/T7 vector digested with *Hind III* and *EcoR1*. For testing the

positive stranded progeny RNA, pT3/T7 sub cloned with the 3’ terminal 207nt of TAV 2 was digested with Hind III and transcribed using T7 RNA polymerase (Ambion); for testing the negaitive stranded progeny RNA, the subcloned plasmid is digested with EcoR1 and transcribed with T3 RNA Polymerase (Ambion). The TAV CP samples were analyzed using Western blots as described previously (Osman et al., 1997). The symptoms exhibited by TAV infected *N.benthamiana* via agroinfiltration or mechanical inoculation were recorded 15 days post infection.

Electron microscopy

TAV virions were purified from *N.benthamiana* as described above and concentrated by high-speed centrifugation at 30,000 RPM for 3 hours and the pellets were re-suspended in TAV resuspension buffer (Peden Kw Fau - Symons and Symons). Approximately 10µl (100ng/µl) of these purified virions were spread on glow-discharged carbon coated grids and negatively stained with 1% uranyl acetate prior to examining with a FEI Tecnai12 transmission electron microscope (Annamalai and Rao, 2005) and images were recorded digitally.

Results

Sub-cloning of TAV 1 and TAV 3 to pCass RZ was successful using DH5α competent cells but sub-cloning of TAV 2 failed repeatedly (12 attempts) with DH5α. Upon changing the competent cell to INVαF’ (Invitrogen) subclones of TAV 2 were recovered.

Northern blot analysis of the total RNA revealed that no TAV RNA was detected in leaves infiltrated with individual TAV genomic RNAs in each *N.benthamiana* plant (lanes 1-3 of Fig 15A). Progeny TAV RNAs 1 and 2 were detected in leaves infiltrated with the TAV 1 & 2 agroconstructs (Lane 4), but no other TAV RNA was detected thus eliminating the possibility of contamination by TAV 3. Progeny RNA 1,2,3 and subgenomic RNAs 4,3a and 5 were detected in *N.benthamiana* leaves infiltrated with pTAV 1,2 and 3 agroconstructs (Lane 6). This proves that the TAV agroconstructs are biologically active in *N.benthamiana* plants 4 days post infiltration. To check whether the subgenomic RNA 4 produced from RNA 3 via replication is capable of translating the coat protein, a western blot analysis on 20 μg of total protein from each of these infiltrated plant was performed using TAV coat protein specific antibodies (Garcia Arenal). TAV coat protein was detected only from leaves infiltrated with all the three of the TAV genomic RNAs (Fig 15B, Lane 6). To check whether the translated coat protein is capable of forming virions, the leaves infiltrated with all three genomic RNAs were subjected to virion extraction and the purified virions were examined under transmission electron microscope (TEM). As expected, virions of 28nm were seen (Fig 15C).

Fifteen days post infiltration, the systemic *N.benthamiana* leaves shows severe blistering and curling associated with stunted growth and reduced flowering (Fig 16) when compared to that of healthy *N.benthamiana* plants (Fig 16A). Fifteen days post mechanical inoculation of the purified virions showed

similar symptoms in the systemic leaves (Fig 16C) confirming that the symptoms were modified by the virus only. Healthy *N.benthamiana* was used a control to compare the symptoms (Fig 16A).

Agroinfiltration system was used to confirm a previous report that when mechanically inoculating RNA transcripts, only RNA 3 of TAV and Q-CMV is readily interchangeable between the two viral genomes to produce infections resulting in lesion development (Rao and Francki, 1981). The three agroconstructs of TAV (TAV 1,2 & 3) were interchanged and co-infiltrated with the three agroconstructs of Q-CMV (Q-CMV1,2 & 3) onto the leaves of *N.benthamiana* and the total RNA of the infiltrated leaves was subjected to Northern blot analysis 6 days post infiltration. Similar results were obtained, onlt the RNA 3 was interchangeable between the two genomes. same (Fig 17, Lanes 4 and 8) while the TAV RNA 3 also made the two novel subgenomic RNA 3B and 5 in the presence of Q-CMV 1 and 2 as previously reported (Shi et al., 1997) (Fig 17, Lane 4 and Lane 8).

To test the replication ability of TAV with CMV Q-Sat and its mutants, the three agroconstructs of TAV is coinfiltrated with the Q-Sat agroconstruct and its six mutants respectively. After 6 days, northern blot analysis using a Q-Sat specific probe revealed that Q-Sat can be replicated by TAV (Fig 18A, Lane 4) but its level of replication is 95% less than its replication by its helper Q-CMV (Fig 18A, Lane 3). Interestingly out of the six Q-Sat mutants, Q-SatΔ3 was the only mutant that replicated in the presence of TAV (Fig 18A, Lane 5) and its replication

was 30% higher than that of the wt Q-Sat with TAV. Correspondingly the level of replication of TAV in the presence of wt Q-Sat (Fig 18B, Lane 4) is reduced by 50% when compared to TAV without Q-Sat (Fig 18B Lane 2) and but it returned to almost wild type levels in the presence of Q-SatΔ3 (Fig 18B, Lane 5). Q-SatΔ7, Δ9, Δ13, Δ18 and Δ23 did not replicate with TAV (Fig 18A, Lanes 6-11).

Discussion

Since the subcloning of TAV 1 and TAV 3 but not TAV2 to pCass RZ were possible by transforming into DH5α cells, it suggests that TAV 2 could be lethal to DH5α. However using INVαF' cells for transformation with TAV2 allowed recovery of viable clones.

A simple, efficient, user-friendly agroinfiltration system for synchronized delivery of DNA-based genomic components of TAV to *N.benthamiana* plants was produced which replicated the results previously performed by using mechanical inoculation. This includes northern blot analysis which were revealed that agroinfiltrated TAV 3 along with TAV 1 and 2 or Q-CMV 1 and 2 in *N.benthamiana* produced subgenomic RNAs 4, 3A and 5 which were previously reported using mechanically inoculation of TAV cDNAs with a 35S promoter (Shi et al., 1997). Subgenomic RNA 4 produced from RNA 3 was capable of translating its coat protein which was competent to form biologically active virions harboring all three genomic and sub-genomic RNAs. Agroinfiltrated TAV was also able to replicate the satellite RNA of Q-CMV but less efficiently than CMV. It was

shown previously that the replication level of TAV B and its symptom modification is strongly mitigated by the satellite RNA of CMV D and K. Here we are reporting that the replication level of V-TAV was reduced on co-infection with Sat RNA of Q-CMV.

Here, for the first time, *Nicotiana benthamiana* was used as a host to test the biological activity of TAV via agroinfiltration and resulted in a severe symptom modification in this species both by agroinfiltration and mechanical inoculation.

Figures:

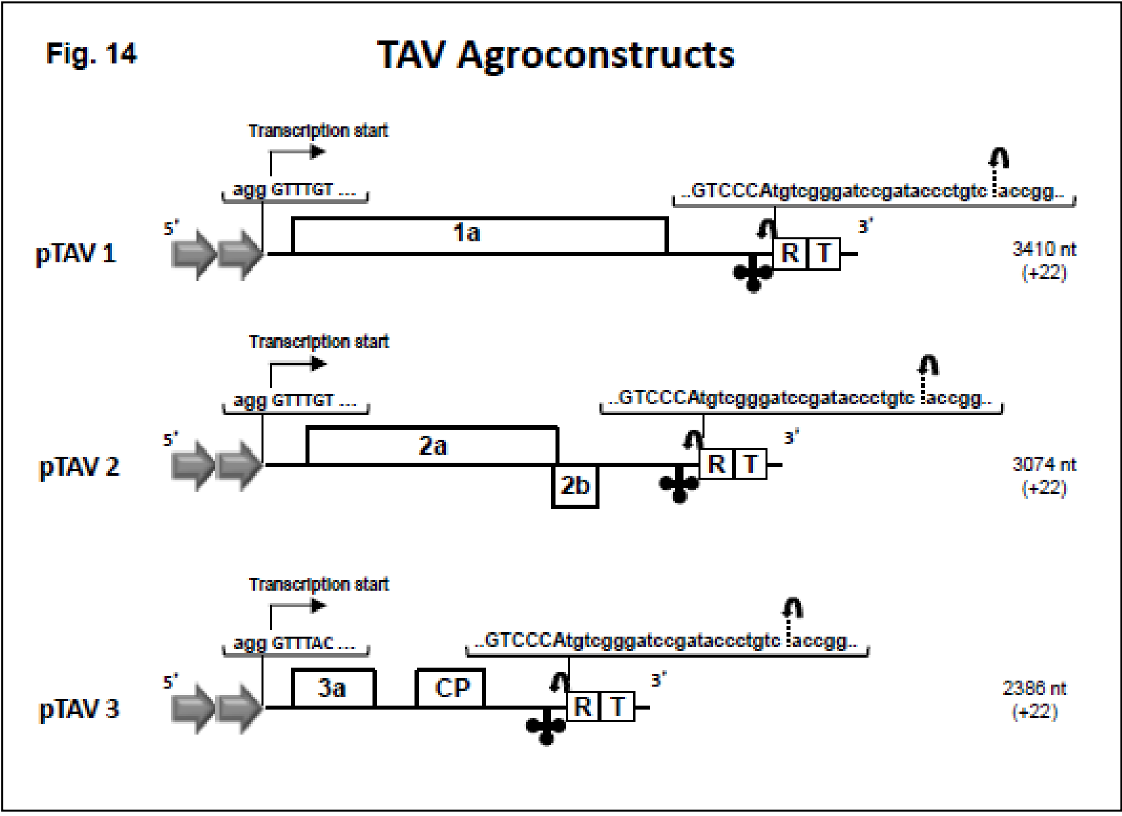


Figure 14: pCass RZ agroconstructs of *Tomato aspermy virus* (TAV) genomic RNAs. TAV RNA 1,2 & 3 cDNA are in pCass RZ which would transcribe the respective RNAs with an extra 22 non-viral nt at the 3' end in vivo

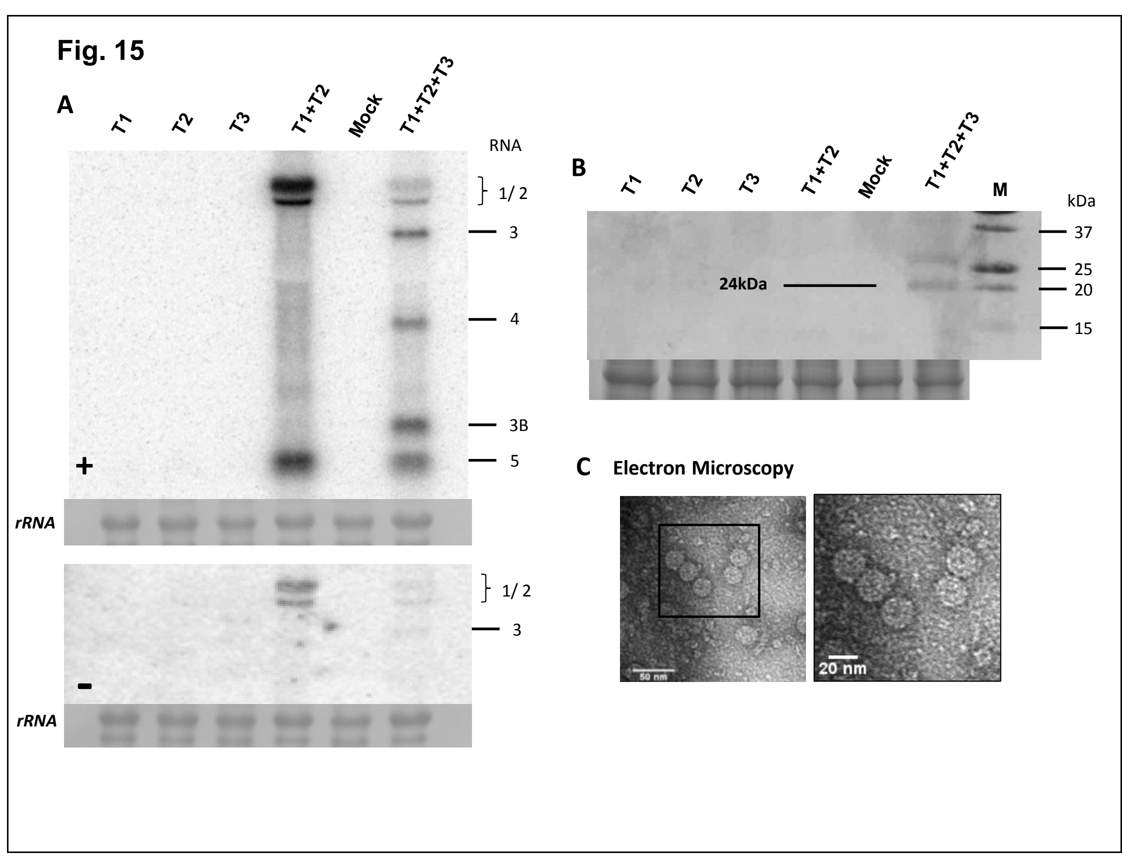


Figure 15: Test for Biological activity of TAV agroconstructs in *N.benthamiana* plant. A: Northern blot analysis, B: Western blot analysis & C: Transmission electron microscopy

Fig. 16

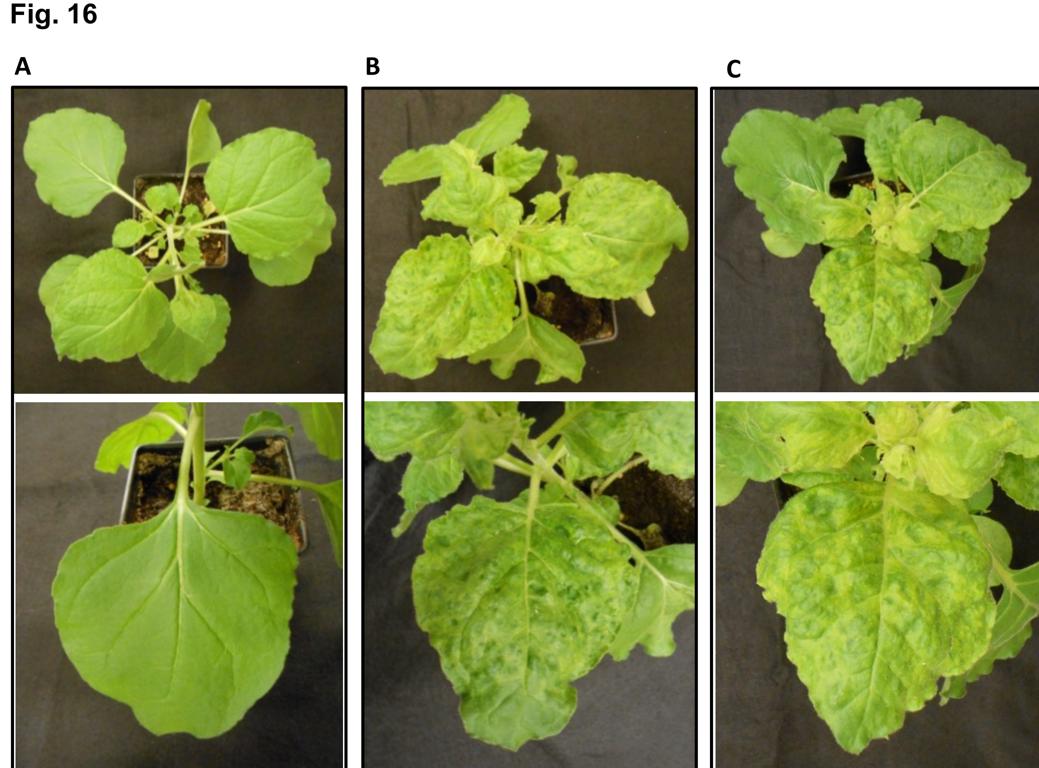


Figure 16: Symptom modification *of Nicotiana benthamiana* 15days post agroinfiltration of TAV 1, 2 &3 (B). Similar symptom modification of *N.benthamiana* 15days post mechanical inoculation of purified virions obtained via agro-infiltration (C). Healthy *N.benthamiana* plant is used as a

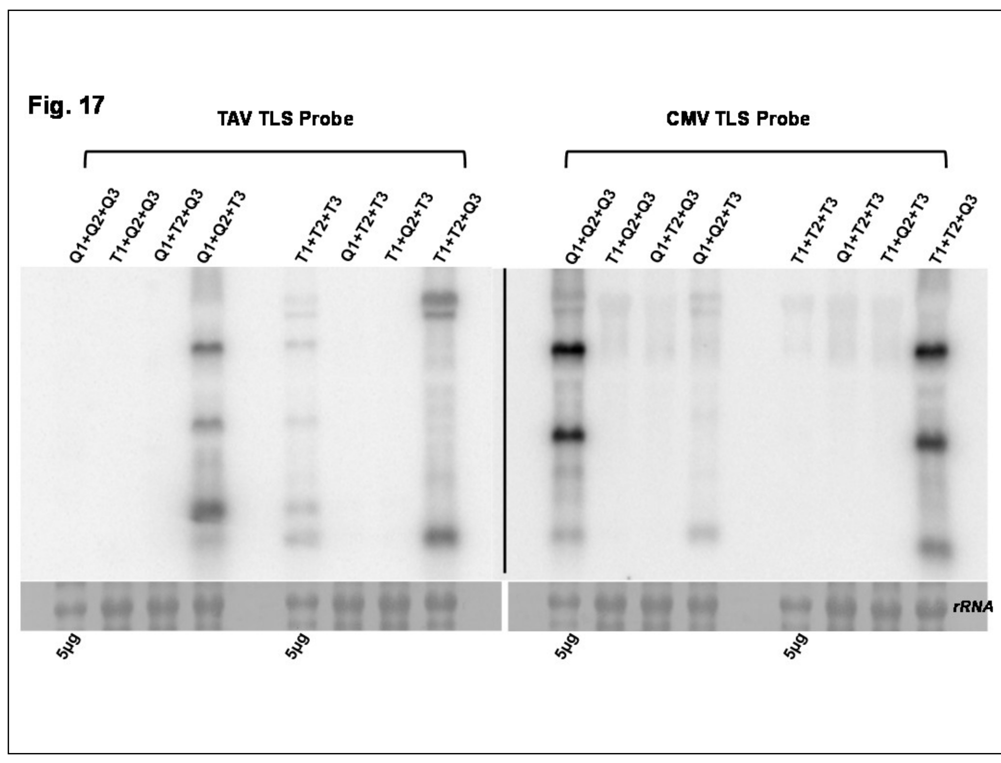


Figure 17: Replication profile of TAV and CMV by reassorting their three genomic RNAs in *N.benthamiana*. 5µg totals RNA of positive controls (T1+T2+T3 & Q1+Q2+Q3) were added while 10 µg of total RNA were added

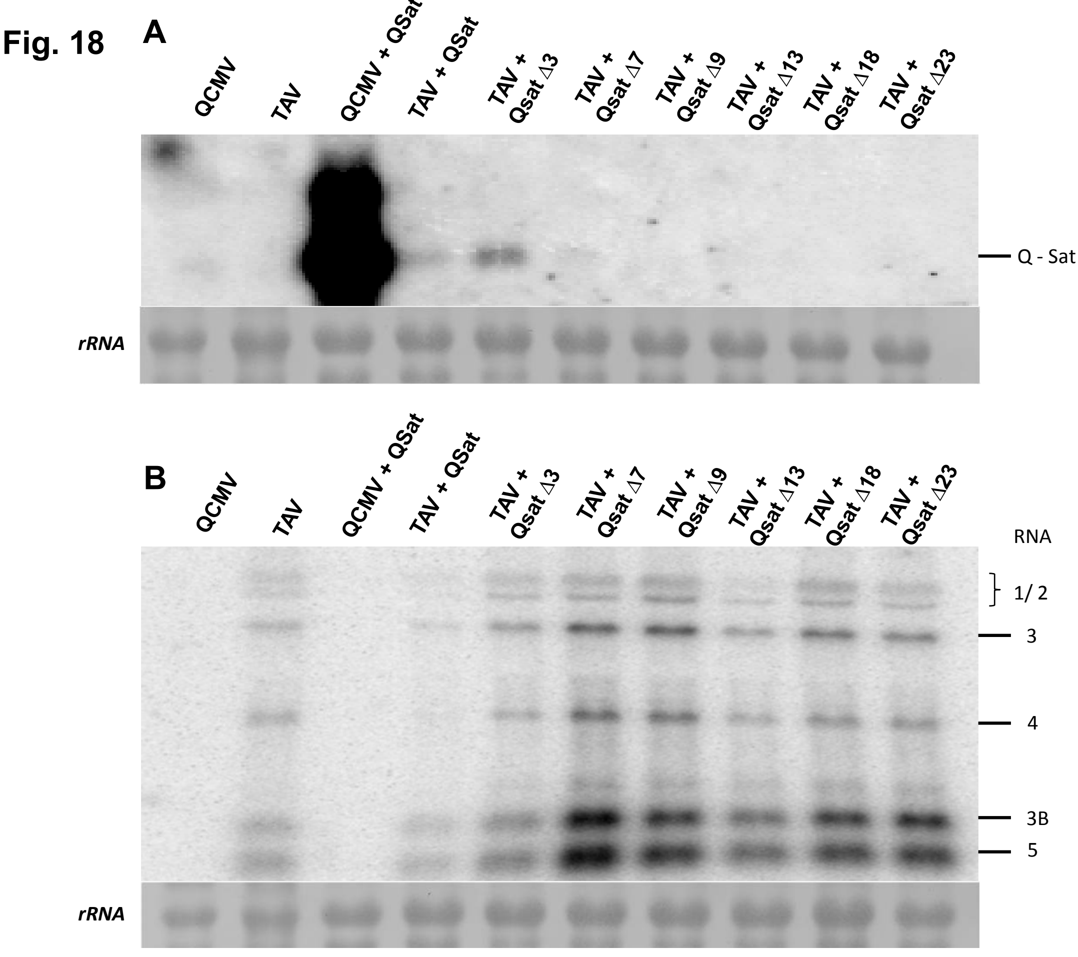


Figure 18: Replication ability of Q-Sat and its six mutants by TAV. Replication of Q-Sat by CMV, TAV and CMV without Q-Sat are used as controls (A). TAV progeny RNA are detected for the same samples(B).

References

Stace-Smith R, Tremaine JH. 1973. Biophysical and biochemical properties of tomato aspermy virus. Virology 51:401-408.

Bernal JJ, Moriones E, García-Arenal F. 1991. Evolutionary relationships in the cucumoviruses: nucleotide sequence of tomato aspermy virus RNA 1. Journal of General Virology 72:2191-2195.

Moriones E Fau - Roossinck MJ, Roossinck Mj Fau - Garcia-Arenal F, Garcia-Arenal F. Nucleotide sequence of tomato aspermy virus RNA 2.

Rezaian Ma Fau - Williams RH, Williams Rh Fau - Gordon KH, Gordon Kh Fau - Gould AR, Gould Ar Fau - Symons RH, Symons RH. Nucleotide sequence of cucumber-mosaic-virus RNA 2 reveals a translation product significantly homologous to corresponding proteins of other viruses.

Rezaian Ma Fau - Williams RH, Williams Rh Fau - Symons RH, Symons RH. Nucleotide sequence of cucumber mosaic virus RNA. 1. Presence of a sequence complementary to part of the viral satellite RNA and homologies with other viral RNAs.

Nitta N, Takanami Y, Kuwata S, Kubo S. 1988. Inoculation with RNAs 1 and 2 of Cucumber Mosaic Virus Induces Viral RNA Replicase Activity in Tobacco Mesophyll Protoplasts. Journal of General Virology 69:2695-2700.

Hayes Rj Fau - Buck KW, Buck KW. Complete replication of a eukaryotic virus RNA in vitro by a purified RNA-dependent RNA polymerase.

Shi Bj Fau - Ding SW, Ding Sw Fau - Symons RH, Symons RH. In vivo expression of an overlapping gene encoded by the cucumoviruses.

O'Reilly D Fau - Thomas CJ, Thomas Cj Fau - Coutts RH, Coutts RH. Tomato aspermy virus has an evolutionary relationship with other tripartite RNA plant viruses.

Salanki K Fau - Balazs E, Balazs E Fau - Burgyan J, Burgyan J. Nucleotide sequence and infectious in vitro transcripts of RNA 3 of tomato aspermy virus pepper isolate.

Schwinghamer Mw Fau - Symons RH, Symons RH. Translation of the four major RNA species of cucumber mosaic virus in plant and animal cell-free systems and in toad oocytes.

Rao ALN, Francki RIB. 1981. Comparative studies on tomato aspermy and cucumber mosaic viruses. VI. Partial compatibility of genome segments from the two viruses. Virology 114:573-575.

Shi B, Ding S, Symons RH. 1997. Two novel subgenomic RNAs derived from RNA 3 of tomato aspermy cucumovirus. Journal of General Virology 78:505-510.

Moriones E, Diaz I, Rodriguez-Cerezo E, Fraile A, Garcia-Arenal F. 1992. Differential interactions among strains of tomato aspermy virus and satellite RNAs of cucumber mosaic virus. Virology 186:475-480.

Garcia-Arenal F Fau - Palukaitis P, Palukaitis P. Structure and functional relationships of satellite RNAs of cucumber mosaic virus.

Simon Ae Fau - Roossinck MJ, Roossinck Mj Fau - Havelda Z, Havelda Z. Plant virus satellite and defective interfering RNAs: new paradigms for a new century.

Lee HS. 1986. Modification of Tomato Aspermy Virus Symptom by Cucumber Mosaic Virus- Associated Satellite RNA. Korean journal of plant pathology 2:145-149.

Choi Sk Fau - Choi JK, Choi Jk Fau - Ryu KH, Ryu Kh Fau - Park WM, Park WM. Generation of infectious cDNA clones of a Korean strain of tomato aspermy virus.

Annamalai P, Rao AL. 2005. Replication-independent expression of genome components and capsid protein of brome mosaic virus in planta: a functional role for viral replicase in RNA packaging. Virology 338:96-111.

Annamalai P, Rofail F, DeMason DA, Rao ALN. 2008. Replication-Coupled Packaging Mechanism in Positive-Strand RNA Viruses: Synchronized Coexpression of Functional Multigenome RNA Components of an Animal and a Plant Virus in Nicotiana benthamiana Cells by Agroinfiltration. Journal of virology 82:1484-1495.

Bamunusinghe D, Seo JK, Rao AL. 2011. Subcellular localization and rearrangement of endoplasmic reticulum by Brome mosaic virus capsid protein. Journal of virology 85:2953-2963.

Annamalai P, Rao AL. 2006. Delivery and Expression of functional viral RNA genomes in planta by agroinfiltration, p. p2.1-2.15. *In* Downey T (ed.), Current Protocols in MIcrobiology, vol. Unit 16B. John Wiley & Sons, New York.

Peden Kw Fau - Symons RH, Symons RH. Cucumber mosaic virus contains a functionally divided genome.

Osman F, Grantham GL, Rao AL. 1997. Molecular studies on bromovirus capsid protein. IV. Coat protein exchanges between brome mosaic and cowpea chlorotic mottle viruses exhibit neutral effects in heterologous hosts. Virology 238:452-459.