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Studies on Replication of Cucumber Mosaic Virus Satellite RNA

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by

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Finally, I remind myself to live daily by the five solas: Sola scriptura (“by Scripture alone”); Sola fide (“by faith alone”); Sola gratia (“by grace alone”); Solus Christus (“Christ alone”); and Soli Deo Gloria (“glory to God alone”).

ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy, Graduate Program in Cell, Molecular, and Developmental Biology
University of California, Riverside, June 2011
Dr. A. L. N. Rao, Chairperson

Satellite RNA variant-Q associated with cucumber mosaic virus (CMV) is a highly structured single-stranded RNA molecule of 336 nucleotides in length. Satellite RNA is an important subviral pathogen in agriculture because it modulates symptom expression in CMV-infected plants. In the agricultural industry, CMV is economically very important since it exhibits a broad host range comprising over 1200 plant species world-wide. Although satellite RNA has no sequence homology with CMV genome, satellite RNA replication is thought to occur entirely in the cytoplasm and is exclusively dependent upon CMV replicase. Chapter 1 of this dissertation describes an *Agrobacterium*-mediated transient gene expression system that allows the expression of individual viral and satellite RNA uncoupled from the virus replication *in vivo*, and by utilizing this system we demonstrate that CMV coat protein expressed independently of

replication can enhance the accumulation of an individual CMV RNA. Virions formed by the CMV coat protein in the absence of virus replication encapsidates some host RNAs, and the satellite RNA can accumulate to a detectable level in the absence of the helper CMV. Chapter 2 describes a novel finding that satRNA expressed in the absence of its helper CMV is amplified by the host. By using immunostaining with antibody against double strand RNA, and confocal microscopy to identify the subcellular distribution of double stranded satellite RNA *in situ*, we have found that in the absence of the helper virus, satRNA can amplify in the nucleus. Furthermore, sequence analysis of satRNA oligomers formed in the absence of CMV replication showed that the junction between two monomeric forms have a unique heptanucleotide sequence GGGAAAA, which is not present in the junction of satRNA oligomers formed in the presence of CMV. Finally in chapter 3, by using agroinfiltration system, we demonstrate that the negative-sense strand of satellite RNA is not amplified by the host cell because it is not recruited into the nucleus. However, consistent with the previously published results, the negative-sense satellite RNA is replicated by the helper CMV but only to a low-level. These novel findings will place the viroids, hepatitis delta virus and satellite RNAs in a closer relationship with each other in terms of their replication mechanisms. Implications of these findings are discussed in Conclusion chapter of this dissertation.

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INTRODUCTION

Subviral agents are the smallest known infectious entities that can have a big impact on the health of the much larger organisms including plants, animals, and humans. Subviral agents are divided into 2 major groups: virus-dependent agents and virus-independent agents. Satellite viruses, satellite nucleic acids and hepatitis delta virus (HDV) are part of the virus-dependent subviral agents, and viroids and prions can be grouped into virus-independent subviral agents. The phylogenetic studies done by Elena *et al*. has shown that viroids, HDV, and some satellite RNAs probably share a common ancestor (Elena et al., 2001; Elena et al., 1991), even though the satellite RNAs, viroids and HDV do not have significant overall sequence similarity.

A novel finding in satellite RNA life cycle will place the viroids, HDV and satellite RNAs in even closer relationship with each other in terms of their replication mechanisms as described in chapter 2 of this dissertation. For the scope of the investigation described in this dissertation, the single-stranded satellite RNA, particularly the satellite RNA associated with cucumber mosaic virus (CMV) is the main focus of this introduction. In addition, the biology of CMV, the replication mechanism of viroids, and HDV will be described briefly.

I. Single-stranded satellite RNAs of plant viruses

A. Types of single-stranded satellite RNAs

Single-stranded RNA satellites of plant viruses are replicated and encapsidated by their associated helper viruses, however, they have little or no sequence homology with the helper viruses and is not required for the helper virus accumulation (Murant and Mayo, 1982). Since the discovery of the tobacco ringspot virus satellite RNA in 1969 (Schneider, 1969), many other satellite RNAs associated with different plant viruses have been described. The single-stranded satellite RNAs, excluding the satellite virus genomic RNA, are classified into three types (Breuning, 2000; Mayo et al., 1995). The first type is B type mRNA satellites, which are 0.8 to 1.5kb in size and synthesize a non-structural protein from a single open reading frame (Roossinck, Sleat, and Palukaitis, 1992). Next type is C type linear RNA satellites. This type of satellite RNAs are less than 0.7 kb in size, no circular forms are found in infected cells, and has no known messenger RNA (mRNA) activity (Mayo et al., 1995). The last type is D type circular RNA satellites (Mayo et al., 1995). Their genomes are about 350 bases in size and as in C type satellite RNAs, they do not have mRNA activity (Breuning, 2000; Mayo et al., 1995). However, they form both circular and linear molecules in their life cycle, which distinguishes them from the C type (Breuning, 2000; Mayo et al., 1995).

B. Origin of single-stranded satellite RNAs

The origin of single-stranded satellite RNA is unknown. However, it is conceivable that the satellite RNA may have been originated from the plant genome since there is a noticeable segmental sequence similarity with the *Arabidopsis thaliana* genome and satellite RNA may be generated from the host plant genome through some unknown mechanism in some unique conditions (Simon, Roossinck, and Havelda, 2004). Viral template-independent 3'-end deletion repair by cucumber mosaic virus (CMV) replicase (Burgyan and Garcia-Arenal, 1998) may also support the idea of satellite RNA origin from the plant genome. In addition, the satellite RNA of peanut stunt virus (PSV) nucleotide sequence study showed that there are structural similarities with some cellular introns in nucleus and mitochondria, and many plant viroids (Collmer, Hadidi, and Kaper, 1985).

C. Sequence variants within the satellite RNAs with a same helper virus

Most sequence variants within the satellite RNAs associated with the same helper virus species do not exhibit biological variation except for the satellite RNAs of CMV (Roossinck, Sleat, and Palukaitis, 1992). The CMV satellite RNA sequence variations can affect pathogenicity (Devic, Jaegle, and Baulcombe, 1990; Garcia-Arenal, Zaitlin, and Palukaitis, 1987; Jaegle et al., 1990; Masuta and Takanami, 1989; Sleat and Palukaitis, 1990) and replication of the satellite RNA (Garcia-Arenal, Zaitlin, and Palukaitis, 1987; Palukaitis, 1988).

The pathogenic effects are due to complex interactions involving specific sequences in the satellite RNA, the strain of helper virus, and the host species (Palukaitis, 1988; Waterworth, Kaper, and Tousignant, 1979). Satellite RNA can bind CMV RNA but this binding does not appear to be the reason for the symptom change (Garcia-Arenal and Palukaitis, 1999). The satellite RNA used in the investigation described in this dissertation is the variant-Q which was first found associated with Q-strain of CMV (Garcia-Arenal and Palukaitis, 1999; Gordon and Symons, 1983; Gould et al., 1978). The satellite variant-Q attenuates symptoms on CMV-infected tomato (Garcia-Arenal and Palukaitis, 1999)

II. Satellite RNAs associated with CMV

A. CMV satellite RNA

The satellite RNA of CMV is a C-type linear RNA satellite that is 332-342 nucleotides in length. There are some that are about 30 to 70 nucleotides longer due to an insertion of sequences at specific sites in smaller satellite RNAs (Garcia-Arenal and Palukaitis, 1999). It is highly structured with about 50% intramolecular base pairing (Garcia-Arenal and Palukaitis, 1999). The existence of satellite RNA of CMV was made known in mid-1970's by J. M. Kaper and colleagues (Kaper, Tousignant, and Lot, 1976) as a result of searching for the causal agent of tomato necrosis (Kaper and Tousignant, 1977; Kaper and Waterworth, 1977). Since then more than 100 variants of CMV satellite RNAs

have been sequenced (Palukaitis and Garcia-Arenal, 2003) and it has been shown that the various CMV satellite RNAs have similar nucleotide sequences varying from 70-99% homology (Palukaitis et al., 1992). They are unusually resistant to nuclease both *in vivo* and *in vitro* probably due to their compact secondary structure (Mossop and Francki, 1978). CMV satellite RNA is not required for CMV infection and replication. CMV satellite RNA has been used as a model for the study of replication, pathogenesis, structure-function relationships, and genetic variation and evolution of plant pathogenic RNAs (Palukaitis and Garcia-Arenal, 2003). All analyzed CMV Sat-RNAs have a 5'-terminal cap and the 3'-end terminates in CCC_{OH} that cannot be aminoacylated (Roossinck, Sleat, and Palukaitis, 1992; Sivakumaran et al., 2000). Despite the lack of recognized homology with the helper virus genomes, satellite RNA is efficiently encapsidated by CMV coat protein, and depends on CMV for the movements from cell to cell, and from one host to another.

B. CMV as a helper virus

CMV belongs to the genus *Cucumovirus* of the family *Bromoviridae* (van Regenmortel et al., 2000). CMV is found world-wide, especially in the temperate zones (Palukaitis et al., 1992). There are many strains of CMV that show differences in host range and symptomatology. By serological relationships, peptide mapping of the coat protein, and nucleic acid hybridization analysis, CMV falls into either the subgroup I or II, and the relationships of virus strains within

each subgroup are very close (Palukaitis and Garcia-Arenal, 2003). In the agricultural industry, CMV is economically very important since it exhibits a broad host range comprising over 1200 plant species (Edwardson and Christie, 1991; Palukaitis and Garcia-Arenal, 2003).

Host range of CMV can be extended to include cereals, forages, woody and herbaceous ornamentals, vegetables, and fruit crops. The most common symptoms caused by CMV are mosaic. The severity of disease may range from symptomless to severe symptoms that lead to the death of the host. CMV has been the causal agent of many disease epidemics, including tomato, banana, legumes, melons, borrage, sweet potato, and many greenhouse crops (Palukaitis et al., 1992). In nature, CMV is transmitted by more than 80 aphid species. CMV is not known to be spread by fungi, nematodes, or insects other than the aphids or by plant-to-plant contact (Kaper and Waterworth, 1981). Efficient transmission of CMV by aphid vectors requires the virus particle (virion) formation (Palukaitis and Garcia-Arenal, 2003). In CMV infection, the presence of satellite RNA can result in symptom modification, and adversely affect the replication of the CMV genomic RNAs by competing for the viral replicase, which results in lowering of the yield of the helper virus. The most common symptom modification associated with the replication of satellite RNA is an attenuation of the virus symptoms (Palukaitis, 1988; Waterworth, Kaper, and Tousignant, 1979). However, in the host species such as tobacco, pepper, and tomato, CMV symptoms can be exacerbated by some variants of satellite RNAs (Palukaitis, 1988; Waterworth,

Kaper, and Tousignant, 1979). Frequency of finding CMV satellite RNAs in agricultural settings or in the wild is relatively rare (Kearney, Zitter, and Gonsalves, 1990; Simon, Roossinck, and Havelda, 2004), although the satellite RNAs are distributed world-wide (Palukaitis et al., 1992).

C. CMV genome organization

CMV genome consists of single-stranded, positive (+)-sense RNAs (i.e. mRNAs). As schematically shown in Fig. A, the entire genome of CMV (~8 kb) is divided among three RNA components (Peden and Symons, 1973). In addition, purified CMV virions also contain two known subgenomic RNAs, RNA4A (Ding et al., 1994) and RNA4 (Peden and Symons, 1973) that are transcribed from the minus (-)-strand progeny of genomic RNAs 2 and 3, respectively. Another RNA molecule referred to as RNA5 is also found to be packaged in virions of subgroup II. Like many other plus (+)-strand RNA viruses, the replication of CMV is asymmetric (*i.e.*, numerous copies of (+)-strands are synthesized from one copy of (-)-strand). The 3'-non-coding regions of CMV genomic and subgenomic RNAs contain a highly conserved tRNA-like structure (TLS) that terminates in CCA_{OH} and can be aminoacylated with tyrosine (Kohl and Hall, 1974). TLS is necessary for initiating (-)-strand synthesis by the CMV replicase.

CMV RNA1 is about 3390 nt-long, and has one open reading frame (ORF) that encodes a non-structural 1a protein which has methyl transferase and helicase functions (Gorbalenya et al., 1988; Habili and Symons, 1989; Hodgman,

1988). RNA2 is about 3050 nt-long and is bicistronic, encoding proteins 2a and 2b in different reading frames (Ding et al., 1994). Non-structural protein 2a has a RNA-dependent RNA polymerase (RdRP) activity (Bruenn, 1991; Habili and Symons, 1989; Poch et al., 1989).

The non-structural proteins 1a and 2a assemble with host factors (Hayes and Buck, 1990), possibly including the tonoplast intrinsic proteins (Kim, Kim, and Paek, 2006), to form the functional viral replicase. The CMV RNA replication site is proposed to be in the tonoplast, which is the localization site of 1a and 2a proteins (Cillo, Roberts, and Palukaitis, 2002). RNA 4A, a subgenomic RNA (sgRNA) transcribed from the (-)-sense RNA2, is 630-702 nt-long (Ding et al., 1994; Palukaitis et al., 1992) and is encapsidated. It expresses the 2b protein that is involved in RNA silencing suppression, movement and symptomatology (Ding et al., 1994; Palukaitis and Garcia-Arenal, 2003). Bicistronic RNA3 is about 2200 nt-long, encoding movement protein (MP) (Boccard and Baulcombe, 1993) and coat protein (CP) (Habili and Francki, 1974a). Only the MP is translated directly from RNA3 while the coat protein of 24.5 kDa is translated from the subgenomic RNA4 that is transcribed from the (-)-sense RNA3 (Schwinghamer and Symons, 1975). RNA5 is about 300 nt-long and is only found in CMV-II subgroups. RNA5 is a mixture of the 3'-300 nt regions from RNAs 1, 2 and 3 encompassing most of 3'-UTR (Blanchard, Boyce, and Anderson, 1996; de Wispelaere and Rao, 2009; Palukaitis et al., 1992). RNA5 is probably a degradation product rather than a subgenomic RNA because it is accumulated

when a CMV genomic RNA is introduced into an intact tobacco leaf in the absence of the functional viral replicase (de Wispelaere and Rao, 2009).

Virions purified from CMV infected plants exhibit homogeneous population of icosahedral virions that are 29nm in diameter with T=3 symmetry (Finch and Bancroft, 1968). In addition to the viral genomic RNAs, satellite RNA can also co-package into CMV virions. Virions consist of about 18% RNA that are encapsidated by 180 coat protein subunits (Finch and Bancroft, 1968; Habili and Francki, 1974b; Kaper and Re, 1974). Based on the biochemical and physical properties of the RNA and the virions, it is hypothesized that genomic RNA1 and RNA2 are packaged into two separate virions while RNA3 and RNA4 are co-packaged into the third virion (Lot and Kaper, 1976). No hypothesis has been proposed for the packaging schemes of RNAs 4A, 5 and satellite RNA (Fig. B). The mechanism involved in distributing the viral RNAs into the morphologically and physically indistinguishable virions remains unknown.

D. Satellite RNA replication by CMV

CMV replicase is required for the replication of the satellite RNA (Francki, 1985). Although some CMV satellite RNAs are found in nucleus of the host, most of them are found in association with vesicular membranes (Diazruiz, Avilarincon, and Garcialuque, 1987) presumably with tonoplast since the CMV replicase is localized on the tonoplast (Cillo, Roberts, and Palukaitis, 2002). Presence of easily detectable level of double-stranded (ds) RNA is a good indicator of the

presence of replicating viral RNA in the host (Dodds, Morris, and Jordan, 1984). Ds-satellite RNA accumulates to a high level in plants infected by CMV harboring satellite RNA (Diaz-Ruiz and Kaper, 1977) and it does not appear to involve rolling circle replication mechanism (Roossinck, Sleat, and Palukaitis, 1992).

CMV-satellite RNA multimers in both polarities are present in infected plants although no single-stranded minus-strand satellite multimers are detected (Kuroda et al., 1997). The dimeric form of the satellite dsRNA can self-cleave to form monomers, and the monomeric form of the dsRNA can self-ligate to form multimers (Roossinck, Sleat, and Palukaitis, 1992; Sleat and Palukaitis). The ratio of the satellite RNA plus-strand to minus-strand is 2-3 to 1 (Garcia-Arenal and Palukaitis, 1999; Piazzolla, Tousignant, and Kaper, 1982). However, during the onset of necrosis caused by programmed cell death on tobacco or tomato, the level of minus-strand increases significantly (Xu and Roossinck, 2000).

CMV strain Ix cannot support the replication of some satellite RNA variants (Kaper, Tousignant, and Geletka, 1990). It was found later that the RNA 1 was responsible for the inability of CMV strain Ix to support the replication (McGarvey et al., 1995). Other studies using pseudorecombinant viruses between the satellite RNA-supporting and non-supporting viruses confirmed that satellite RNA replication support maps to RNA 1 (Hu, Sanger, and Ghabrial, 1998; Roossinck, Kaplan, and Palukaitis, 1997).

Inoculating minus-strand satellite RNA transcript with CMV onto tomato plant resulted in accumulation of the satellite RNA although the level of

accumulation was much lower than inoculating with the plus-strand (Tousch, Jacquemond, and Tepfer, 1994). *In vitro* replication study with CMV replicase showed that the satellite RNA was not a template for the viral replicase without an extra guanosin residue on the 3'-end of the minus-strand (Wu, Kaper, and Kung, 1993; Wu, Kaper, and Jaspars, 1991). However, *in vivo* study showed that the minus-strand satellite RNA with 16Gs on the 3'-end was not a better template than the one with other nucleotides on the 3'-end (Tousch, Jacquemond, and Tepfer, 1994).

Satellite RNA replication must involve more than just CMV replicase because temperature affects replication of satellite RNA more than that of CMV RNAs in tomato (White et al., 1995). Also, Sny-strain of CMV cannot support certain variant of satellite RNA in squash. However, in tobacco it is able to support the satellite RNA although CMV replicase activity is the same in both squash and tobacco (Gal-On, Kaplan, and Palukaitis, 1995). Furthermore, in the absence of the helper virus, the mechanically inoculated satellite RNA was shown to be surviving in the plant for about 10 days in one study (Mossop and Francki, 1978) and 25 days in another study (Jacquemond and Lot, 1982).

III. Viroid replication

Viroids are circular naked RNAs of about 250 to 400 nucleotides in length, which do not encode any proteins. They are replicated autonomously, move systemically to cause diseases in certain plants, and move from one host to

another without requiring helper viruses (Ding, 2009). They are classified into two families. One is *Pospiviroidae* which replicates in the host nucleus, and the other is *Avsunviroidae* which replicates in the chloroplast (Flores et al., 2000). The members of *Pospiviroidae* are not known to have ribozyme activities, however, all members of *Avsunviroidae* have ribozyme activities (Flores, Daros, and Hernandez, 2000). Both families replicate through rolling circle mechanisms (Branch and Robertson, 1984; Daros et al., 1994)

It is shown that the satellite variant-Q of CMV shares some striking similarities in replication mechanism with *Potato spindle tuber viroid* (PSTVd) in chapter 1 of this dissertation. PSTVd, a type member of *Pospiviroidae*, replicates in the nucleoplasm through an asymmetric rolling circle mechanism (Branch and Robertson, 1984) and eventually accumulates in the nucleolus as a mature positive-sense circular single-stranded RNA (Qi and Ding, 2003; Sanger, 1987). *In vitro* transcription using purified tomato DNA-dependant RNA polymerase II (Rackwitz, Rohde, and Sanger, 1981) and alpha-amanitin induced inhibition of replication (Muhlbach and Sanger, 1979; Schindler and Muhlbach, 1992) indicate that PSTVd is replicated by host DNA-dependant RNA polymerase II.

Not much is known about the sequence and structural features that are important for the nuclear import/export, and the cellular proteins that interacts with PSTVd. Expression library screening has led to isolating the tomato protein Virp1 (viroid RNA-binding protein 1) that contains the bromodomain and interacts with PSTVd (Gozmanova et al., 2003; Maniataki, Tabler, and Tsagris, 2003).

Virp1 localizes in the nucleus; furthermore, PSTVd was no longer supported in the protoplasts derived from transgenic *N. benthamiana* with Virp1 gene knocked-down (Kalantidis et al., 2007).

Some studies have found that tomato plants infected with CMV harboring satellite RNA show only mild symptoms when challenged with a severe strain of PSTVd. In addition, PSTVd accumulation in the plant infected with CMV satellite RNA is significantly lower than the positive-control plants. They have shown that the satellite RNA, not CMV, is responsible for making the tomato plant resistant to PSTVd infection (Montasser, Kaper, and Owens, 1991; Yang, Kang, and Tien, 1996). These studies support the finding in chapter 2 of this dissertation that CMV satellite RNA has a nuclear phase in its life cycle. The reduction of the PSTVd accumulation may have occurred due to the satellite RNA competing with the PSTVd for some proteins such as VirP1 for transport into the nucleus or for the host polymerase in order to replicate.

IV. Hepatitis delta virus replication

Hepatitis delta virus (HDV) is a human pathogen that is a satellite associated with hepatitis B virus (HBV) (Diener and Prusiner, 1985; Rizzetto et al., 1977). HDV genome is a circular single-stranded RNA (Wang et al., 1986) of about 1.7kb, which has some striking similarities to viroids of plants in terms of its replication mechanism (Taylor et al., 1990). However, unlike viroids, it encodes

hepatitis delta antigen which occurs as 2 species, and needs proteins from HBV for obtaining its outer membrane (Lai, 1995).

HDV replicates in the nucleus via rolling circle mechanism during which a messenger RNA for hepatitis delta antigen is produced as an antigenomic-strand by the host DNA-dependent RNA polymerase II (Lai, 1995). The delta antigen is involved in stabilizing the HDV RNA (Lazinski and Taylor, 1994) and nuclear import of HDV (Chou et al., 1998). The replication involves a ribozyme (Jeng, Daniel, and Lai, 1996) and the synthesis of genomic and anti-genomic strand is performed by host DNA-dependent RNA polymerase II and I, respectively (Macnaughton et al., 2002). HDV is the only known animal virus that has a circular RNA genome and is a satellite of another virus. One characteristic it shares with plant satellite RNA is that it depends on its helper virus for the movement from cell to cell.

V. Scope of dissertation

In Chapter 1, agroconstructs for QSatRNA replication study will be described and their biological activities are tested. Experiments in chapter 2 investigate satellite RNA accumulation in the absence of its helper CMV in tobacco plants. In Chapter 3, QSatRNA replication mechanism will be further investigated by initiating replication with the negative-sense strand QSatRNA. Finally a new model for the satellite RNA variant-Q replication cycle will be proposed.

Figure A. CMV genome organization.

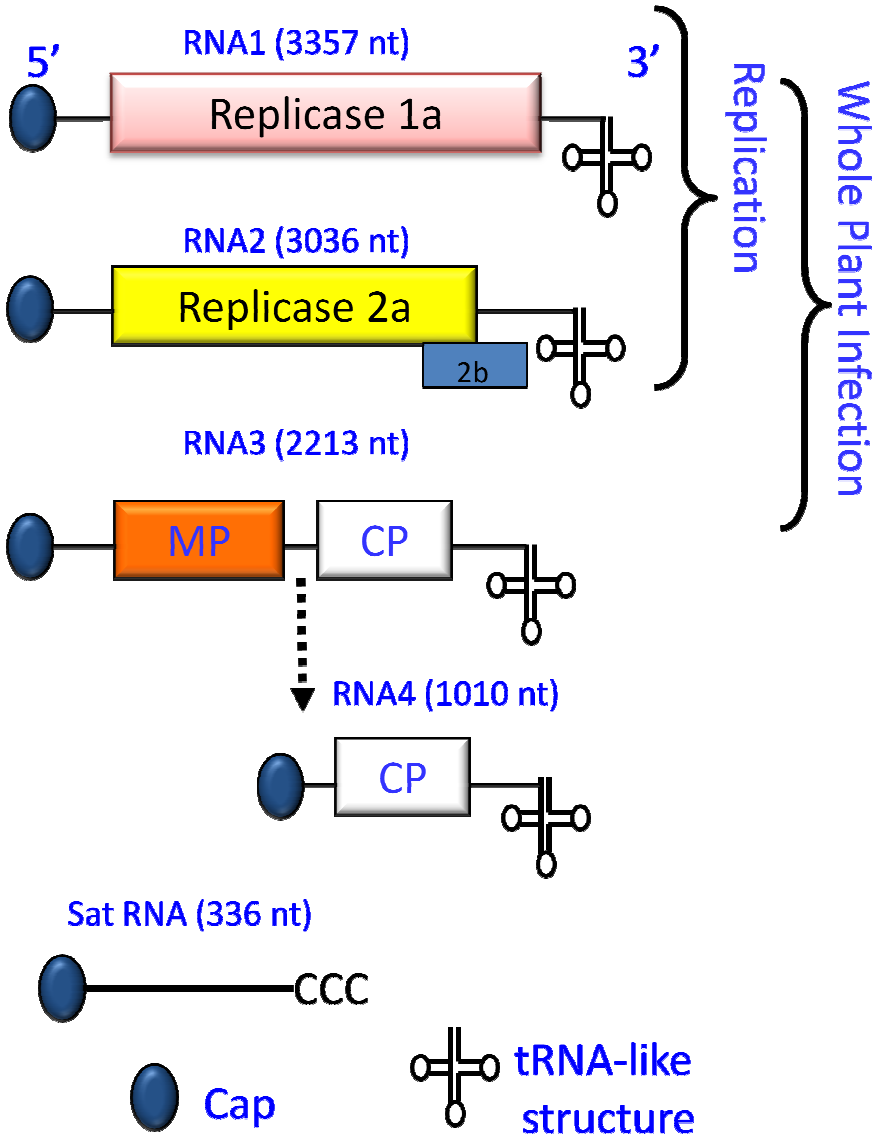
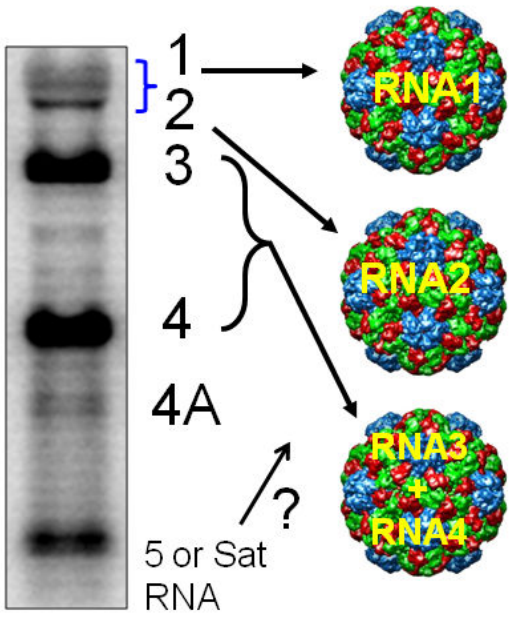


Figure B. CMV packaging scheme.



CHAPTER 1

Study of cucumber mosaic virus associated satellite RNA replication *in vivo* using agroinfiltration

ABSTRACT

Satellite RNA variant-Q associated with cucumber mosaic virus (CMV) is a highly structured single-stranded RNA molecule of 336 nucleotides in length. SatRNA is an important subviral pathogen in agriculture because it modulates symptom expression in CMV-infected plants. Although satRNA has no sequence homology with CMV genome, it is replicated by CMV replicase accumulating in monomeric and oligomeric forms. Previous studies on CMV satellite RNA replication involved using mechanical inoculation and/or transgenic plants. We used agroinfiltration methods to study the replication mechanism of the satellite RNA. This method allows the expression of individual RNA components of the virus uncoupled from virus replication. By using agroinfiltration method, we demonstrate that CMV coat protein expressed independently of replication can enhance the accumulation of individual CMV RNAs but not the satellite RNA. Virions formed by the CMV coat protein in the absence of virus replication

encapsidates some host RNA, and the satellite RNA can accumulate to a detectable level in the absence of the helper CMV.

INTRODUCTION

Satellite RNA (SatRNA) associated with cucumber mosaic virus (CMV) is a linear single-stranded RNA of 330-405 nucleotides in length (Simon, Roossinck, and Havelda, 2004) that has a highly structured secondary structure with about 50% intramolecular base pairing (Garcia-Arenal and Palukaitis, 1999). It does not encode any protein, and it is encapsidated by its helper CMV although it has no sequence homology with its helper. The specific satRNA used in this study is a variant-Q (QSat RNA) associated with CMV Q-strain (Q-CMV) (Gordon and Symons, 1983). The helper CMV belongs to family *Bromoviridae*. It is an economically important plant virus with icosahedral morphology (29 nm in diameter), and its genome consists of 3 single-stranded messenger-sense RNAs. CMV is transmitted by over 80 species of aphids (Palukaitis and Garcia-Arenal, 2003), and can infect over 1,200 species of various kinds of plants (Edwardson and Christie, 1991; Xu and Roossinck, 2000), thus it can have devastating effects in agriculture. The satRNA was discovered due to the tomato-necrosis epidemics in the early 1970's in French Alsace, where the satRNA aggravated the symptoms caused by CMV infection (Kaper, Tousignant, and Lot, 1976; Kaper and Waterworth, 1977). However, the association of SatRNA with CMV

usually attenuates the CMV infection symptoms. SatRNA of CMV is very useful for virus replication study because it does not encode any proteins necessary for its replication (Garcia-Arenal and Palukaitis, 1999).

The helper CMV produces only 5 proteins. 1a and 2a proteins are the major components of CMV replicase, and they are produced from RNA1 and 2, respectively (Habili and Symons, 1989; Hayes and Buck, 1990; Hodgman, 1988; Poch et al., 1989). 1a and 2a proteins play critical role in satRNA replication. 3a protein from RNA3 is the movement protein of CMV (Boccard and Baulcombe, 1993). CMV coat protein, and 2b protein which is a suppressor of RNAi, are produced from the subgenomic RNAs 4 and 4A, respectively (Ding et al., 1994; Habili and Francki, 1974a), and they will be described more in detail.

Coat protein

CMV particles are stabilized by RNA-protein interactions and disassembled easily at a high concentration of neutral chloride salts or in the presence of sodium dodecyl sulfate (Kaper, 1975). RNA-protein interaction is necessary for CMV coat protein (CP) assembly to form a virion, and the N-terminal arginine-rich RNA-binding motif (ARM) is the RNA binding site of the CP (Schmitz and Rao, 1998). In addition to its role in protecting viral RNAs from degradation (Palukaitis and Garcia-Arenal, 2003), the CP plays a role in the viral movement since it is required for CMV movement from cell to cell (Boccard and

Baulcombe, 1993; Scotti, Dearing, and Mossop, 1983). The CP also presents determinants for the transmission by the aphid vectors (Chen and Francki, 1990).

CP is expressed from CMV subgenomic RNA4 which is produced from replicating RNA 3. Dr. Padmanabhan in our lab has shown in an *in vivo* experiment that CP expressed *in trans* (CP^{trans}) enhances accumulation of individual CMV RNAs in the absence of viral replication (data not shown). Whether the CP has same effect on QSat RNA accumulation will be investigated.

2b protein

CMV 2b protein is a viral suppressor of RNAi (VSR) that binds to dsRNA (Brigneti et al., 1998; Ding et al., 1994; Li et al., 1999; Qi et al., 2004). Although it does not seem to affect CMV RNA accumulation in tobacco, CMV RNAs do not accumulate well without 2b in cucumber plants (through communication with Dr. Shou-wei Ding). Absence of 2b protein does not affect Sat RNA replication in the presence of CMV (Ding et al., 1995). In Figure 1-5, various VSRs including QCMV 2b (Q2b) are co-expressed with QSat RNA to study accumulation of QSat RNA independent from its helper CMV.

Agrobacterium-mediated gene transfer as a tool to study satellite RNA replication. Transient *in vivo* assay mediated by *Agrobacterium tumefaciens* (Agroinfiltration) is employed in this study. This method produces rapid results

(Janssen and Gardner, 1990), and this method is superior to mechanical inoculation in following ways: (i) vector expressing the gene of interest (GOI) can be delivered efficiently to all the cells in the infiltrated leaf; (ii) it ensures synchronized delivery of multiple plasmids to the same cell (Annamalai and Rao, 2005b; Marillonnet et al., 2004); (iii) it provides steady supply of GOI *in vivo* for several days; and (iv) it enables the expression of CMV RNA and/or mRNA along with the satRNA in the absence of the viral replicase to study the effect of CMV protein expressed *in trans* on the satellite RNA survival without the helper virus.

Binary vector plasmid used in this system (Table 1.1) has a multiple cloning site (MCS) that is preceded by two consecutive Cauliflower Mosaic Virus (CaMV) 35S promoters, and is followed by a a terminator sequence (Fig. 1.1). The delivery of the T-DNA vector to the nucleus via *Agrobacterium* results in the synthesis of RNAs by the host polymerase II. The resulting RNAs having a 5'-cap are exported to the cytoplasm.

By utilizing agroinfiltration system, QSatRNA accumulation has been studied in the presence and/or absence of the viral replicase and/or CP and described in this chapter. The implications of the new finding will be discussed.

MATERIALS AND METHODS

Constructs and primers: (See Tabe 1.1 and 1.2)

Agroinfiltration method for in vivo expression of genes of interest

Agrotransformation and agroinfiltration was done as described previously (Annamalai and Rao, 2006). Briefly, the *Agrobacterium* strain EHA105 (Fig. 1.2-6) or GV3101 (Fig. 1.7) were transformed with desired binary vector construct by freeze and thaw method (Annamalai and Rao, 2006). The transformed agrobacteria were grown at 28ºC for 48 hours on LB plates containing the desired antibiotics. A single colony was inoculated into a 2-5ml LB medium with the antibiotics, and grown at 28ºC for 48 hours with vigorous shaking. 0.5 mL of the culture was transferred to 50mL medium containing the appropriate antibiotics, 10mM MES (pH 5.6) and 40µM acetosyringone. After 14-18 hour incubation at 28ºC with vigorous shaking, the agrobacteria were washed and resuspended in 10mM $MgCl_2$ to reach the OD_{600} of 0.8. Acetosyringone was added to final concentration of 0.25mM. For co-infiltration involving more than one kind of agrotransformant, equal volumes of each culture were mixed. The acetosyringone added agrotransformant cultures were kept at room temperature for at least 3 hour without shaking. The cultures were infiltrated using 1cc syringe without a needle into the abaxial surface of either *N. bentamiana* or *N. clevelandii* leaves. The leaves were harvested 4 days post infiltration, unless otherwise specified.

Total RNA extraction from leaves

Total RNA extraction from leaves were essentially done as described (Annamalai and Rao, 2005b). Briefly, 1 gram of leaf tissue was ground with liquid nitrogen, and then 1ml of hot RNA extraction buffer (86ºC; 100mM Tris pH9.0, 10mM EDTA, 100mM LiCl, 1% SDS, and equal volume of phenol) and 0.5 ml chloroform-isoamyl alcohol (24:1, v/v) were added and further ground until the mixture was homogenized. The homogenized sample was transferred to a microcentrifuge tube, vortexed for 5 min and centrifuged at 13,000 x *g* for 5 min. The aqueous layer was transferred along with an equal volume of 4M LiCl to a fresh microcentrifuge tube and kept at -80ºC for at least 2 hours to precipitate. After thawing, it was centrifuged at 13,000 x *g* for 20 min at 4ºC. The pellet was washed with 70% ethanol, dried and resuspended in desired volume of RNase free water.

Virion purification

For each gram of the infected leaf material, 2ml of the citrate buffer (0.5M Sodium Citrate, 5mM EDTA, and 0.5% TGA (v/v)) was used. Ground desired amount of the infected leaf material with sterile sand in appropriate volume of the citrate buffer. The ground leaf material was strained through double layer cheesecloth into a flask. The sample was centrifuged at 13,000 x *g* (low speed centrifugation). The supernatant was transferred to a new centrifuge tube and

equal volume chloroform was added, shaken, and then centrifuged by low speed centrifugation. The aqueous layer was saved and triton-X 100 was added (2% v/v) and dissolved. The sample was spun by low speed centrifugation to get rid of the cell debris further and the resulting supernatant was placed in a new centrifuge tube with a 10-12% sucrose-cushion. The sample was centrifuged at ≥30,000 x *g* (high-speed centrifugation) for 3 hours and the virus pellet was resuspended in desired amount of CMV suspension buffer (5mM Sodium Borate and 0.5mM EDTA pH 9.0). The sample was stored at 4ºC and used within 3 days after the preparation. If further purification was needed, the sample was subjected to 5%-25% sucrose gradient.

Virion RNA extraction

To the purified virus sample, bentonite (final conc. 5mg/ml), SDS (final conc of 2%), and equal volume of phenol/chloroform were added, and emulsified. The sample was centrifuged and the aqueous phase was transferred to a clean tube. Phenol/chloroform (P/C) extractions were repeated as needed. The P/C extracted sample was subjected to ethanol precipitation, centrifuged, and the RNA pellet was washed with 70% ethanol. The washed RNA pellet was resuspended in desired volume of RNase-free water.

Electron microscopy

Purified virus preparation at a concentration of approximately 50µg/ml was placed on a glow-discharged carbon coated copper grid and stained with 1% uranyl acetate for negative staining (Annamalai and Rao, 2005a). FEI Tecnai12 transmission electron microscope was used to examine the grid and images were recorded.

Northern blot analysis

For Northern blot analysis, specified amount of RNA sample in 10 ul of RNase-free water was mixed with 10µl of sample buffer (10x MOPS buffer/formaldehyde/formamide in the ratio of 1:1.8:5, respectively), heated at 65ºC for 15 minutes and electrophoresed in 1.5% agarose-formaldehyde gel (Sambrook and Russell, 2001) until desired fractionation was achieved. The fractionated RNA was transferred to a nylon membrane with a VacuGene XL blotting unit (Pharmacia Biotech). The nylon membrane was pre-hybridized and hybridized using the appropriate buffers as described (Rao et al., 1994) and appropriate ^{32}P-labelled riboprobes.

Synthesis of the ^{32}P-labeled riboprobes.

For preparing strand-specific Q-Sat riboprobes a PCR amplified cDNA clone corresponding Q-Sat was subcloned to a *Hin*dIII/*Eco*RI treated pT7/T3 vector. ^{32}P-labeled T3 RNA polymerase transcripts from *Eco*RI linearized plasmids were used to specifically detect (+)-Q-Sat progeny whereas T7 RNA polymerase transcripts from *Hin*dIII linearized plasmids were used specifically detect (-)-Q-Sat progeny.

CMV probes were prepared in two ways: (1) For preparing (+)-strand, or (-)-strand specific CMV probe, *Bam*HI-digested pUCT7Q2-TLS #2, or #11 (a gift from Dr. Shou-wei Ding), respectively, was used to produce ^{32}P-labeled T7 RNA polymerase transcripts. (2) T7 RNA polymerase transcripts from *Hin*dIII linearized pT7/T3 kin-CMV RNA3 plasmids were used to specifically detect (+)-CMV RNA progeny whereas T7 RNA polymerase transcripts from *Eco*RI linearized plasmids were used specifically detect (-)-CMV RNA progeny.

Host RNA probe

Host cellular RNA probe was prepared as described (Annamalai and Rao, 2005b). Briefly, total RNA from healthy leaves were prepared by using hot-phenol method (Verwoerd, Dekker, and Hoekema, 1989). RNAs were de-capped using tobacco acid pyrophosphate (Epicenter, Madison, WI), and dephosphorylated

with alkaline phosphatase. The resulting RNAs were end-labeled with γ-[^{32}P]-ATP using T4 polynucleotide kinase (Sambrook and Russell, 2001).

Electrophoresis of virion RNA

Purified virion RNAs were denatured in urea solution (approximately 10% v/v) at 50 °C for 5 minutes. subjected to electrophoresis in 1.5% agarose gels prepared and electrophoresed in TBE buffer (1X, pH 7.0) (Sambrook and Russell, 2001). Gels are stained with ethidium bromide and photographed under UV light.

Western blot analysis

Proteins were extracted from the infected leaves by grinding in extraction buffer (final concentration: 125mM Tris, pH 6.8, 2.5% [w/v] dithiothretol (DDT), 2% SDS, 0.01%). Samples were suspended in SDS-PAGE sample buffer (final concentration: 125mM Tris, pH 6.8, 10%[w/v] glycerol, 2.5% [w/v] dithiothretol (DDT), 2% SDS, 0.01% bromphenol blue), and denatured at 100ºC for 5 minutes. The proteins were separated according to the size on 15% SDS-PAGE according to Laemmli (Laemmli, 1970). The separated proteins were transferred to a nitrocellulose membrane and probed with CMV CP antibody (1:1000 dilution). The secondary antibody conjugated with alkaline phosphatase was used, and

visualized with the combination of NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt).

RESULTS

Characteristic features and biological activities of T-DNA-based constructs of Q-CMV and Q-satellite RNA.

To study replication mechanism involved in satRNA variant-Q (QSat RNA) associated with Q-CMV using agroinfiltration, it was necessary to make T-DNA based constructs for expressing CMV genomic RNAs and QSat RNA. The T-DNA based Q-CMV RNA1 was already available (gift from Dr. Shou-wei Ding in the Plant Pathology Department at UCR). The constructs for expressing Q-CMV RNAs 2 and 3, QSat RNA, and Q-CMV coat protein (CP) were made and described in Fig. 1-1 A-D and Table 1.1 and 1.2. Q-CMV RNAs 1, 2, 3, and Q-Sat constructs are named pQ1, pQ2, pQ3, and pQSat, respectively, They were engineered to initiate transcription precisely at the authentic 5'-end by the cauliflower mosaic virus 35S promoter and terminated by 35S terminator which gives 3'-polyA tail (Fig. 1-1A-D). pQ2 and pQ3, however, contained tobacco ringspot virus (TRSV) ribozyme (RZ) sequence following the Q-CMV cDNA so that the expressed Q-CMV RNAs would have polyA tails cleaved out to mimic more closely to the wild-type Q-CMV RNAs (Fig. 1-1 B and C). The construct expressing Q-Sat RNA contained hepatitis delta virus (HDV) RZ after the Q-Sat

cDNA so that the correctly initiated Q-Sat RNA transcript would have a correctly terminated 3'-end (Fig 1-1D).

The biological activities of the above constructs (Fig. 1-1 A-D) were evaluated. Four days after the agroinfiltration for co-expressing the constructs pQ1, pQ2, and pQ3 (hereafter denoted as Q1+Q2+Q3 or CMV) the infiltrated leaves were harvested, and the virions were purified. Using transmission electron microscopy (TEM), the wild-type CMV particles were observed (Fig. 1-2A, left panel). The viral RNAs were extracted from the virions and subjected to Northern blot analysis. The Q-CMV RNA, or QSat RNA specific ^{32}P-UTP labeled riboprobe was used to detect the CMV RNAs, or QSat RNA, respectively. As shown in Fig. 1-2B, both genomic and subgenomic RNAs were detected which means that the agroconstructs were biologically active to make the wild type CMV. Agroinfiltration with the combination of Q1+Q2+Q3+QSat (CMV+QSat) resulted in formation of virions indistinguishable from the CMV without QSat RNA (Fig. 1-2A, right panel). The Northern blot analysis with the virion RNA showed all CMV genomic and subgenomic RNAs with the QSat RNA in both monomeric and dimeric forms, which is a characteristic of replicated QSat RNA (Roossinck, Sleat, and Palukaitis, 1992). In the earlier studies, a series of satRNA multimers were found in plants infected with CMV carrying satRNA (Kuroda et al., 1997; Linthorst and Kaper, 1985; Young, Palukaitis, and Zaitlin, 1987). SatRNA multimers were probably produced by the reinitiation of replication on the 3'-end before the nascent SatRNA molecule is released (Kuroda et al., 1997). In order to confirm

the Northern blot results further, urea-denatured virion RNAs of CMV and CMV+QSat along with BMV RNA as a marker were run on 1.2% agarose gel electrophoresis in 1x TBE buffer (Fig. 1-2C), and the result was consistent with the Northern blots in Fig. 1-2B.

Transient expression of Q CMV coat protein via agroinfiltration

SatRNAs are dependent on its helper CMV for replication and encapsidation (Gould et al., 1978; Linthorst and Kaper, 1985). In order to study SatRNA encapsidation uncoupled from virus replication, we engineered pQCP to express Q CMV coat protein (QCP) *in vivo* independently from CMV RNAs.

The coat protein of Q-CMV can only be translated from the subgenomic RNA 4 which is produced from replicating RNA 3. In order to uncouple CP production from CMV replication, a construct was designed that makes Q-CMV CP mRNA (pQCP; Fig. 1-3A). The construct expressing Q-CMV CP mRNA was designed so that it would provide the viral CP *in trans* (CP^{TRANS}). The 5'-end of Q-CP ORF was fused to *Tobacco etch virus* (TEV) translational enhancer sequence and subcloned into the binary vector pCass4 (Fig. 1-3A). It was anticipated that following agroinfiltration, the mRNA synthesized from pQCP agrotransformant would result in efficient translation of wild type Q-CP. Thus, plants infiltrated with pQCP agrotransformant were processed for the detection of QCP expression. Northern blot analysis with QCP specific probe showed that CPmRNA was

expressed in the cell and also encapsidated (Data not shown). Western blot analysis demonstrated that efficient expression of Q-CP with predicted molecular weight (~24.5 kDa) had occurred in infiltrated plants (Fig. 1-3B). To verify whether transiently expressed QCP is competent for virion assembly, virions were purified from infiltrated plants and subjected to TEM. Although fewer particles were detected under EM compared to the wild-type, their morphology and appearance resembled those of CMV (Fig. 1-3C). CMV virions are stabilized by RNA-protein interactions (Kaper and Geelen, 1971). Therefore virus assembly occurs only in the presence of RNA. To verify packaging of individual CMV genomic RNAs by CP^{Trans}, plants were co-infiltrated with inoculum containing agrotransformants of Q-CP and a desired genomic RNA component (e.g. Q-CP+Q1). It showed that CP^{Trans} is competent to package each of CMV genomic RNA (data not shown).

Average diameter of the virions formed by CMV only, CMV harboring QSatRNA (CMV+QSatRNA) and CP^{Trans} are shown in histogram (Fig. 1-3D). Diameters of 49 CMV particles, 77 CMV+QSatRNA particles, and 93 CP^{Trans} particles were measured and their average diameters were 29.2 nm (standard deviation (s.d.) =1.9), 27.6 nm (s.d.=4.6), and 30.8 nm (s.d.=2.1), respectively. By using Student's t-test, CMV and CP^{Trans} virions had diameters that are statistically the same. However, the diameter of the virions formed by CMV with SatRNA varied more compared to the virions formed by CMV or CP^{Trans}, and the average diameter compared to the other two virions types was statistically

different. If it is true, increased size variation resulting from SatRNA encapsidation may imply how the SatRNAs are packaged. The bigger virions may contain SatRNAs along with the CMV RNAs and the smaller virions may have resulted from multiple SatRNAs packaged without any CMV RNAs.

Effect of QCP on SatRNA accumulation *in vivo*.

The CMV constructs described above were used in various combinations to study the effects of CP^{TRANS}, Q-Sat, and CMV RNAs on each other (Fig. 1-4). Western blot analysis was done to confirm the expression of QCP (Fig. 1-4A, bottom). As described previously (Wu and Kaper, 1995), CMV RNA accumulation was reduced in the presence of Q-Sat (Fig. 1-4A, lanes 12 and 13, 2nd blot from top). QSatRNA accumulation was reduced by co-expression with CP^{TRANS} in the presence of the viral replicase (Fig. 1-4A, lanes 10 and 11, top blot), however, CMV RNAs 1 and 2 were accumulated to a higher level in the presence of CP^{TRANS} (Fig. 1-4A, lanes 8 and 9, 2nd and 3rd blot from the top). Since the negative-sense CMV RNAs 1 and 2 accumulated to about equal levels to its corresponding positive-sense RNA, the accumulation must had been due to some interaction with the CP. The QSat RNA in the absence of CMV replicase (Q1+Q2) did not accumulate to a detectable level, and co-expression with CP did not affect it (Fig. 1-4A, lanes 4 and 5, top blot). However, QSatRNA accumulated to a detectable level in the presence of p19, a viral suppressor of RNAi (VSR; Fig. 1-4A, lanes 6 and 7, top blot). P19 is from *Tombusvirus* (tomato bushy stunt

virus; cymbidium ringspot virus), and it preferably binds to 19nt RNA duplex (Qi et al., 2004; Silhavy et al., 2002; Voinnet, Pinto, and Baulcombe, 1999). Further investigation on QSatRNA accumulation in the absence of CMV replicase will be shown in Fig. 1-5.

Replication-independent encapsidation by Q-CP

The presence of QCP in the samples used in Fig. 1-4A resulted in virion formation (EM data not shown). The virion RNAs were extracted and Northern blot analysis was done to check the RNA contents of the virions (Fig. 1-4B). The QSatRNA and CMV RNAs that accumulated to a detectable levels in the Fig. 1-4A were packaged (Fig 1-4B, top and middle blots). When the virion RNAs were probed with host RNA riboprobe, CP^{Trans} virions contained a host RNA of a size between 1 kb to 1.5kb, whereas in the presence of the viral replicase (Q1+Q2), the virions did not contain such host cellular RNA (Fig. 1-4B, bottom gel). Further investigation will be done to identify the encapsidated cellular RNA.

Affect of 2a *in trans* in QSat replication

CMV produces 2a protein from its genomic RNA 2. This protein is an integral part of viral replicase because it has RNA-dependent RNA polymerase function (Hodgman, 1988). We wanted to see whether 2a expressed independent of RNA2 ($2a^{trans}$) effects Q-Sat RNA accumulation. The T-DNA constructs that express CMV 2a (made by Dr. de Wispelaere) was used. When

RNA 2 was substituted with 2a (viral polymerase), Q-Sat RNA replication was not affected (data not shown). However, 2a alone did not assist in QSat RNA accumulation (data not shown).

Autonomous amplification of QSat RNA

In the absence of its helper virus, QSatRNA accumulated to a detectable level when co-expressed with p19 (Fig. 1-4, lanes 6 and 7, top blot). We tested whether other VSRs can produce the same results. Q2b and T2b protein are VSRs from *cucumoviruses* cucumber mosaic virus and tomato aspermy virus, respectively, and has roles in dsRNA binding (Brigneti et al., 1998; Li et al., 1999; Qi et al., 2004). HC-Pro is a VSR of *Potyvirus* (tobacco etch virus; potato virus Y) (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998; Kasschau et al., 2003). In the absence of the helper viral replicase, Q-Sat RNA accumulated to a detectable level in the presence of any of the 4 VSRs (Fig. 1-5A). However, there was a distinctive difference between the Q-Sat accumulated in the presence or absence of the helper viral replicase. In the absence of the helper viral replicase, Q-Sat RNAs were mostly in dimeric and trimeric forms, whereas in the presence of the viral replicase, they were mostly in monomeric forms (Fig. 1-5A).

To ensure that QSatRNA multimers formed in the absence of its helper CMV was not an artifact of agroinfiltration, we used T-DNA based Q-CMV RNA5 (Q5) construct. Unlike Q-Sat, Q5 did not form multimeric forms in the absence of

the viral replicase and the presence of VSR did not have any effect (Fig.1-5B). When the Q-Sat was co-expressed with Q-CP using agroinfiltration, and the virions were purified, we found that Q-Sat expressed in the absence of the viral replicase were encapsidated into the virions (Fig. 1-5C).

Above experiments were done with the *Agrobacterium* strain EHA105. We tested another *Agrobacterium* strain, GV3101 for agroinfiltration of Q-Sat. With GV3101 we found that Q-Sat can be expressed without co-infiltration with VSR (Fig. 1.7). GV3101 probably has some RNAi suppressing activity and this will be discussed more in detail in the discussion section.

DISCUSSION

In this chapter, the experiments were carried out by using agroinfiltration system instead of the mechanical inoculation method utilized in previous studies on SatRNA replication done by other groups. It allowed us to perform experiments that could not have been done previously. The agroconstructs described in this chapter were biologically active to express CMV RNAs and QSatRNA (Figs. 1-1 and 1-2). Furthermore, it made *in vivo* expression of QCP uncoupled from CMV replication possible, which allowed *in vivo* analysis of the virions formed by CP^{Trans} (Fig. 1-3). In this study, we demonstrated that QCP can enhance the accumulation of CMV RNAs (Fig. 1-4A), and QSatRNA can be accumulated to a detectable level in the absence of the helper virus when co-expressed with a VSR (Fig 1-4 and 1-5A).

As mentioned earlier, agroinfiltration is a transient assay mediated by *Agrobacterium tumefaciens* that produces rapid result (Janssen and Gardner, 1990). Most of the *A. tumefaciens* strains used in the laboratory originate from either C58 isolate (Hamilton and Fall, 1971) or Ach5 isolate (LBA4404; (Hoekema et al., 1983)). The strain designations come from what specific Ti plasmid derivatives, which are available in various forms, the *A. tumefaciens* were engineered to carry (Hellens, Mullineaux, and Klee, 2000). The *A. tumefaciens* strains we used in this study were EHA105 (Hood et al., 1993) and GV3101 (Koncz and Schell, 1986). Using EHA105 strain, detection of autonomously accumulated Q-Sat RNA was successful only when VSR was co-expressed (Fig. 1-4 and 1-5). It may be due to RNAi elicited by agroinfiltration with EHA 105 since agroinfiltration can cause RNAi (Voinnet, 2008). This was resolved by providing VSR *in trans*. However, GV3101 did not require VSR co-expression (Fig. 1-6). This may imply the location of bacterial suppressor of RNAi, or BSR (Mosher and Baulcombe, 2008) on the resident Ti plasmid in GV3101. Interestingly, *in vivo* expression of individual QCMV RNA results in a very low accumulation. However, when CP^{trans} is co-expressed, it accumulates to a higher level (Dr. Annamalai, unpublished data). It is probably because of CP^{trans} binds and protects it from degradation, or it has a role in viral replication since it helps the negative sense RNAs to accumulate also.

Finally, it must be tested whether the accumulation of QSatRNA was just a product of agroinfiltration or it was a result of amplification ((-)-sense strand

synthesis) by the host. If it was amplified by the host, it raises two more questions, namely: (1) what is the subcellular location of the amplification site; and (2) how is the SatRNA amplified by the host different from the CMV replicated ones. These questions will be answered in the next chapter of this dissertation.

Figure 1-1 Schematic diagram of T-DNA based construct to study QSat RNA replication *in vivo* via agroinfiltration. (see Tables 1.1 and 1.2)

The binary vectors used are pCass4 (A), pCassRZ (B and C), and pCassHDV (D). They all have a double 35S promoter (35S), a multiple cloning site (MCS) where a cDNA can be inserted so that the transcripts with a correctly initiated 5'-end with a cap would be made. Following MCS is the 35S terminator. LB and RB denotes left border and right border of T-DNA, respectively.

(A) Q-CMV RNA 1 (Q1) cDNA is in pCass4 which would express Q1 with a poly A tail *in vivo*.

(B and C) Q-CMV RNA2 and 3 (Q1 and Q2, respectively) cDNAs are in pCassRZ vector. The pCassRZ vector contains tobacco ringspot virus (TRSV) ribozyme (RZ) down stream of MCS so that the expressed Q2 or Q3 would have a poly A tail cleaved out to mimic more closely to the authentic viral 3'-end.

(D) QSatRNA cDNA is in pCassHDV which has HDV ribozyme sequence. This construct was engineered so that, in addition to the correctly initiated 5'-end, the Q-Sat transcript would have correctly terminated 3'-end.

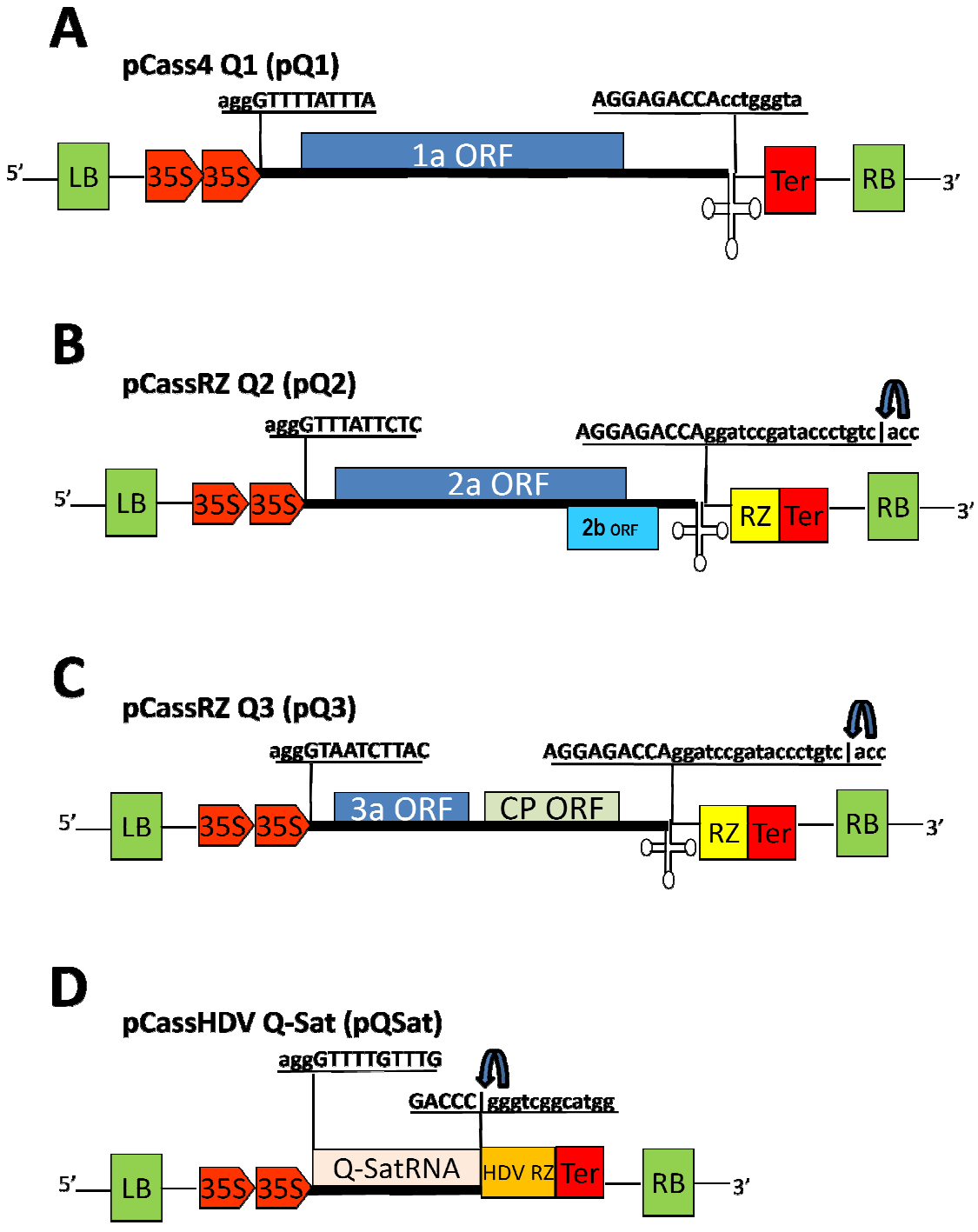
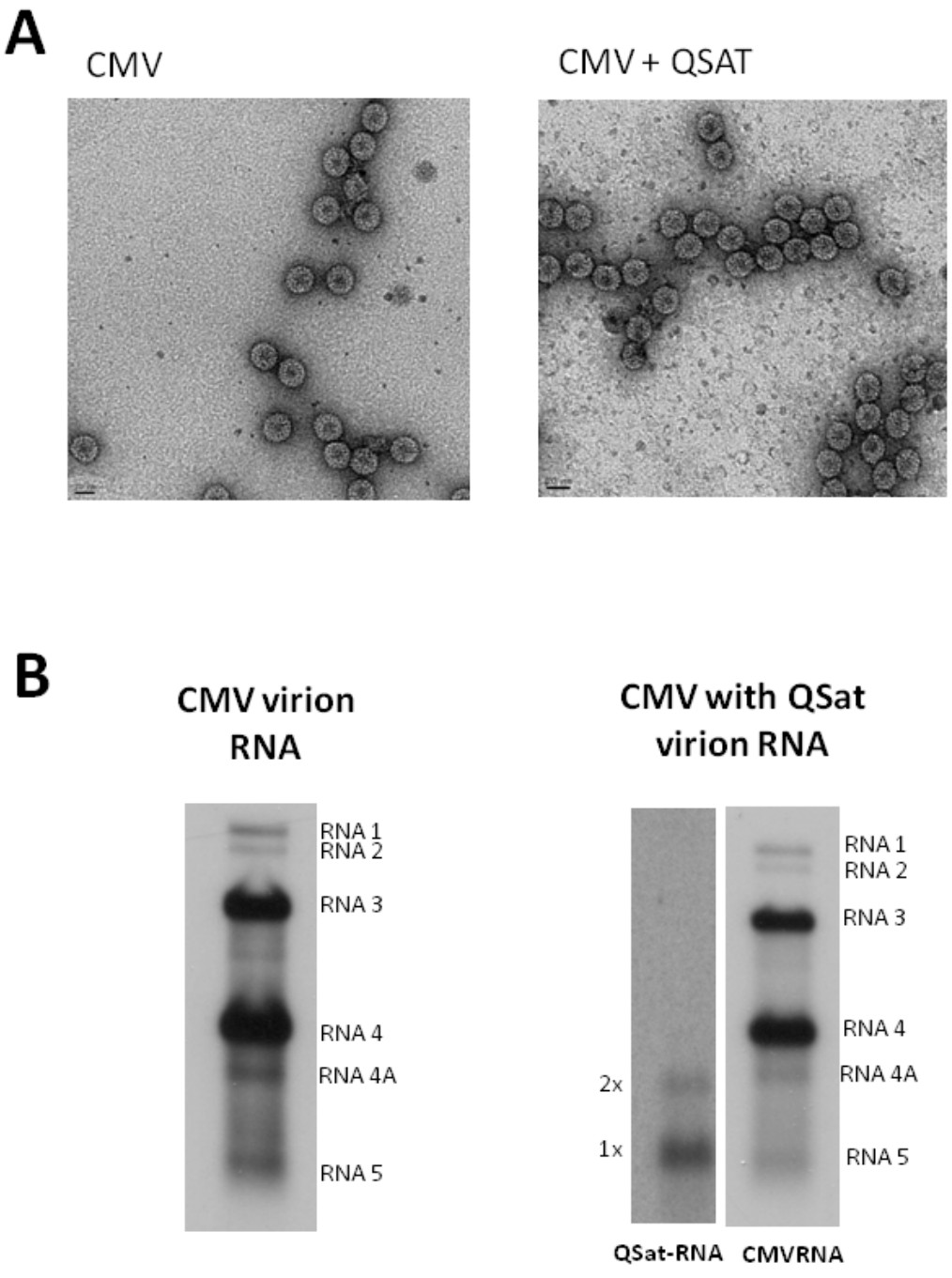

A
pCass4 Q1 (pQ1)
aggGTTTTATTTA
AGGAGACCAcctgggta
5'
LB
35S
35S
1a ORF
Ter
RB
3'
B
pCassRZ Q2 (pQ2)
aggGTTTATTCTC
AGGAGACCAggatccgataccctgtc|acc
5'
LB
35S
35S
2a ORF
2b ORF
RZ
Ter
RB
3'
C
pCassRZ Q3 (pQ3)
aggGTAATCTTAC
AGGAGACCAggatccgataccctgtc|acc
5'
LB
35S
35S
3a ORF
CP ORF
RZ
Ter
RB
3'
D
pCassHDV Q-Sat (pQSat)
aggGTTTTGTTTG
GACCC|gggtcggcatgg
5'
LB
35S
35S
Q-SatRNA
HDV RZ
Ter
RB
3'

Figure 1-2 Biological activity of the agroconstructs for *in planta* expression of CMV and QSat RNA.

(A) Transmission electron microscopy image of the virions produced from agroinfiltration with pQ1+pQ2+pQ3, which results in Q-CMV production *in vivo* (left) or pQ1+pQ2+pQ3+pQSat, which results in production of Q-CMV with QSatRNA production in vivo (right). Bar: 20nm.

(B) Northern blot analysis of virion RNA extracted at 4 days post agroinfiltration with pQ1+pQ2+pQ3 (left). The membrane was hybridized with riboprobes that detect CMV RNAs. On the right side, duplicate Northern blot membranes were made with virion RNA from pQ1+pQ2+pQ3+pQSat infiltrated leaf and probed with riboprobes that detect CMV RNAs or QSat RNA. 1x and 2x denote QSat RNA monomer and dimer, respectively.

(C) RNAs were extracted from the virions purified from pQ1+pQ2+pQ3 (CMV) or pQ1+pQ2+pQ3+pQSat (CMV + QSat) infiltrated leaves, denatured with urea, and subjected to electrophoresis (1% agarose in 1x TBE buffer). Brome mosaic virus (BMV) virion RNA is in the first lane as a control.


A
CMV
CMV + QSAT
B
CMV virion RNA
CMV with QSat virion RNA
RNA 1
RNA 2
RNA 3
RNA 4
RNA 4A
RNA 5
2x
1x
QSat-RNA
CMVRNA

C

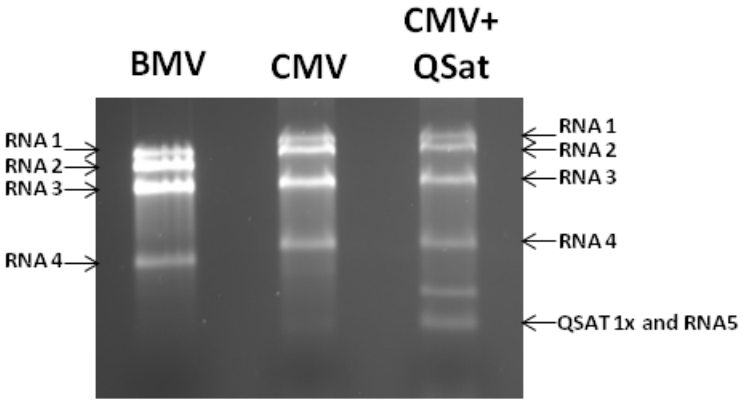

BMV
CMV
CMV+
QSat
RNA 1
RNA 2
RNA 3
RNA 4
RNA 1
RNA 2
RNA 3
RNA 4
QSAT 1x and RNA5

Figure 1-3 Biological activity of the agroconstruct for *in planta* expression of Q-CMV coat protein *in trans*.

(A) Schematic diagram of a T-DNA based Q-CMV coat protein (QCP) construct for *in planta* expression of Q-CP via agroinfiltration. pCass4 vector is used. The QCP open reading frame is preceded by tobacco etch virus (TEV) translation enhancer sequence.

(B) Virions were purified from pQCP agroinfiltrated leaf and subjected to Western blot analysis along with CMV virion and protein ladder. CMV CP specific antibody was used. The position of 27kDa is indicated.

(C) Transmission electron microscopy image of the virions formed in the leaf infiltrated with pQCP agrotransformant. The blue arrows point to the virions. Bar: 50nm.

(D) Virion diameter comparison between CMV, CMV harboring QSatRNA and CP^{Trans} virions. Average diameters of each type of virions in nanometer (nm) are shown in colored columns with the error bars indicating the corresponding standard deviation.

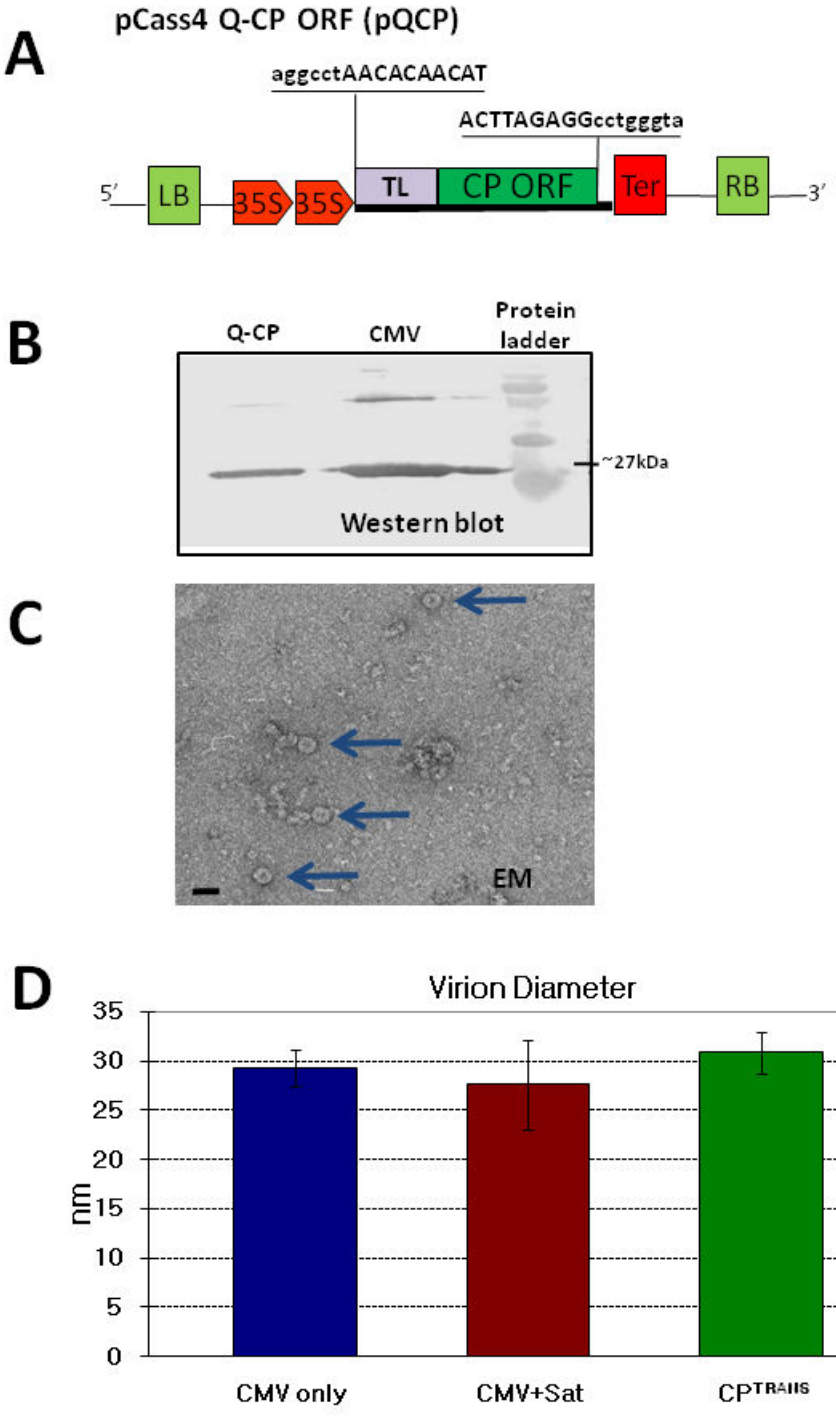
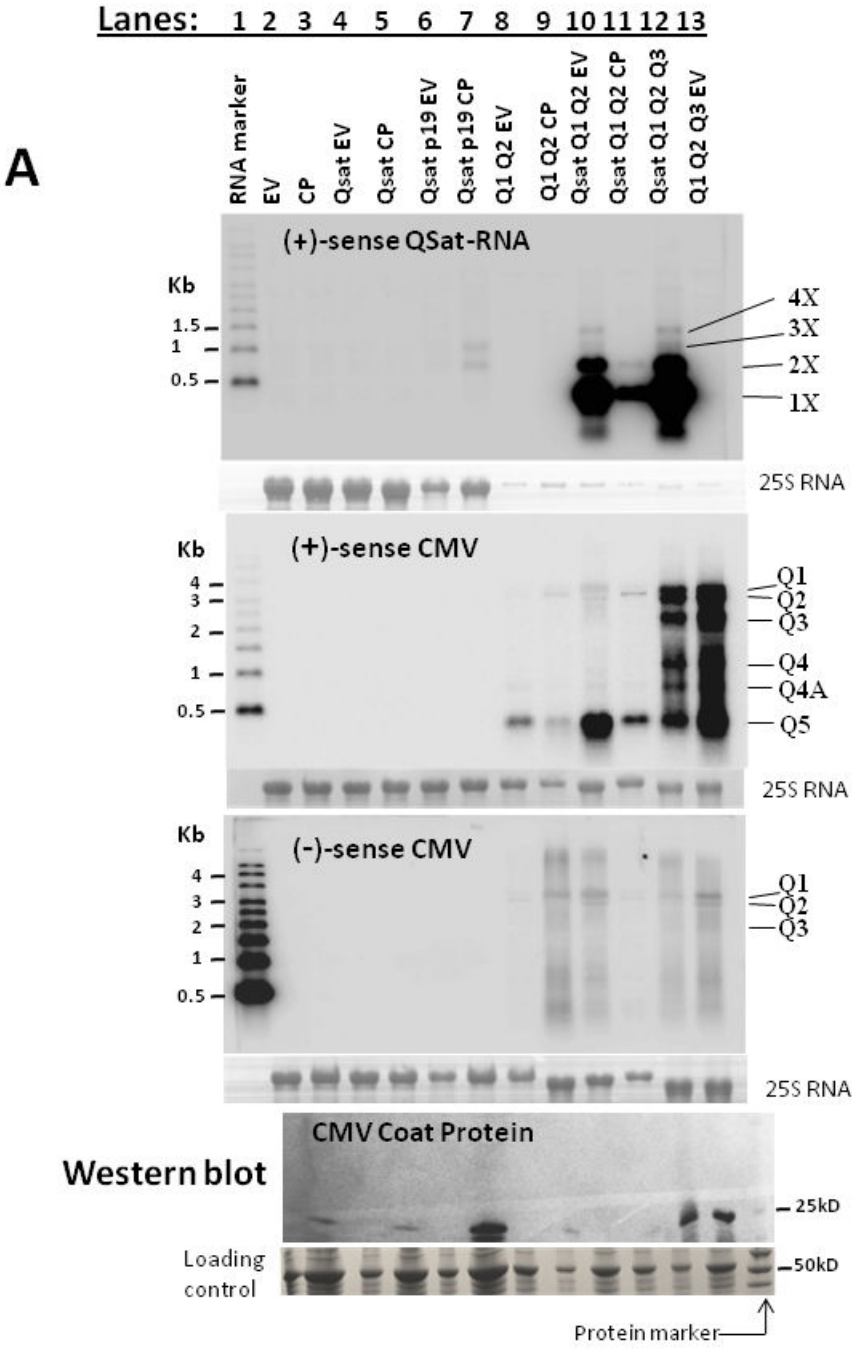

A
pCass4 Q-CP ORF (pQCP)
aggcctAACACAACAT
ACTTAGAGGcctgggta
5′
LB
35S
35S
TL
CP ORF
Ter
RB
3′
B
Q-CP
CMV
Protein ladder
~27kDa
Western blot
C
EM
D
Virion Diameter
35
30
25
20
15
10
5
0
nm
CMV only
CMV+Sat
CP TRAIIS

Figure 1-4 Northern blot analysis to test the effect of QCP on QSatRNA accumulation *in planta*.

(A) Northern blot analysis of total RNA recovered at 4 days post infiltration (dpi) from *N. clevelandii* leaves agroinfiltrated with indicated cultures. Triplicate Northern membranes were generated and each one was prehybridized with riboprobes specific for either (+)-sense QSat RNA (top blot), (+)-sense CMV RNAs (2nd blot from the top), or (-)-sense CMV RNAs (3rd blot from the top). The positions of CMV RNAs, QSat RNA monomer (1x) and dimer (2x) are indicated on the right. Methylene blue stained 25S rRNA shown on the bottom of each Northern blot image as a loading control. Lane numbers are shown on the top. For all the Northern gels, 20 µg of total RNA of each sample was used except for the top blot lanes 8-13 where 1 µg of total RNAs were used. The bottom panel is the Western blot to show QCP expression. RNA marker (RNA Millenium^{TM} Marker, Ambion) was loaded to measure the sizes of the RNA bands.

(B) Northern blot analysis of virion RNAs recovered from the same leaf samples in panel A. Triplicate Northern blots were generated and each one was hybridized with riboprobes specific for either (+)-sense QSatRNA (top blot), (+)-sense CMV RNA (middle blot), or Host cellular RNA (bottom blot) as indicated. Lane numbers are shown on the bottom. 500 ng of virion RNA from each sample was used, except for the lanes 6-9 on the top blot, where 100 ng of virion RNA was used. 1 µg of total RNA (TRNA) from empty vector (EV)-infiltrated leaves were loaded on lane 2 as a control.


A
Lanes: 1 2 3 4 5 6 7 8 9 10 11 12 13
RNA marker
EV
CP
Qsat EV
Qsat CP
Qsat p19 EV
Qsat p19 CP
Q1 Q2 EV
Q1 Q2 CP
Qsat Q1 Q2 EV
Qsat Q1 Q2 CP
Qsat Q1 Q2 Q3
Q1 Q2 Q3 EV
(+)-sense QSat-RNA
Kb
1.5
1
0.5
4X
3X
2X
1X
25S RNA
(+)-sense CMV
Kb
4
3
2
1
0.5
Q1
Q2
Q3
Q4
Q4A
Q5
25S RNA
(-)-sense CMV
Kb
4
3
2
1
0.5
Q1
Q2
Q3
25S RNA
Western blot
CMV Coat Protein
25kD
Loading control
50kD
Protein marker

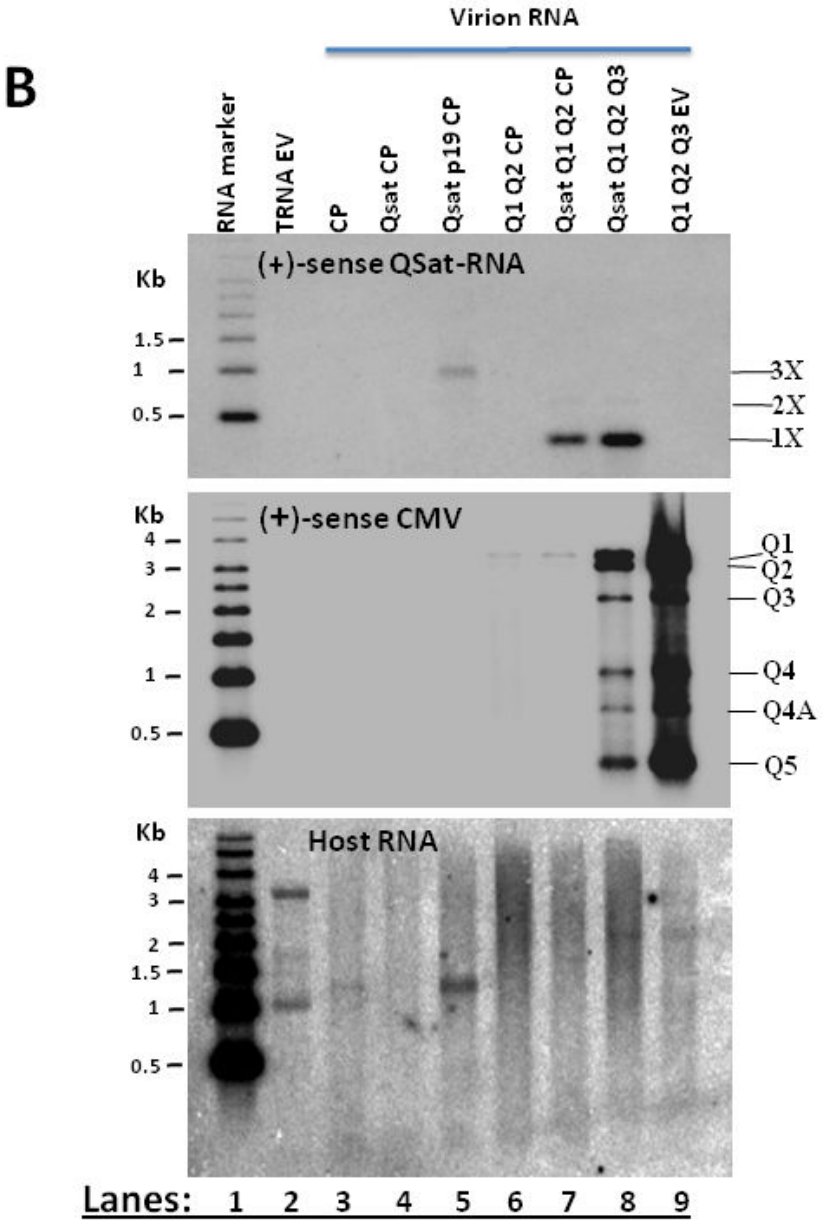

B
Virion RNA
RNA marker
TRNA EV
CP
Qsat CP
Qsat p19 CP
Q1 Q2 CP
Qsat Q1 Q2 CP
Qsat Q1 Q2 Q3
Q1 Q2 Q3 EV
Kb
(+)-sense QSat-RNA
1.5
1
0.5
3X
2X
1X
Kb
(+)-sense CMV
4
3
2
1
0.5
Q1
Q2
Q3
Q4
Q4A
Q5
Kb
Host RNA
4
3
2
1.5
1
0.5
Lanes: 1 2 3 4 5 6 7 8 9

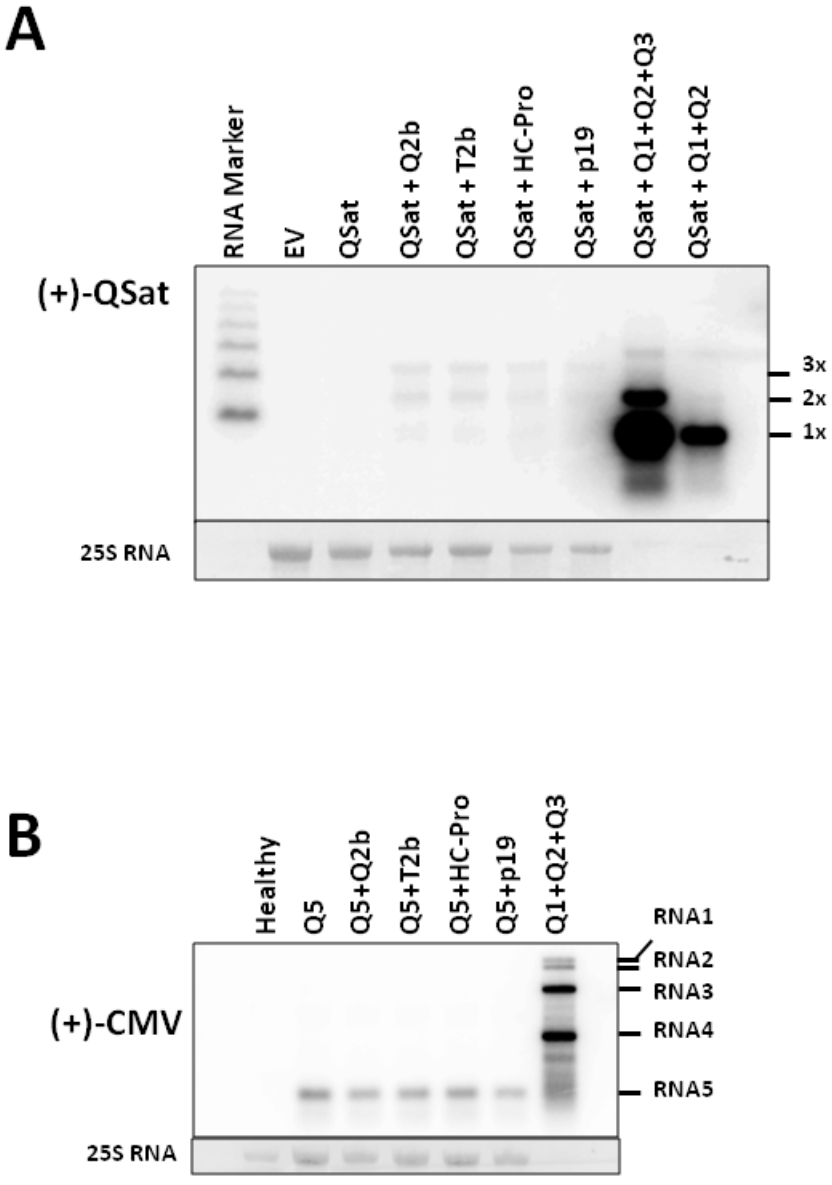
Figure 1-5 Detection of autonomously accumulated QSatRNA *in vivo* via agroinfiltration with EHA 105 agrotransformants.

(A and B) Northern blot analysis of total RNA recovered at 4 days post infiltration (dpi) from *N. clevelandii* leaves agroinfiltrated with indicated cultures. Q2b, T2b, HC-Pro, p19 refers to VSRs from *cucumber mosaic cucumovirus, tomato aspermy cucumovirus, potyvirus, and tombusvirus*, respectively. The positions of CMV RNAs, QSat RNA monomer (1x), dimer (2x), and trimer (3x) are indicated on the right. Q5 is the RNA5 expressed from pQ5 (Table 1.1). RNA marker (RNA Millenium^{TM} Marker, Ambion) was loaded to measure the sizes of the RNA bands. Methylene blue stained 25S rRNAs are shown as a loading control.

(A) (+)-QSat RNAs are probed. 20 µg of total RNAs were used except for QSat +Q1+Q2+Q3 and QSat+Q1 +Q2 where 1 µg was used.

(B) (+)-CMV RNAs are probed. 10 µg of total RNAs were used except for Q1+Q2+Q3 where 1 µg was used.

(C) Northern blot of virion RNAs extracted from the virions formed by indicated cultures. The positions of QSat RNA monomer (1x), dimer (2x), and trimer (3x) are indicated on the right. (+)-QSat RNAs are probed. 500 ng of virion RNAs were loaded except for the 100 ng of RNAs loaded on the last 3 lanes.


A
RNA Marker
EV
QSat
QSat + Q2b
QSat + T2b
QSat + HC-Pro
QSat + p19
QSat + Q1+Q2+Q3
QSat + Q1+Q2
(+)-QSat
3x
2x
1x
25S RNA
B
Healthy
Q5
Q5+Q2b
Q5+T2b
Q5+HC-Pro
Q5+p19
Q1+Q2+Q3
RNA1
RNA2
RNA3
RNA4
RNA5
(+)-CMV
25S RNA

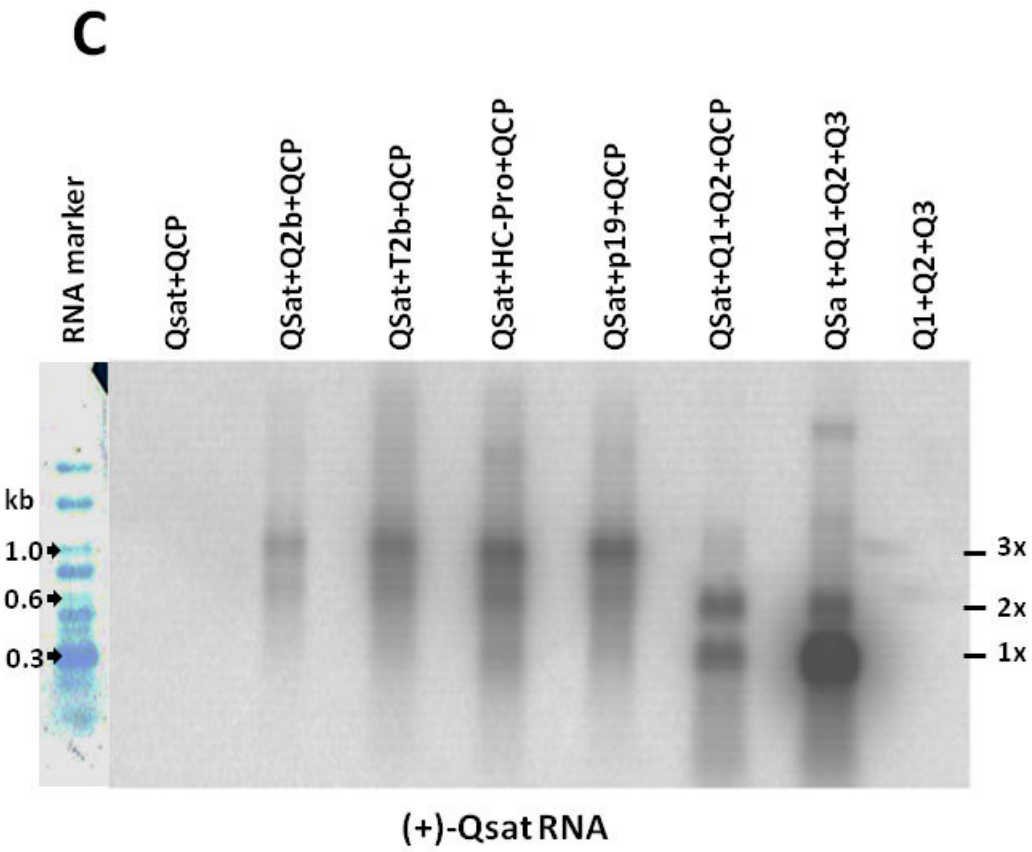

C
RNA marker
Qsat+QCP
QSat+Q2b+QCP
QSat+T2b+QCP
QSat+HC-Pro+QCP
QSat+p19+QCP
QSat+Q1+Q2+QCP
QSa t+Q1+Q2+Q3
Q1+Q2+Q3
kb
1.0
0.6
0.3
3x
2x
1x
(+)-Qsat RNA

Figure 1-6 Detection of autonomously acculated QSatRNA in vivo via agroinfiltration with GV3101 agrotransformants.

Northern blot analysis of total RNA recovered at 4 days post infiltration (dpi) from *N. clevelandii* leaves agroinfiltrated with indicated cultures. The positions of CMV RNAs, QSat RNA monomer (1x) and dimer (2x) are indicated. Methylene blue stained 25S rRNA shown on the bottom of each Northern blot image as a loading control.

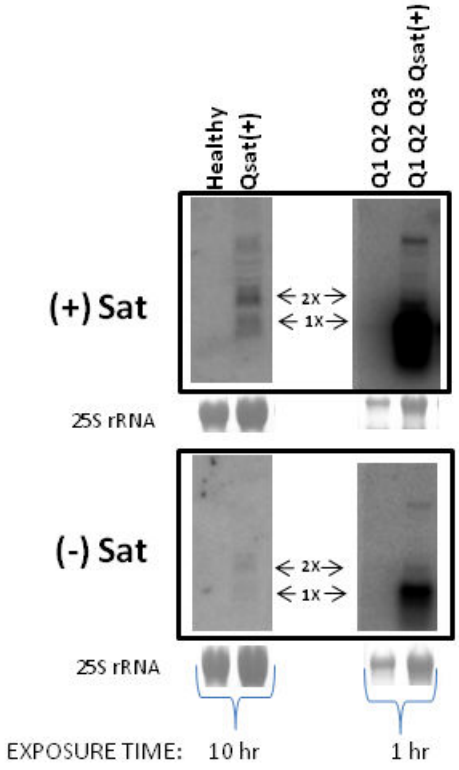

Healthy
Qsat(+)
Q1 Q2 Q3
Q1 Q2 Q3 Qsat(+)
(+) Sat
2X
1X
25S rRNA
(-) Sat
2X
1X
25S rRNA
EXPOSURE TIME:
10 hr
1 hr

Table 1.1 List of agro-plasmids and riboprobe templates			
Name of the construct	**vector type**	**RNA expressed (abbreviation)**	**Proteins expressed**
pQ1	pCass4 binary	Q-CMV RNA 1 (Q1)	1a of CMV
pQ2	pCassRZ binary	Q-CMV RNA 2 (Q2)	2a and 2b of CMV
pQ3	pCassRZ binary	Q-CMV RNA 3 (Q3)	MP and CP of CMV
pQ5	pCassRZ binary	Q-CMV RNA 5 (Q5)	none
pQCP	pCass4 binary	Q-CMV coat protein mRNA	CP of CMV
pQSat	pCassHDV binary	Q-Sat RNA (a variant Q)	none
Q2b	pCass4 binary	Q-CMV 2b mRNA	Viral suppressor of RNAi (VSR) 2b of CMV
T2b	pCass4 binary	TAV (*cucumovirus*) 2b mRNA	Viral suppressor of RNAi (VSR) 2b of TAV
HC-Pro	pCass4 binary	*Potyvirus* HC-Pro mRNA	Viral suppressor of RNAi (VSR) HC-Pro
p19	pCass4 binary	*Tombusvirus* p19 mRNA	Viral suppressor of RNAi (VSR) p19
pT7T3 Qsat	pT7T3 riboprobe template	either (+)-Qsat or (-)-Qsat full-length transcript	none
pT7T3 Q3TLS	pT7T3 riboprobe template	either (+)-Q3 or (-)-Q3 tRNA like structure transcript	none

Table 1.2 Primers for constructing agro-plasmids, riboprobe templates, and for divergent PCR

Name of the construct	Designated primer number	Oligonucleotide sequence	Comments
pCassRZ Q2 (pQ2)	1	GTTTATTCTCAAGAGCGTATGGT	(see de Wispelaere et al., 2009. Table 1)
	2	ACGGATCCTGGTCTCCTTATGGAGAA	(see de Wispelaere et al., 2009. Table 1)
pCassRZ Q3 (pQ3)	3	GTAATCTTACCACTTTCTTTCACG	(see de Wispelaere et al., 2009. Table 1)
	4	ACGGATCCTGGTCTCCTTATGGAGAA	(see de Wispelaere et al., 2009. Table 1)
pCassHDV Qsat (pQSat) or pCassHDV (-)Qsat (p(-)Qsat)	Qsat Fwd Blunt	GTTTTGTTTGTTAGAGAATTG	The cDNA was kinased and cloned. Orientation of QSat checked by sequencing
	Qsat Rev Blunt	GGGTCCTGGTAGGGAATGATA	
pCass4 TEV QCP ORF (pQCP)	5'-TEV	ggagaggaccaggcctAACACAACATATACAAAA	joint PCR method (cite); *Stu*I underlined.
	CMV CP ORF TEV REV	GGGAGATCCAGATTTGTCCATGGCTATCGTTCGTAAATGG	
	CMV CP ORF REV	AACACACGGAAGGCCTCTAAGTCGGGAGCATCCGT	
pT7/T3 Qsat	*Hin*dIII Qsat Fwd	AAA AAA AAG CTT GTT TTG TTT GTT AGA GA ATT	
	EcoRI Qsat Rev	AAA AAA GAA TTC GGG TCC TGG TAG GGA ATG AT	
For divergent PCR	Qsat Rev 151-193	GTTTTGCTAGCGAACTGAGCGGGGGCTCAAATG	correct sequence location is 162-194; this primer was used for reverse transcription and sequencing
	Qsat 217-236 FWD	GCG GAA TTT CGA AAG AAA CA	correct sequence location is 218-237

CHAPTER 2

Satellite RNA associated with Q-CMV is amplified in the nucleus of the host in the absence of its helper virus.

ABSTRACT

Satellite RNA replication is thought to occur entirely in the cytoplasm and is exclusively dependent upon CMV replicase. However, we discovered that satRNA expressed in the absence of its helper CMV is amplified by the host. By using immunostaining with antibody against double strand RNA, and confocal microscopy to identify the subcellular distribution of double stranded satellite RNA *in situ*, we have found that in the absence of the helper virus, satRNA can amplify in the nucleus. Furthermore, sequence analysis of satRNA oligomers formed in the absence of CMV replication showed that the junction between two monomeric forms had a unique heptanucleotide sequence GGGAAAA, which is not present in the junction of satRNA oligomers formed in the presence of CMV. These novel findings will place the viroids, hepatitis delta virus and satellite RNAs in a closer relationship with each other in terms of their replication mechanisms.

INTRODUCTION

A study on survival of SatRNA in the absence of its helper virus has been done by Mossop *et al*., and Jacquemond et al., which showed that it can survive up to 10 days and 25 days, respectively, in plants (Jacquemond and Lot, 1982; Mossop and Francki, 1978). Other than their reports, no study has been done to investigate the biological activity of the satRNA in the absence of its helper CMV, since satRNA replication has been believed to be completely dependent on its helper CMV in the cytoplasm of the host cell. In this chapter, the accumulation of Q-satRNA in the absence of its helper virus will be investigated more closely.

We have observed that Q-satellite RNA (QSatRNA) accumulates to a detectable level in the absence of its helper virus in Chapter 1 of this dissertation. Steady supply of QSatRNA expression *in vivo* by agroinfiltration method and co-expression with viral suppressors of RNAi made the discovery possible. Three important questions are raised from this observation: (1) was the satRNA replicated by the host; (2) if it was replicated, what is the subcellular location for the replication; and (3) is the host replicated satRNA different from the helper virus replicated satRNA.

Replication of Q-satellite RNA (QSatRNA) requires CMV replicase which is only present in cytoplasm of the infected cell. Thus, it can be assumed that satellite replication in the presence of its helper CMV occurs only in the cytoplasm. However, if the host replicated the satRNA, this has occurred in either

cytoplasm or even nucleus. Host RNA-dependent RNA polymerases involved in RNA silencing are present in the cytoplasm. However, they do not replicate RNAs to a high level (cite Dr. Dodds). Furthermore, it has been shown that RDRs 1, 2 (resides in the nucleus), and 6 do not amplify QSatRNA (Wang et al., 2010). Other RNA polymerases are in the nucleus, chloroplasts and mitochondria. Different subviral pathogens such as PSTVd and HDV are known to replicate in the nucleus of the host cell. The phylogenetic studies done by Elena *et al*. has shown that viroids, HDV, and some satellite RNAs probably share a common ancestor (Elena et al., 2001; Elena et al., 1991) even though the satellite RNAs, viroids and HDV do not have significant overall sequence similarity. Although Q-satRNA was not included in their study, their report showed that some SatRNAs, viroids and HDV may be related. If QSatRNA can be found in the nucleus for replication, it would be another evidence that the 3 subviral pathogens are related.

In the plants, ectopic expression of satRNA in the absence of its helper CMV will result in a very low level of accumulation that it is difficult to detect. To circumvent the problem of low accumulation, we have employed viral suppressors of RNAi, and provided steady supply of satRNA in the cell by *agrobacterium*-mediated gene delivery system (agroinfiltration). SatRNAs are known to form double-stranded (ds) form during replication (Diaz-Ruiz and Kaper, 1977). In this study, we will use a mouse monoclonal antibody against dsRNA (J2mAb; Scicon) that has been successfully used for detection of dsRNA

(Schonborn et al., 1991; Weber et al., 2006) to find the location of dsSatRNA in the cell. dsSatRNA in the absence of its helper CMV would mean that SatRNA indeed is amplified by the host. Moreover, the subcellular location of dsSatRNA can lead us to identify the cellular RNA polymerase that amplified QSatRNA.

MATERIALS AND METHODS

Constructs and primers

The constructs used are described in Table 2.1. The primers are listed in Table 1.2 with brief descriptions.

Agroinfiltration method for in vivo expression of genes of interest

Agrotransformation and agroinfiltration was done as described previously (Annamalai and Rao, 2006). Briefly, the *Agrobacterium* strain EHA105 were transformed with desired binary vector construct by freeze and thaw method (Annamalai and Rao, 2006). The transformed agrobacteria were grown at 28ºC for 48 hours on LB plates containing the desired antibiotics. A single colony was inoculated into a 2-5ml LB medium with the antibiotics, and grown at 28ºC for 48 hours with vigorous shaking. 0.5 mL of the culture was transferred to 50mL medium containing the appropriate antibiotics, 10mM MES (pH 5.6) and 40µM

acetosyringone. After 14-18 hour incubation at 28ºC with vigorous shaking, the agrobacteria was washed and resuspended in 10mM $MgCl_2$ to reach the OD_{600} of the culture at 0.8. Acetosyringone was added to final concentration of 0.25mM. For co-infiltration involving more than one kind of agrotransformant, equal volumes of each culture were mixed. The acetosyringone added agrotransformant cultures were kept at room temperature for at least 3 hour without shaking. The cultures were infiltrated using 1cc syringe without a needle into the abaxial surface of either *N. clevelandii* (Fig. 2-1 and 2-3) or *N. bentamiana* (Fig. 2-2) leaves. The leaves were harvested 4 days post infiltration, unless otherwise specified.

Mechanical inoculation method for in vivo expression of Q-Sat RNA

To deliver Q-Sat via mechanical inoculation, agarose gel purified *Sma*I linearized cDNA clone of Q-Sat (Wang et al., 2010) was used as template for synthesizing capped transcripts using MEGAscript T7 kit (Ambion Inc., Austin, Texas). Specified amount of Q-Sat RNA transcripts only or a mixture containing virion RNA of CMV and transcripts of Q-Sat (~100 μg/ml) were used for mechanical inoculation.

Total RNA extraction from leaves

Total RNA extraction from leaves were essentially done as described (Annamalai and Rao, 2005b). Briefly, 1 gram of leaf tissue was ground with liquid nitrogen, and then 1 ml of hot RNA extraction buffer (86ºC; 100mM Tris pH9.0, 10mM EDTA, 100mM LiCl, 1% SDS, and equal volume of phenol) and 0.5 ml chloroform-isoamyl alcohol (24:1, v/v) were added and further ground until the mixture was homogenized. The homogenized sample was transferred to a microcentrifuge tube, vortexed for 5 min and centrifuged at 13,000 x *g* for 5min. The aqueous layer was transferred along with an equal volume of 4M LiCl to a fresh microcentrifuge tube and kept at -80ºC for at least 2 hours to precipitate. After thawing, it was centrifuged at 13,000 x *g* for 20 min at 4ºC. The pellet was washed with 70% ethanol, dried and resuspended in desired volume of RNase-free water.

Northern blot analysis

For Northern blot analysis, specified amount of RNA sample in 10 ul of RNase-free water was mixed with 10µl of sample buffer (10x MOPS buffer/formaldehyde/formamide in the ratio of 1:1.8:5, respectively), heated at 65ºC for 15 minutes and electrophoresed in 1.5% agarose-formaldehyde gel (Sambrook and Russell, 2001) until desired fractionation was achieved. The fractionated RNA was transferred to a nylon membrane with a VacuGene XL

blotting unit (Pharmacia Biotech). The nylon membrane was pre-hybridized and hybridized using the appropriate buffers as described (Rao et al., 1994) and appropriate ^{32}P-labelled riboprobes.

Synthesis of the ^{32}P-labeled riboprobes.

For preparing strand-specific Q-Sat riboprobes a PCR amplified cDNA clone corresponding Q-Sat was subcloned to a *Hin*dIII/*Eco*RI treated pT7/T3 vector. ^{32}P-labeled T3 RNA polymerase transcripts from *Eco*RI linearized plasmids were used to specifically detect Q-Sat (+) progeny whereas T7 RNA polymerase transcripts from *Hin*dIII linearized plasmids were used specifically detect Q-Sat (-) progeny.

For preparing (+)-strand specific CMV probe, *Bam*HI-digested pUCT7Q2-TLS #2 (a gift from Dr. Shou-wei Ding) was used to produce ^{32}P-labeled T7 RNA polymerase transcripts.

***In situ* detection of dsRNA**

Immunofluorescence labeling of satRNA agroinfiltrated leaf segments was essentially done as described (Bamunusinghe et al., 2009). Briefly, very thin leaf segments were made using a razor blade, and fixed in a solution of 3.7% (v/v)

formaldehyde, 5% (v/v) dimethyl sulfoxide, and PHEM buffer (60 mM PIPES, 25mM HEPES, 5 mM EGTA, 2 mM $MgCl_2$, pH 6.9) for 2 hours (Liu, Blancaflor, and Nelson, 2005). Fixed segments were washed with PHEM (3 times, 3 min/wash), and digested on microscope slides for 2 hours with cell wall degrading enzymes (1% cellulase, 0.1% pectinase, and 0.1% bovine serum albumin fraction V in PHEM buffer). The samples were washed with PHEM (3 times, 3 min/wash), and incubated with 1% (v/v) Triton X-100 for 20 min. Then the samples were incubated with ice-cold methanol at room temperature, and then washed with PHEM (3 times, 3 min/wash). Specified samples were treated with 4 units of RNaseIII (Ambion®) in the supplied buffer and incubated for 2 h at 37ºC, then washed with PHEM (3 times, 3 min/wash). All the samples were incubated with 1:200 diluted J2 mouse anti-dsRNA mAb (Scicons, Hungary) overnight in a moisture chamber at 4ºC. Samples were washed 5 times with PHEM for 5 min/wash, and then incubated with 1:100 diluted Alexa Fluor 633 Goat antimouse antibody (Invitrogen) for 2 h at room temperature, and then washed with PHEM (5 times, 5 min/wash). To stain the nucleus, the samples were incubated with 300 nM of DAPI in PHEM for 5 minutes, and then washed with PHEM (5 times, 5 min/wash). Commercially available Vectashield™ mounting media (Vector Laboratories, Inc., Burlingame, CA) was added to the samples and cover glasses were placed on the sample. Confocal microscopy was performed using Leica DMRE microscope equipped with Leica TCS SP2

confocal imaging system. He/Ne lasers were used to detect Alexa Fluor 633 fluorescence, and UV laser was used for detecting DAPI staining.

Sequencing oligomeric satRNA junction

Divergent PCR method was done (Albarino et al., 2001) by synthesizing satRNA cDNA with Protoscript® II RT-PCR kit (NEW ENGLAND BioLabs). The forward primer spans nucleotides 218 to 237 (GCGGAATTTCGAAAGAAACA) and the reverse primer spans 162-194 (GTTTTGCTAGCGAACTGAGCGGGGGCTCAAATG) of satRNA.

RESULTS

Autonomous amplification of satRNA.

A T-DNA based satRNA construct, pQSat, was made for transient expression *in vivo* (Fig. 1-1D). It was designed to produce Q-satRNA with correctly initiated 5'-end with a cap and correctly terminated 3'-end due to the activity of HDV ribozyme (RZ). *Nicotiana clevelandii* plants were agroinifltrated with constructs expressing CMV genomic RNAs (Q1+Q2+Q3) plus empty vector (EV), CMV genomic RNAs plus satRNA (QSat), empty vector (EV), satRNA plus p19 (a viral suppressor of RNAi), or CMV RNA 5 plus p19 (Q5+p19). 4 days after

the agroinfiltration, total RNA was extracted from 1 gram of each infected leaves and subjected to Northern blot analysis (Fig. 2-1A). We used riboprobes specific for (+)-satRNA, (-)-satRNA, or conserved 200nt 3'-end of CMV RNA2 (top, middle, or bottom, respectively in Fig.2-1A) for the detection of the corresponding RNAs. 20μg of total RNAs extracted from healthy leaves, empty vector infiltrated leaves, QSat+p19 infiltrated leaves and Q5+p19 infiltrated leaves were loaded on the corresponding lanes (Fig. 2-1A). Since the satRNA with CMV helper accumulates much more robustly than satRNA in the absence of its helper virus, we loaded 0.4 μg (1/50 of satRNA lane) of the total RNA extracted from CMV + QSat infiltrated leaves and 4 μg of total RNA extracted from CMV infiltrated leaves (top and middle blots in Fig. 2-1A). For the blots probed with CMV probe, 4 μg of total RNA extracted from CMV or CMV+QSat infected leaves were loaded for visual comparison of the CMV RNA bands in the presence or the absence of the satRNA (bottom blot in Fig. 2-1B). RNA marker (RNA Millenium^{TM} Marker, Ambion) was loaded to measure the sizes of the RNA bands. The 2 lanes for QSat+p19 and Q5+p19 represent total RNAs extracted from 2 separate plants. As expected, autonomously expressed QSat accumulated to a detectable level and is in both (+)- and (-)-strands, which means it was amplified by the host.

By using Typhoon scanner and ImageQuant software to quantify the intensity of the dimeric and trimeric forms, we found that in positive-sense Q-Sat, the dimeric forms were 40% more abundant compared to the trimeric form. In the

case of negative-sense Q-Sat, however, the dimeric forms were about 8% more abundant than the trimeric form.

Replication of satRNA does not involve DNA intermediates. Therefore, to verify the data shown in Fig. 2-1A, we mechanically inoculated with specified concentrations of *in vitro* transcribed satRNA transcripts. 20 µg of total RNA extracted from each inoculated plant was subjected to Northern blot analysis with riboprobes specific for (+)- and (-)– satRNA (Fig. 2.1B). In the membrane hybridized for (+)-satRNA, dimeric forms were detected in plants inoculated with 1mg/ml and 0.2 mg/ml (Fig. 2-1B top) whereas in the Northern hybridization of satRNA transcripts used to inoculate showed no dimers (Fig. 2-1B bottom) demonstrating that there were no oligomeric forms in the input satRNA transcripts. The oligomeric forms of satRNA detection in the blot hybridized for (-)-satRNA (Fig. 2-1B middle) showed that satRNA is replicated by host in the absence of the helper CMV.

In situ detection of dsQSatRNA in the nucleus

In plants infected with CMV carrying satRNA, over 90% of the RNAs accumulated were ds-satRNA (Diaz-Ruiz and Kaper, 1977). It is known that, in many single-strand (+)-sense RNA viruses, viral (-)-sense RNA synthesis leads to formation of partially and/or completely double-stranded RNA (dsRNA) structures, commonly referred to as replicative intermediates (RI). Thus *in situ*

detection of dsRNA in cells infected with a given RNA virus can be effectively used as a marker to provide insight into the localization and sites of replication. A monoclonal antibody J2 (J2mAb; Scicons, Hungary) that recognizes RNA duplexes, has been successfully used as a tool in characterizing replication sites for a wide-range of RNA viruses (Targett-Adams, Boulant, and McLauchlan, 2008; Weber et al., 2006). To determine the subcellular localization and replication sites of satRNA when expressed independent of CMV, leaf sections at almost 3 days (~69 hours) and 4 days post infiltration were labeled with J2 as described in the Materials and Method.

Results of the confocal microscopy shown in Fig. 2.2 demonstrate the subcellular sites of satRNA replication, when expressed autonomously. In the cells that were agroinfiltrated, DAPI specifically stained the nucleus emitting strong blue fluorescence (Fig. 2-2 column DAPI). Localization of the J2 specific signal in the nucleus was only found in cells expressing satRNA alone (Fig. 2-2 3dpi) but not in CMV infected control (Fig. 2-2, both 3 and 4 dpi) that had signals outside of the nucleus consistent with CMV replication in the cytoplasm. Merged images showing magenta fluorescence in the nucleus (resulting from blue and red fluorescence overlap) confirmed that replication of satRNA occurs in the nucleus similar to viroids and hepatitis delta virus (HDV) (Fig. 2-2, 3 dpi). The samples treated with RNaseIII which digests ds-RNAs did not show J2 signal. As for 4dpi, dsRNA forms of SatRNA are seen in the cytoplasm. No J2 signals are seen in the nucleus anymore probably because the overwhelmingly large

number of dsSatRNA accumulated for 4 days in the cytoplasm sequestered all J2mAb (Fig. 2-2, 4 dpi).

Host cell adds a unique heptanucleotide sequence in the oligomeric form of satellite RNA.

It was reported that in the oligomeric form of satRNA replicated in the presence of the helper virus, the oligomers often contain deletions at the junction regions Kuroda, et al. (Kuroda et al., 1997). In order to compare and characterize junction sequences of satRNA oligomers formed in the presence and absence of its helper, we employed an RT-PCR approach that was successfully used for determining the junction sequences of oligomers formed during replication of flock house virus (Albarino et al., 2001). We produced RT-PCR products encompassing the junction regions of the satRNA oligomers (Fig. 2-3 A). The RT-PCR products were sequenced directly (Fig. 2-3 C and D). In the junction of the oligomeric satRNA formed in the presence of the helper virus, the 3'-end C-residue was missing in the first monomeric unit joined to the second unit (Fig. 2-3C). However, the oligomeric forms formed in the absence of the helper virus, a unique heptamer, namely GGGAAAA, was joining the two monomeric units (Fig. 2-3D). This heptamer sequence is found neither in QSatRNA nor in CMV, so it must have originated from the host cell.

DISCUSSION

By using these tools, this study demonstrates 3 novel discoveries: (i) during *in vivo* satRNA expression in the absence of its helper CMV, the complementary strand (negative (-)-strand) is synthesized by the host; (ii) satRNA can be localized in the nucleus of the host cell; and (iii) the satRNAs in the absence of its helper virus exist mostly as oligomeric forms with the unit-length satRNAs joined by a unique junction hepta-nucleotide sequence originated from the host cell, which is not seen in the satRNA oligomers formed in the presence of the helper virus.

By studying the satRNA *in vivo* in the absence of the helper CMV, we have witnessed for the first time, a virus-dependent subviral agent Q-Sat behaving like a viral-independent subviral agent. It has been unknown until now that satRNA can be transcribed by host to synthesize the negative-strand in the absence of the helper CMV (Fig. 2-1). The reason for the wide acceptance of the theory that satRNA cannot replicate autonomously have been probably because satRNA accumulates to an undetectable level in the absence of its CMV helper. We have overcome this obstacle by using agroinfiltration, and viral and bacterial suppressor of RNAi. Agroinfiltration provides a steady supply of satRNA transcripts in the cell for about 4 days (Annamalai and Rao, 2005b). In addition, viral or bacterial suppressor of RNAi protects the single-stranded satRNA

transcripts produced by the T-DNA because the single-stranded satRNA can be subjected to RNAi (Du et al., 2007).

It is interesting that the satRNA accumulated in the absence of the helper virus are mostly in oligomeric forms while the satRNA replicated in the presence of the helper CMV exists predominantly in monomeric form (Fig. 2-1A). There has been unpublished report that ds-satRNA may self-ligate (Roossinck, Sleat, and Palukaitis, 1992). It is possible that in the absence of the helper virus, the ds-satRNAs made by the host rapidly self-ligate. Therefore, the monomeric forms of (+)-satRNA (Fig. 2-1 A, top) may represent the starting satRNA transcripts and the oligomers are from the self-ligated dsRNAs. The Northern membrane probed for the (-)-satRNA supports this hypothesis because the monomeric form of (-)-satRNA is hardly seen (Fig. 2-1A, middle).

The satRNAs accumulated in the presence of its helper CMV, however, are mostly in monomeric form (Fig. 2-1A, top and bottom). It is possible that the junction hepta-nucleotide plays a role here (Fig. 2-3). This hepta-nucleotide sequence GGGAAAA is reminiscent of the slippery hepta-nucleotide sequence located upstream of a pseudoknot that can trigger -1 frame shift in gag/gag-pol translation in certain viruses (Dinman and Wickner, 1992). Although the slippery sequence participates in translation involving the translational machinery to slip, it is possible that GGGAAAA may cause the helper viral replicase complex to fall off and re-start the transcription in the next initiation site producing satRNA

monomers. Furthermore, the satRNA oligomers formed in the presence of its helper are missing 3'-end C residue, and it may indicate that the transcription initiation site for satRNA may be the 3'- penultimate C as in bromome mosaic virus. We can envision that some of the ds-satRNA replication intermediates are ligated before the addition of the 3'-end C residue. Therefore, the nuclear phase (viroid/HDV-like phase) in which the hepta-nucleotides are presumed to be added may be a necessary step in robust replication of satRNA.

The nuclear phase of the satRNA may indicate that satRNA associated with CMV, some viroids such as PSTVd, and HDV are evolutionarily related, because they share the nuclear phase as a common feature. The presence of the unique heptameric junction sequence GGGAAAA inserted by the host strongly supports the hypothesis that satRNA is originated from the host cell. The unique heptameric junction sequence may have been originated from "escaped introns" (Elena et al., 1991), or other small RNAs, or satRNA resembling the host telomere to be used as an acceptor by telomerase, as viroids and HDV mimic DNA to be transcribed by the host polymerase II (Feldstein, P. and Bruening, G. through personal communication), or some other host enzyme that has similar activity as telomerases. Whether or not the sequence GGGAAA present within PSTVd loopE motif that functions in RNA-RNA and RNA-protein interactions and processing of longer-than-unit-length PSTVd (Tabler and Tsagris, 2004) and the satRNA junction sequence GGGAAAA are related needs a closer look. Furthermore, whether other variants and species of satellite RNAs can be

transcribed by the host in the nucleus and have the unique junction sequence of the host origin needs to be studied.

The nucler phase in the life cycle of the satRNA may also be beneficial to the helper CMV because satRNA is a better template than CMV RNAs for the viral replicase (Wu and Kaper, 1995). If satRNA upon entry into the host cell inside the CMV immediately becomes a template for the viral replicase, not much of CMV RNAs will be replicated and it is also unbeneficial to satRNA. It may provide a head start for the CMV RNAs to replicate. Further support that nuclear-phase is a necessary step comes from previous reports demonstrating that tomato plants infected with CMV harboring satellite RNA show only mild symptoms when challenged with a severe strain of PSTVd. Furthermore, PSTVd accumulation in the plant infected with CMV satellite RNA is significantly lower than the positive-control plants. They have shown that the satellite RNA, not CMV, is responsible for making the tomato plant resistant to PSTVd infection (Montasser, Kaper, and Owens, 1991; Yang, Kang, and Tien, 1996). The reduction of the PSTVd accumulation may have occurred due to the satellite RNA competing with the PSTVd for the host polymerase (we already know that satRNA is not transcribed by the host RDR 2 which resides in the nucleus; data not shown) in the nucleus in order to replicate, or competing for some protein(s) involved in PSTVd translocation between the nucleus and the cytoplasm. One candidate protein that may be involved in satRNA nuclear translocation is VirP1, which is the first identified host factor necessary for PSTVd infection (Kalantidis

et al., 2007). Moreover, it has been reported that up to seven nucleotides deletion in the 3'-end of (+)-sense satRNA can be repaired *in planta* by CMV in template independent manner (Burgyan and Garcia-Arenal, 1998). This repair maybe taking place in the nucleus.

The satRNA transcribed in the nucleus does translocate into the cytoplasm. Packaging study with CMV CP expressed *in trans* has shown that the oligomeric forms of autonomously accumulated satRNAs are packaged (Chapter 1, Fig. 1-5C). In addition, it seems that satRNA that accumulates in the cytoplasm is in dsRNA form because MS2-tagging study done by Dr. Seo in our lab showed that satRNAs are not seen in the cytoplasm (data not shown), whereas J2 labeled ds-satRNAs are seen in the cytoplasm in 4 days post infiltration (Fig. 2-2). Whether the satRNA packaged by the CP expressed *in trans* is dsRNA remains to be studied. It is also not known whether or not the ds-satRNA can be re-imported into the host nucleus. It is possible that ds-satRNA in the cytoplasm in the absence of the helper virus may not be transcribed again.

From the data shown in this study, we propose a hyotheical model of SatRNA activity in the absence of the helper CMV (Fig. 2-4). Positive-sense strand QSatRNA in the cytoplasm would be recruited to the nucleus of the host and then imported. Some nuclear polymerase would transcribe it and oligomerization occurs in the nucleus through some unknown mechanism. The double stranded form would be exported out into the cytoplasm. This provides an

ideal situation for SatRNA awaiting its helper virus in the cell because dsRNA is more resistant to host RNase activity and ds-satRNA is not subjected to RNAi (Du et al., 2007) as a replicating HDV RNA is not a substrate to dicer activity (Chang, Provost, and Taylor, 2003). Also, it may increase the chance to be rapidly replicated when CMV enters the cell for it has been suspected that the optimal template for synthesizing the positive-strand satellite RNA may be a double-stranded form (Hayes et al., 1992; Kuroda et al., 1997; Tousch, Jacquemond, and Tepfer, 1994).

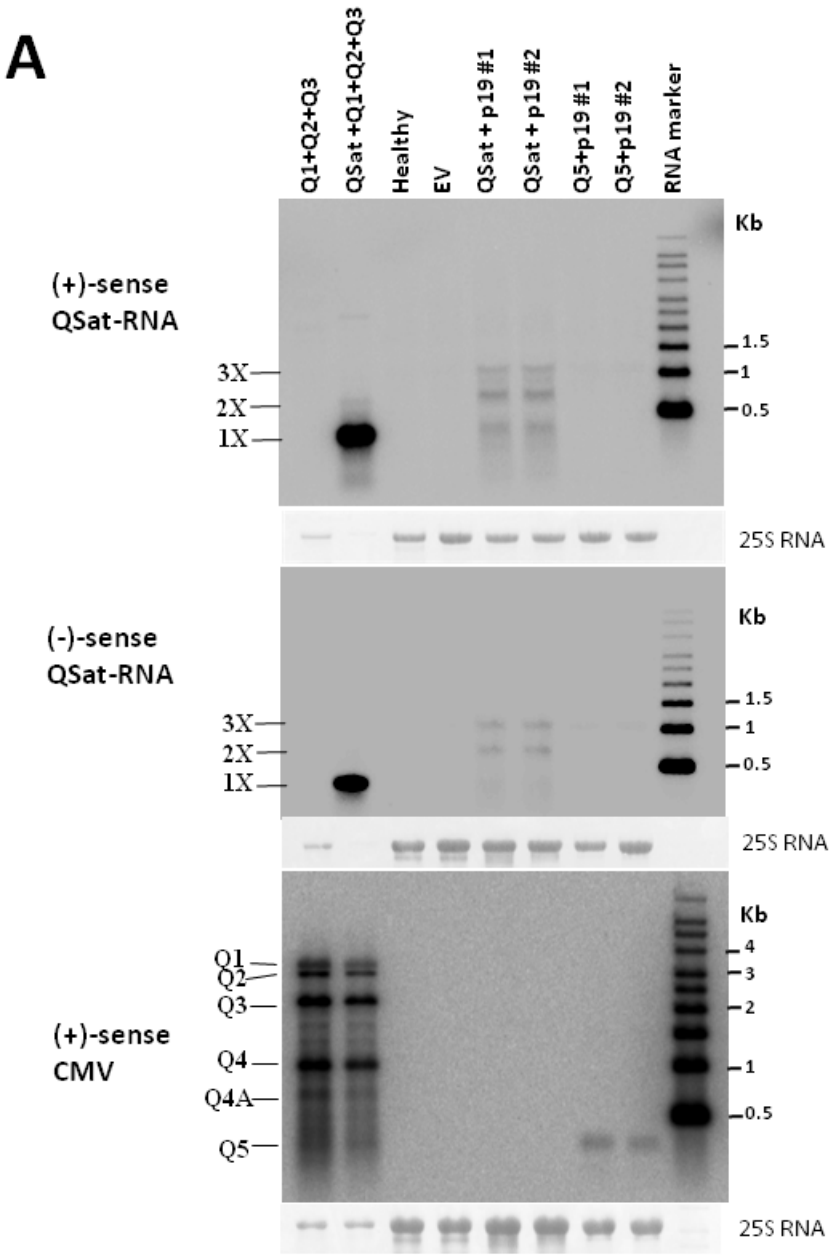
A new hypothetical model for overall QSatRNA replication is proposed in Figure 2.5. Q-CMV harboring QSatRNA enters the host cell and QSatRNA is released into the cytoplasm. QSatRNA is then imported into the nucleus, transcribed by an unknown host polymerase, and then exported into the cytoplasm. ds-QSatRNA is recruited by CMV replicase to the virus-induced vesicle on vacuolar membrane (tonoplast) where it is replicated. The newly synthesized QSatRNA is then either packaged by CMV or recruited into the host nucleus for transcription.

The novel findings in this report will place satellite RNAs in even closer relationship with viroids and HDV. The implications of the findings will shed light into the mysteries that have been revolving around the satRNA replication, its evolutionary relationship with other subviral pathogens, and the origin of the subviral agents in general.

Figure 2-1 Helper virus independent amplification of QSat RNA

(A) Northern blot analysis of total RNA from the infiltrated leaves at 4 days post infiltration (dpi) from *N. clevelandii* leaves. Indicated agroconstruct cultures were agroinfiltrated and hybridized with indicated specific riboprobes. The positions of Q-Sat monomeric (1x), dimeric (2x) and trimeric (3x) forms are indicated on the left of each panel. The positions of CMV RNAs (Q1-Q5) are indicated on the left of the bottom panel. All samples contained 20 μg of total RNA except the following: In top and middle panels, in lanes indicated with Q1+Q2+Q3 and QSat+Q1+Q2+Q3, respectively contained 4 μg and 0.4μg of total RNA. In the bottom panel these two lanes contained 4 μg of total RNA. 25S RNA represents loading control. In each panel a radiolabeled RNA size marker is shown on the right.

(B) Northern blot analysis of total RNA recovered 4 days after mechanical inoculation of *N. clevelandii* plants with indicated concentrations of *in vitro* synthesized Q-Sat transcripts and probed with indicated riboprobes (Top and Middle panels). Bottom panel shows the Northern blot hybridization of Q-Sat transcripts used for mechanical inoculation.


A
Q1+Q2+Q3
QSat +Q1+Q2+Q3
Healthy
EV
QSat + p19 #1
QSat + p19 #2
Q5+p19 #1
Q5+p19 #2
RNA marker
Kb
1.5
1
0.5
(+)-sense
QSat-RNA
3X
2X
1X
25S RNA
Kb
1.5
1
0.5
(-)-sense
QSat-RNA
3X
2X
1X
25S RNA
Kb
4
3
2
1
0.5
(+)-sense
CMV
Q1
Q2
Q3
Q4
Q4A
Q5
25S RNA

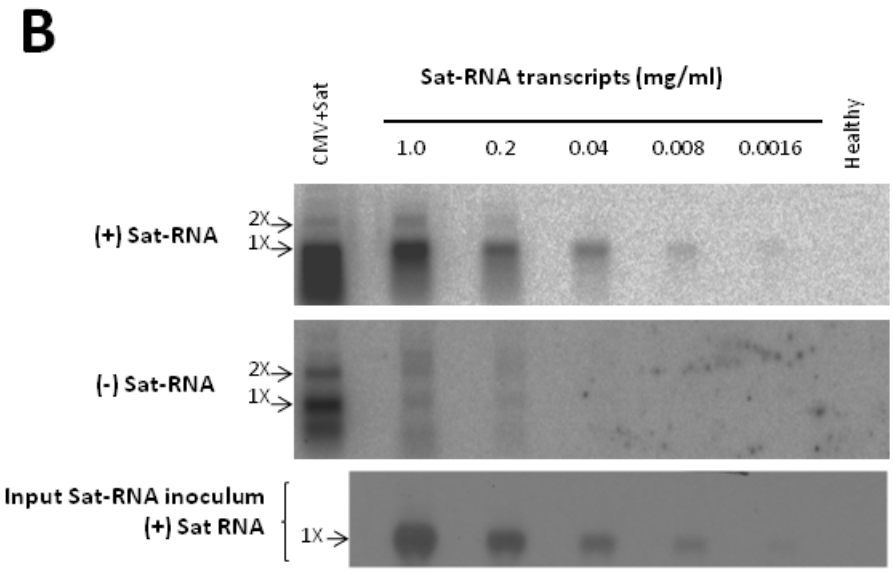

B
Sat-RNA transcripts (mg/ml)
CMV+Sat
1.0
0.2
0.04
0.008
0.0016
Healthy
(+) Sat-RNA
2X
1X
(-) Sat-RNA
2X
1X
Input Sat-RNA inoculum
(+) Sat RNA
1X

Fig 2-2 Nuclear localization of QSat RNA in the absence of its helper virus.

Immunolabeling of dsRNA in leaf segments of *N. clevelandii* prepared 3 and 4 days after infiltration with the following agro cultures: Empty vector (EV); QCMV RNA 5 (Q5); QSat RNA (SatRNA) only and CMV. To visualize the nuclei, the leaf segments were treated with DAPI (blue). Leaf segments were probed for the presence of dsRNA using J2mAb and then treated with AlexaFluor 633 (red fluorescence) conjugated secondary antibody. Leaves infiltrated with cultures of SatRNA only and CMV were treated with RNase III prior to immunolabelling with J2-Ab. Bar, 10 μm, except for SatRNA for 3 dpi (9.37μm).

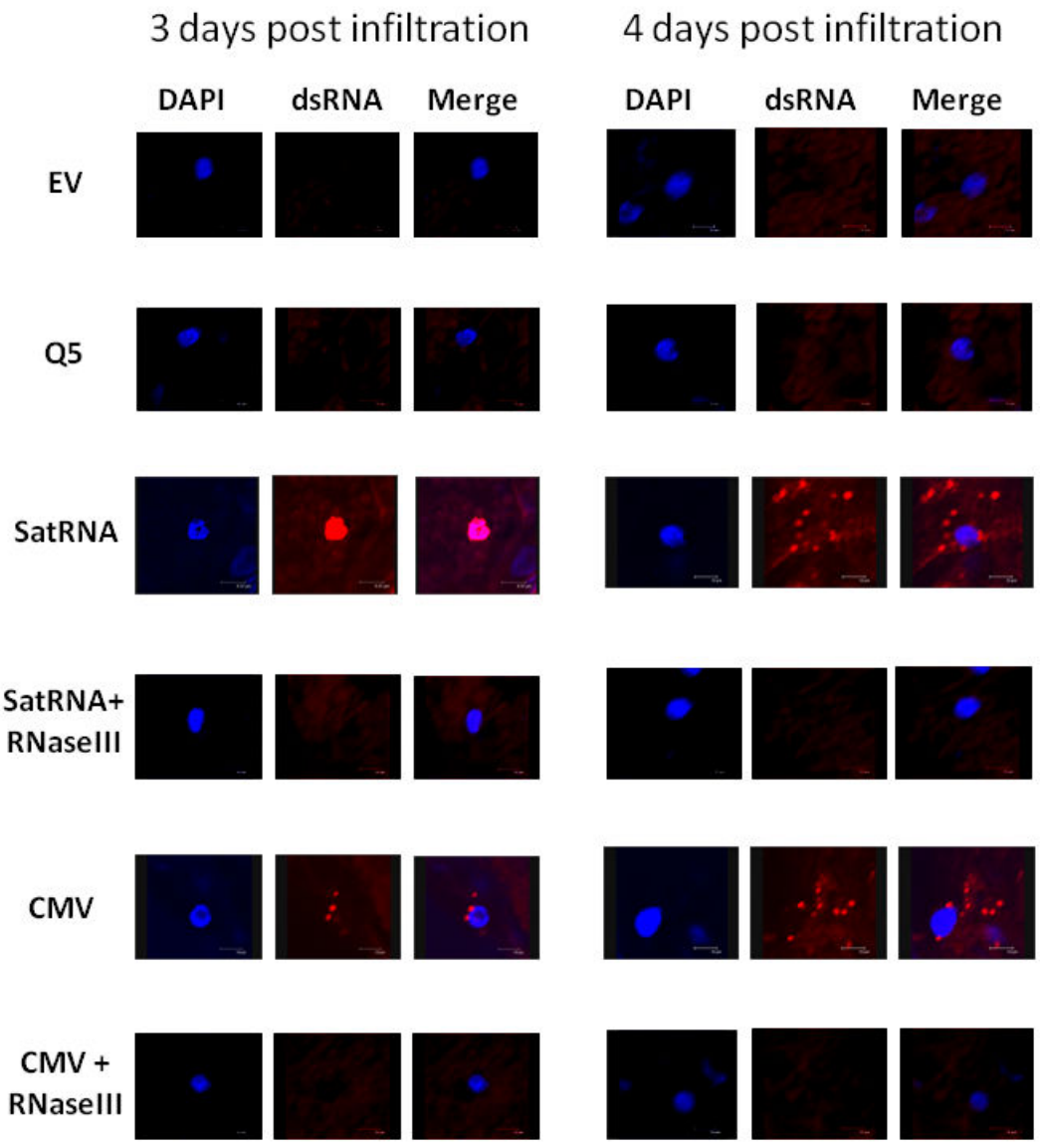

3 days post infiltration
4 days post infiltration
DAPI
dsRNA
Merge
DAPI
dsRNA
Merge
EV
Q5
SatRNA
SatRNA+
RNaseIII
CMV
CMV +
RNaseIII

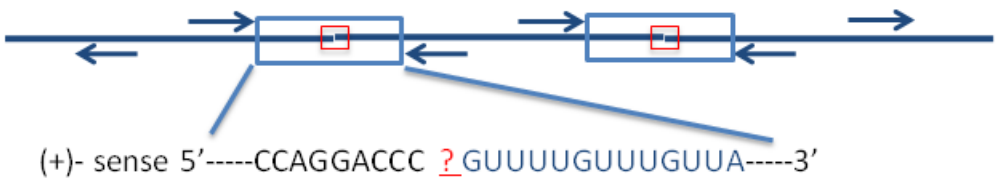
Figure 2-3 Analysis of junction sequences in QSat RNA oligomers formed in the presence and absence of its helper virus

(A) Strategy for divergent PCR. Primers described in Table 1.2 for divergent PCR are used.

(B) Perfectly joined satellite multimer junction sequence

(C and D) Summary of sequence analysis of the junction region of Q-Sat oligomers from agroinfiltrated leaves. The numbers on the right indicate the number of cDNAs with a specific sequence over total number of cDNAs used for sequencing.

A



B Expected Satellite multimer junction sequence

(+)- sense 5’-----CCAGGACCCGUUUUGUUUGUUA-----3’

C Satellite multimer in the presence of the helper virus

(+)- sense 5’-----CCAGGACC Δ GUUUUGUUUGUUA-----3’ ***(23/23)***

D Satellite multimer in the absence of the helper virus

(+)- sense 5’-----CCAGGACCCGGGAAAAGUUUUGUUUGUUA-----3’

(***48/48)***

(-)- sense 5’-----UAACAAACAAAACUUUUCCCGGGUCCUGG-----3’

(6/6)

Figure 2-4 Hypothetical model of QSat RNA amplification in the absence of its helper virus

QSatRNA is imported into the nucleus of the host. It is transcribed by an unknown host polymerase (Viroid/HDV-like phase), then the double-stranded (ds) QSatRNAs are exported into the cytoplasm.

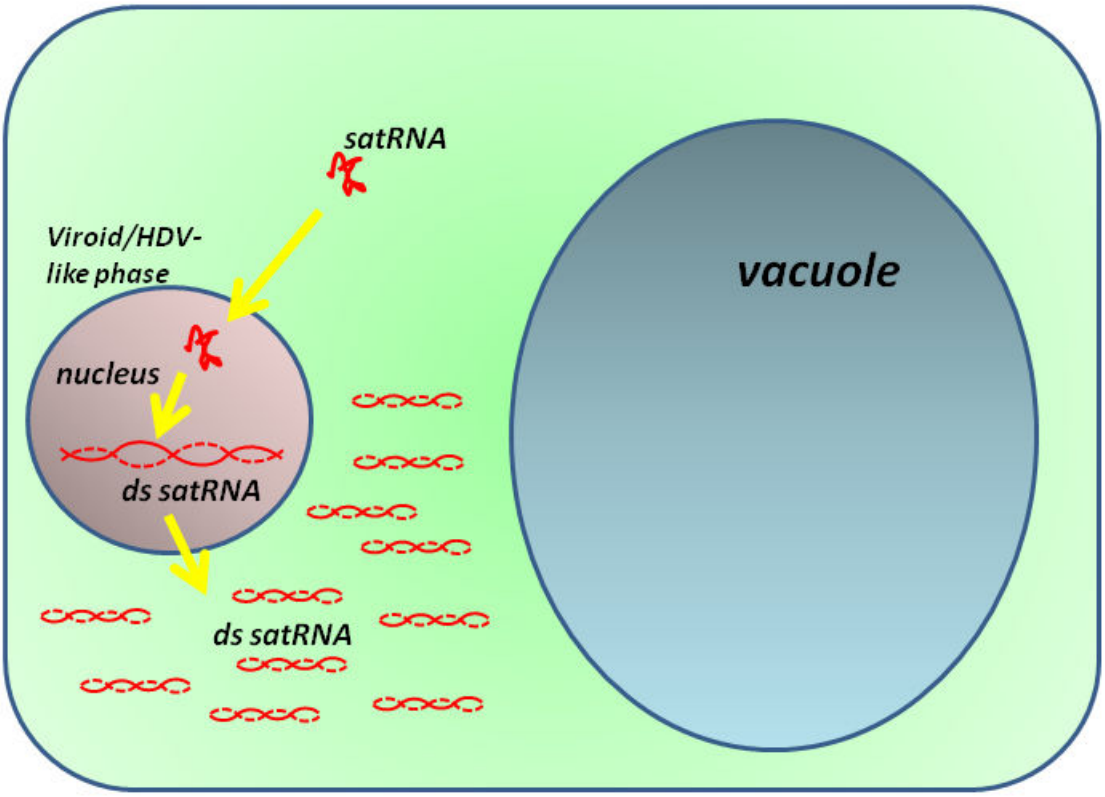

satRNA
Viroid/HDV-
like phase
nucleus
ds satRNA
ds satRNA
vacuole

Figure 2-5 Hypothetical model of QSat RNA replication in the presence of its helper virus

Q-CMV harboring QSatRNA enters the host cell and QSatRNA is released into the cytoplasm. QSatRNA is then imported into the nucleus, transcribed by an unknown host polymerase (Viroid/HDV-like phase), and then exported into the cytoplasm. ds-QSatRNA is recruited by CMV replicase to the helper virus replication site on vacuolar membrane (tonoplast) where it is replicated (Virus-like phase). The newly synthesized QSatRNA is then either packaged by CMV or recruited into the host nucleus for transcription.

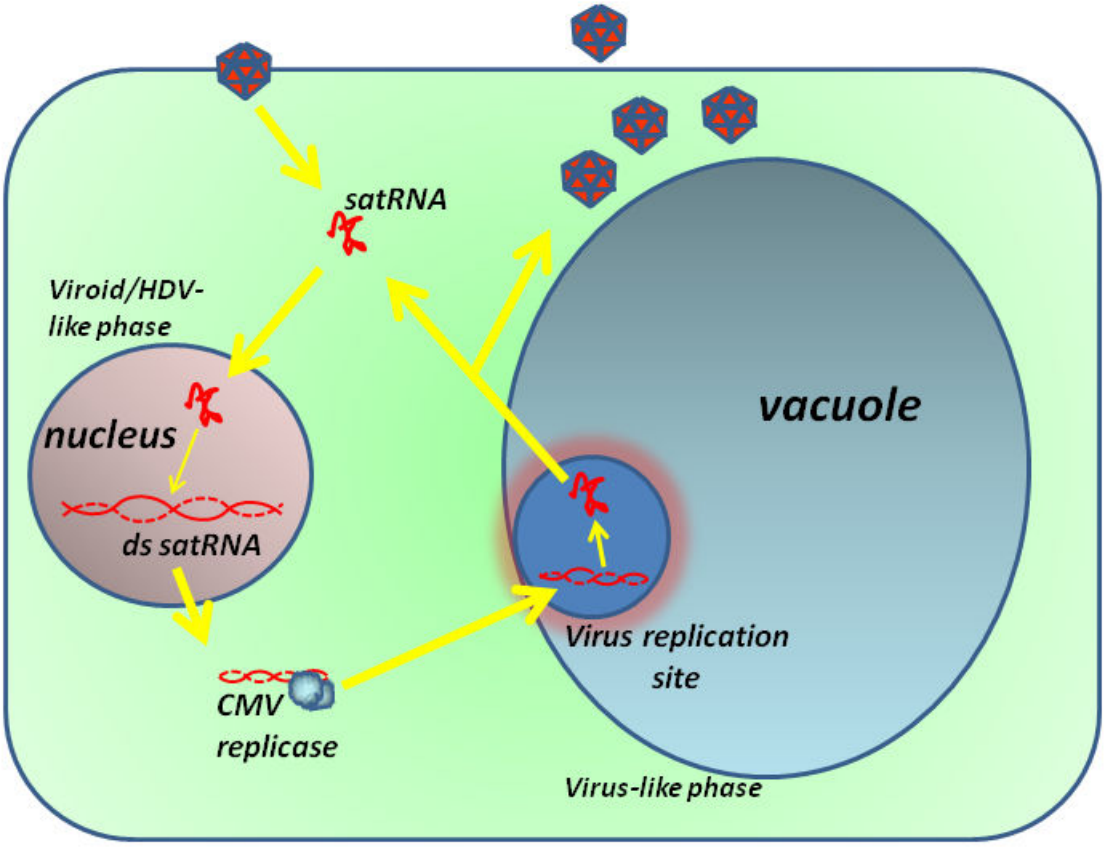

satRNA
Viroid/HDV-
like phase
nucleus
ds satRNA
vacuole
Virus replication
site
CMV
replicase
Virus-like phase

Table 2.1 List of agro-plasmids and riboprobe templates			
Name of the construct	**vector type**	**RNA expressed (abbreviation)**	**Proteins expressed**
pQ1	pCass4 binary	Q-CMV RNA 1 (Q1)	1a of CMV
pQ2	pCassRZ binary	Q-CMV RNA 2 (Q2)	2a and 2b of CMV
pQ3	pCassRZ binary	Q-CMV RNA 3 (Q3)	MP and CP of CMV
pQ5	pCassRZ binary	Q-CMV RNA 5 (Q5)	none
pQSat	pCassHDV binary	Q-Sat RNA (a variant Q)	none
pCass4 (or EV)	pCass4 binary	none	none
pUC T7 Q-Sat	pUC19 for making QSatRNA transcript	QSat RNA	none
p19	pCass4 binary	*Tombusvirus* p19 mRNA	Viral suppressor of RNAi (VSR) p19
pT7T3 Qsat	pT7T3 riboprobe template	either (+)-Qsat or (-)-Qsat full-length transcript	none
pUC T7 Q2-TLS #2	pUC19 riboprobe template	(-)-Q2 tRNA like structure transcript	none
pUC T7 Q2-TLS #11	pUC19 riboprobe template	(+)-Q2 tRNA like structure transcript	none

CHAPTER 3

***In vivo* study of satellite RNA replication initiated with negative-sense strand.**

ABSTRACT

It has been previously demonstrated by other research groups that satellite RNA of CMV does not accumulate to a high level when the replication is initiated with the negative-sense strand. By using agroinfiltration system, we demonstrate that the negative-sense strand of satellite RNA is not amplified by the host because it is not recruited into the host nucleus. However, consistent with the previously published results, the negative-sense satellite RNA is replicated by the helper CMV but only to a low-level. These results may implicate that the initial nucleus-phase is an important step in satellite replication for a robust accumulation.

INTRODUCTION

Subviral pathogens that are replicated in the host nucleus include PSTVd of plants and HDV of human. In Chapter 2 of this dissertation, it was shown that QSat RNA is also able to localize in the nucleus of the host and gets amplified (negative-sense strand produced). Prior to this study, there has been no report of nuclear phase involvement in satRNA replication. For PSTVd and HDV, it is the genomic, or positive-sense strand that enters the host nucleus to be replicated. For PSTVd and HDV, the negative-sense strand only exists in the nucleus as a replication intermediate. The protein that recruits PSTVd can only bind to the genomic, or (+)- sense strand. Thus, as with PSTVd or HDV, it is conceivable that for QSat RNA to be recruited to the nucleus, it must be in (+)-sense strand form. In this chapter, the study of QSatRNA replication initiated with (-)-sense strand is described. This study may shed a light in answering the question posed in the Chapter 2 of this dissertation that asked whether the nuclear phase is a necessary step when QSatRNA is replicated by its helper CMV. To study this, we used T-DNA based construct expressing negative-sense strand of QSat RNA (p(-)QSat RNA).

There have been several attempts to study CMV SatRNA replication with the negative-sense strand as a starting material. (-)-sense satRNA transcripts were shown to be not infectious in two studies (Collmer and Kaper, 1986; Kurath and Palukaitis, 1987). Only one study has successfully demonstrated that

QSatRNA replication can be initiated with (-)-sense strand by using transgenic plants (Tousch, Jacquemond, and Tepfer, 1994). They suggested that (-)-satRNA was a poor template for (+)-satRNA synthesis *in vivo* since it accumulated to a low level (Tousch, Jacquemond, and Tepfer, 1994). In the review paper published in 1999, Garcia-Arenal and Palukaitis suggested that it is due to either the requirement for a single addition of guanosine residue at the 3'-end of the (-)-satRNA (Wu and Kaper, 1994) or (+)-satRNA has a role in biosynthesis of (+)-sat RNA (Garcia-Arenal and Palukaitis, 1999).

We hypothesize that the reason for the low accumulation of QSat RNA when the replication is initiated with (-)-sense strand is that it is not recruited to the nucleus although it is a template for the CMV replicase. If that is the case, then it would imply the importance of the initial nuclear phase (going through the nuclear phase before going through the virus-like phase) during the QSat RNA replication in the presence of the helper virus. In this study, we attempt to explain why a unit-length (-)QSat RNA, which is a reverse complementary strand to the (+)-sense QSat RNA, as a starting material does not result in robust accumulation of QSat RNA, and ultimately provide a feasible answer to the question posed in Chapter 2 of this dissertation, namely: Is the initial nuclear phase a necessary part of robust QSat RNA replication cycle in presence of its helper CMV?

MATERIALS AND METHODS

Constructs and primers

See Table 3.1 for the constructs used and Table 1.2 for the primers and comments.

Agroinfiltration method for in vivo expression of genes of interest

Agrotransformation and agroinfiltration was done as described previously (Annamalai and Rao, 2006). Briefly, the *Agrobacterium* strain EHA105 (Fig. 1-2~6) or GV3101 (Fig. 1-7) were transformed with desired binary vector construct by freeze and thaw method (Annamalai and Rao, 2006). The transformed agrobacteria were grown at 28ºC for 48 hours on LB plates containing the desired antibiotics. A single colony was inoculated into a 2-5ml LB medium with the antibiotics, and grown at 28ºC for 48 hours with vigorous shaking. 0.5 mL of the culture was transferred to 50mL medium containing the appropriate antibiotics, 10mM MES (pH 5.6) and 40μM acetosyringone. After 14-18 hour incubation at 28ºC with vigorous shaking, the agrobacteria was washed and resuspended in 10mM $MgCl_2$ to reach the OD_{600} of the culture at 0.8. Acetosyringone was added to final concentration of 0.25mM. For co-infiltration involving more than one kind of agrotransformant, equal volumes of each culture were mixed. The acetosyringone added agrotransformant cultures were kept at room temperature for at least 3 hour without shaking. The cultures were

infiltrated using 1cc syringe without a needle into the abaxial surface of either *N. bentamiana* or *N. clevelandii* leaves. The leaves were harvested 4 days post infiltration for the infiltrated leaves and 10 days post infiltration for the systemic leaves.

Total RNA extraction from leaves

Total RNA extraction from leaves were essentially done as described (Annamalai and Rao, 2005b). Briefly, 1 gram of leaf tissue was ground with liquid nitrogen, and then 1ml of hot RNA extraction buffer (86ºC; 100mM Tris pH9.0, 10mM EDTA, 100mM LiCl, 1% SDS, and equal volume of phenol) and 0.5 ml chloroform-isoamyl alcohol (24:1, v/v) were added and further ground until the mixture was homogenized. The homogenized sample was transferred to a microcentrifuge tube, vortexed for 5 min and centrifuged at 13,000 x *g* for 5min. The aqueous layer was transferred along with an equal volume of 4M LiCl to a fresh microcentrifuge tube and kept at -80ºC for at least 2 hours to precipitate. After thawing, it was centrifuged at 13,000 x *g* for 20 min at 4ºC. The pellet was washed with 70% ethanol, dried and resuspended in desired volume of RNase free water.

Northern blot analysis

For Northern blot analysis, specified amount of RNA sample in 10 ul of RNase-free water was mixed with 10µl of sample buffer (10x MOPS buffer/formaldehyde/formamide in the ratio of 1:1.8:5, respectively), heated at 65ºC for 15 minutes and electrophoresed in 1.5% agarose-formaldehyde gel (Sambrook and Russell, 2001) until desired fractionation was achieved. The fractionated RNA was transferred to a nylon membrane with a VacuGene XL blotting unit (Pharmacia Biotech). The nylon membrane was pre-hybridized and hybridized using the appropriate buffers as described (Rao et al., 1994) and appropriate ^{32}P-labelled riboprobes.

Synthesis of the ^{32}P-labeled riboprobes.

For preparing strand-specific Q-Sat riboprobes a PCR amplified cDNA clone corresponding Q-Sat was subcloned to a *Hin*dIII/*Eco*RI treated pT7/T3 vector. T3 RNA polymerase transcripts from *Eco*RI linearized plasmids were used to specifically detect (+)-Q-Sat progeny whereas T7 RNA polymerase transcripts from *Hin*dIII linearized plasmids were used specifically detect (-)-Q-Sat progeny. *BamH*I-digested pUC19-T7Q2TLS #2 construct (gift from Dr. Shou-wei Ding) was used as a template for generating the riboprobe to detect CMV genomic and subgenomic RNAs.

In situ detection of dsRNA

Immunofluorescence labeling of satRNA agroinfiltrated leaf segments was essentially done as described (Bamunusinghe et al., 2009). Briefly, very thin leaf segments were made using a razor blade, and fixed in a solution of 3.7% (v/v) formaldehyde, 5% (v/v) dimethyl sulfoxide, and PHEM buffer (60 mM PIPES, 25mM HEPES, 5 mM EGTA, 2 mM $MgCl_2$, pH 6.9) for 2 hours (Liu, Blancaflor, and Nelson, 2005). Fixed segments were washed with PHEM (3 times, 3 min/wash), and digested on microscope slides for 2 hours with cell wall degrading enzymes (1% cellulase, 0.1% pectinase, and 0.1% bovine serum albumin fraction V in PHEM buffer). The samples were washed with PHEM (3 times, 3 min/wash), and incubated with 1% (v/v) Triton X-100 for 20 min. Then the samples were incubated with ice-cold methanol at room temperature, and then washed with PHEM (3 times, 3 min/wash). Specified samples were treated with 4 units of RNaseIII (Ambion®) in the supplied buffer and incubated for 2 h at 37ºC, then washed with PHEM (3 times, 3 min/wash). All samples were incubated with 1:200 diluted J2 mouse anti-dsRNA mAb (Scicons, Hungary) overnight in a moisture chamber at 4ºC. Samples were washed 5 times with PHEM for 5 min/wash, and then incubated with 1:100 diluted Alexa Fluor 633 Goat antimouse antibody (Invitrogen) for 2 h at room temperature, and then washed with PHEM (5 times, 5 min/wash). To stain the nucleus, the samples were incubated with 300 nM of DAPI in PHEM for 5 minutes, and then washed with PHEM (5 times, 5 min/wash). Commercially available Vectashield™

mounting media (Vector Laboratories, Inc., Burlingame, CA) was added to the samples and cover glasses were placed on the sample. Confocal microscopy was performed using Leica DMRE microscope equipped with Leica TCS SP2 confocal imaging system. He/Ne lasers were used to detect Alexa Fluor 633 fluorescence, and UV laser was used for detecting DAPI staining.

RESULTS

Negative-sense QSatRNA is not amplified by the host

A T-DNA based (-)-QSat RNA construct was made for studying (-)-Q-SatRNA replication in vivo (Fig. 3-1). Just as the pQSat described in Chapter 1, it is preceded by a double 35S promoter and followed by HDV RZ so that the exact reverse complementary Q-Sat nucleotide sequence would be produced *in vivo* (Fig 3-1A). To test the biological activity of p(-)QSat, *N. clevelandii* leaves were coinfiltrated with the following agrotransformants: (i) (-)QSat; (ii) (-)QSat +HC-Pro; (iii) (-)QSat+CMV (Q1+Q2+Q3), and (iv) CMV+QSat. For (i)-(iv), the agrotransformants were infiltrated into 3 different plants. Four days post infiltration, the total RNA was extracted from the infiltrated leaves and 20 µg of each sample was used except for the CMV+QSat (10 µg), a positive control. Northern blot analysis of the extracted RNA samples were done (Fig. 3-1B). Duplicate Northern blots were made and hybridized with specific riboprobes for either (+)QSat or (-)QSat. The (-)QSat accumulated (Fig 3-1B, bottom) but it was

not replicated (Fig. 3-1 B, top), even in the presence or absence of HC-Pro. In the presence of CMV, however, it was replicated although to a lower level than the wild-type control. This result demonstrates that the unit-length (-)QSat is a template for CMV replicase but it is not amplified autonomously.

To test for autonomous (-)-QSat RNA replication using another method, immunostaining to visualize the subcellular localization of dsRNA was done. *N. Benthamiana* leaves that were infiltrated with specified agrotransformants along with healthy leaf were sectioned and subjected to confocal microscopy as described in the materials and methods section. DAPI stained nucleus in blue color as seen in all 3 samples (Fig. 3-2). 4 days after infiltration, the fluorescence signals specific for J2 mAb were seen in (+)-QSat and CMV+QSat as expected but not in (-)QSat and Healthy samples. This further confirms that (-)-QSatRNA is not amplified by the host (Fig 3-2).

(-)QSat as a parental strand results in low accumulation of satRNA in infiltrated leaf but accumulates well in the systemic leaf.

Tousch et al (Tousch, Jacquemond, and Tepfer, 1994) reported that (-)-sense strand satellite RNA as a parental RNA (starting RNA) resulted in a low accumulation. However, in their study, transgenic plant expressing (-)-sense satellite RNA was used. This means that in all leaves, the replication was initiated with the (-)-sense strand satellite RNA. If the lack of nuclear phase involvement is the reason for the low accumulation, the virions produced in the

infiltrated leaf that moved to the systemic leaf should result in wild type level accumulation of the satellite RNA, since the virus only packages (+)SatRNA (Simon, Roossinck, and Havelda, 2004) and the virions infecting systemic leaf would release (+)SatRNA that would go through the nuclear phase. In fact, that is what is shown in Fig 3-3. The (-)QSat RNA as a starting material in the presence of CMV accumulated to a low level compared to (+)QSat RNA as a starting material in the infiltrated leaf (Fig. 3-3). However, in the systemic leaf where the virions carrying (+)QSat RNA had reached, showed comparable level of (+)QSat RNA accumulation (Fig. 3-3).

DISCUSSION

One advantage of using agroinfiltration system over transgenic plant system in doing the experiments shown in Fig. 3-3, other than being more convenient, quicker and inexpensive, is that while you can distinguish the inoculated leaves from the systemic leaves with agroinfiltration assay, you cannot do so with the transgenic plants. By initiating satRNA replication with (-)QSatRNA, which is a template for CMV replicase and not amplified by the host in the nucleus, and agroinfiltration assay, we hypothesize the following scenario for (-)QSat replication by CMV *in vivo*. (-)QSatRNA cannot go through the nuclear phase. It is probably inefficiently (or slowly) recruited to the viral replication site and then inefficiently (or slowly) replicated. The positive-sense QSatRNAs would appear in the cytoplasm to be packaged by the CMV CP and some of them

would be imported into the nucleus to be amplified. By the time the double-stranded forms of QSatRNAs are exported to the cytoplasm, it is late in the virus replication cycle and the virus no longer replicates it or only a few QSatRNAs are replicated. It further demonstrates that the initial nuclear phase is required for robust replication of QSatRNA. The requirement of the initial nuclear phase for robust replication may further strengthen the probability of the close evolutionary link with viroids and HDV.

The studies described in this chapter support the hypothetical model for QSatRNA replication cycle described in Fig. 2-5. The initial nuclear phase before the recruitment to the virus replication site is necessary for robust replication of QSat RNA by the helper CMV. The robust replication is probably due to the double stranded multimeric QSatRNAs that are better templates than the single stranded QSatRNA. More will be discussed in the Conclusion Chapter of this dissertation.

Figure 3-1 Biological activity of T-DNA based (-)QSat construct

(A) Schematic diagram of a T-DNA based (-)QSat construct for *in planta* expression via agroinfiltration. The cDNA of Q-SatRNA is cloned into pCass HDV binary vector in reverse orientation. The reverse complementary RNA sequence to QSatRNA is expressed *in planta* from this construct due to the correctly initiated 5'-end driven by a double 35S promoter and correctly terminated 3'-end produced by HDV ribozyme activity.

(B) Northern blot analysis of total RNA recovered at 4 days post infiltration (dpi) from *N. clevelandii* leaves agroinfiltrated with indicated agrotransformant cultures (-)QSat (p(-)QSat agrotransformant); (-)QSat+HC-Pro; CMV+(-)QSat (pQ1+pQ2+pQ3+p(-)QSat); and CMV+QSat (pQ1+pQ2+pQ3+pQSat)
The positions of CMV RNAs, QSat RNA monomer (1x), dimer (2x), and trimer (3x) are indicated on the right. Methylene blue stained 25S rRNA shown on the bottom of each Northern blot image as a loading control.

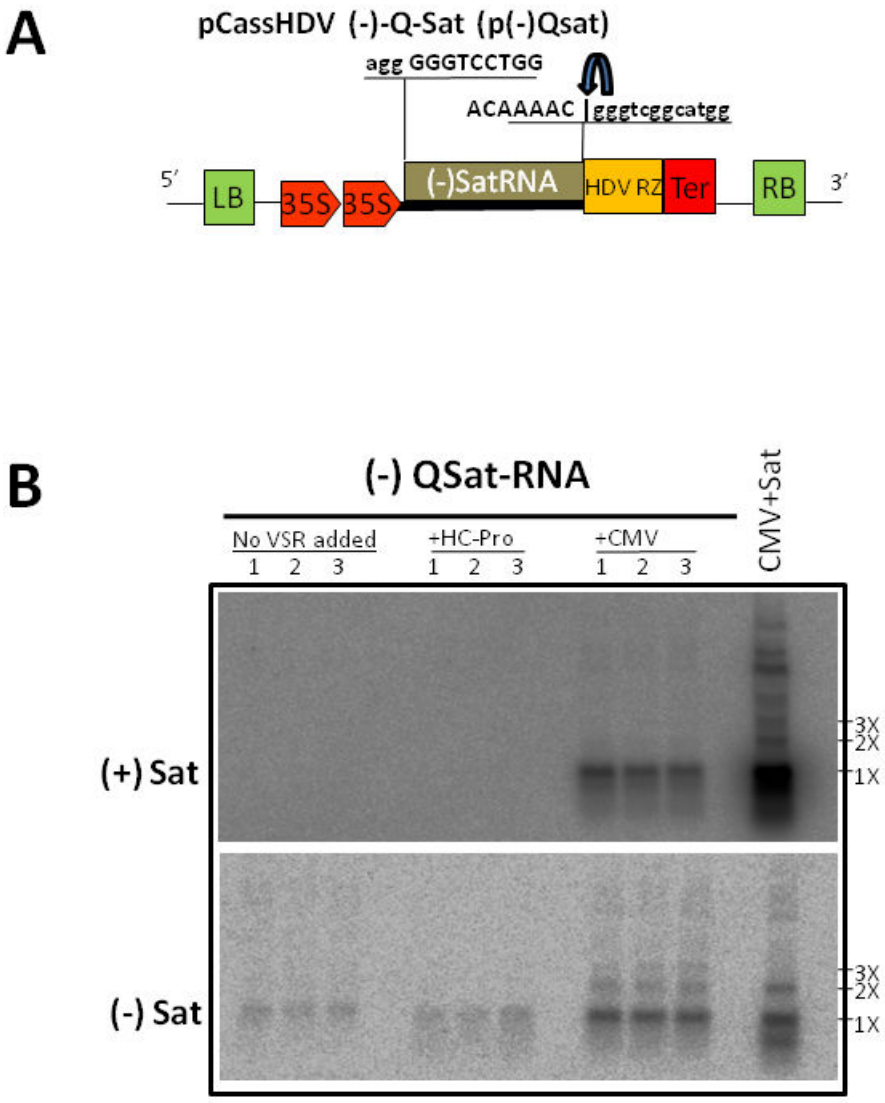

A
pCassHDV (-)-Q-Sat (p(-)Qsat)
agg GGGTCCTGG
ACAAAAC | gggtcggcatgg
5′
LB
35S
35S
(-)SatRNA
HDV RZ
Ter
RB
3′
B
(-) QSat-RNA
CMV+Sat
No VSR added
1 2 3
+HC-Pro
1 2 3
+CMV
1 2 3
(+) Sat
3X
2X
1X
(-) Sat
3X
2X
1X

Figure 3-2 Another demonstration showing that autonomously expressed (-)QSat RNA is not amplified by the host.

Immunolabeling of dsRNA in leaf segments of *N. Benthamiana* prepared 4 days post agroinfiltration. Thin sections of healthy leaf, p(-)QSat, pQSat, and pCMV+pQSat infiltrated leaves are used. DAPI stained nuclei are shown in blue. dsRNAs probed with J2 mAb and then with AlexaFluor 633 conjugated secondary antibody are shown in red.

Bar: 10μm.

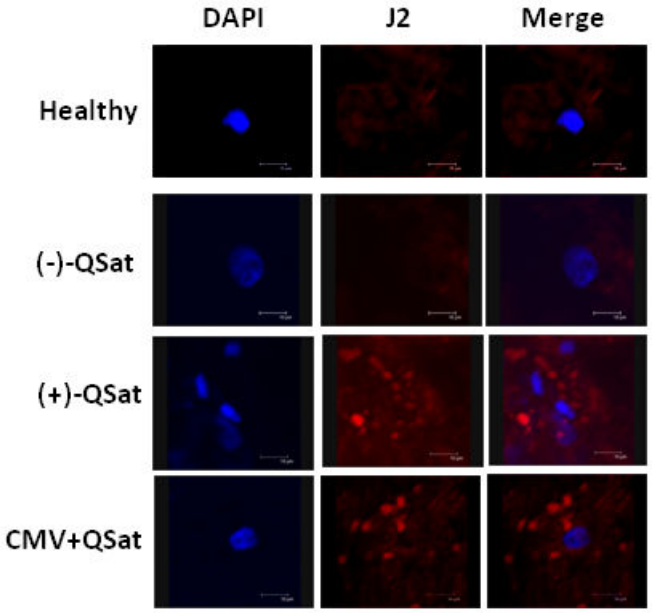

DAPI
J2
Merge
Healthy
(-)-QSat
(+)-QSat
CMV+QSat

Figure 3-3 Replication initiated with (-)QSat RNA: Comparison of QSat RNA accumulation in infiltrated leaf vs. systemic leaves.

Northern blot analysis of total RNA recovered at 4 days post infiltration (dpi) from *N. clevelandii* leaves agroinfiltrated with pQ1+pQ2+pQ3+pQSat (designated as +) or pQ1+pQ2+pQ3+p(-)QSat (designated as -). I: agroinfiltrated leaf; S: systemic leaf; H: healthy. The positions of QSat RNA monomer (1x) and dimer (2x) are indicated on the right. Methylene blue stained total RNA shown on the bottom of the Northern blot image as a loading control.

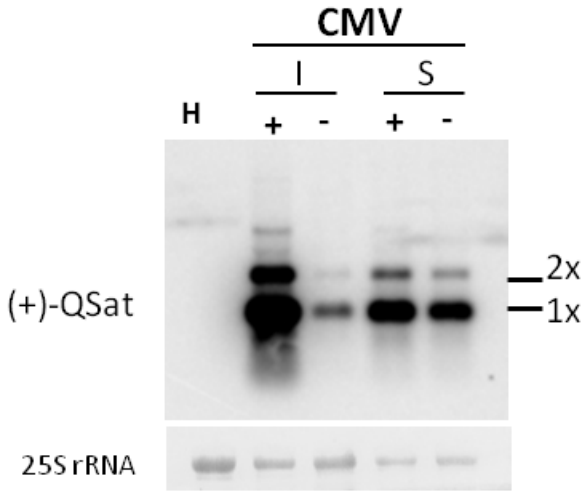

CMV
I
S
H
+
-
+
-
(+)-QSat
2x
1x
25S rRNA

Table 3.1 List of agro-plasmids and riboprobe templates			
Name of the construct	**vector type**	**RNA expressed (abbreviation)**	**Proteins expressed**
pQ1	pCass4 binary	Q-CMV RNA 1 (Q1)	1a of CMV
pQ2	pCassRZ binary	Q-CMV RNA 2 (Q2)	2a and 2b of CMV
pQ3	pCassRZ binary	Q-CMV RNA 3 (Q3)	MP and CP of CMV
pQSat	pCassHDV binary	Q-Sat RNA (variant Q)	none
p(-)QSat	pCassHDV binary	negative-sense Q-Sat RNA	none
HC-Pro	pCass4 binary	*Potyvirus* HC-Pro mRNA	Viral suppressor of RNAi (VSR) HC-Pro
pT7T3 Qsat	pT7T3 - riboprobe template	either (+)-Qsat or (-)-Qsat full-length transcript	none
pUC T7 Q2-TLS #2	pUC19 - riboprobe template	(-)-Q2 tRNA like structure transcript	none

CONCLUSION

Uniqueness of this study on SatRNA replication

The discovery of the involvement of nucleus in QSatRNA replication cycle described in this dissertation has been possible because of several reasons. First, we have expressed the satRNA in the absence of its helper virus *in vivo*. No one except two groups (Jacquemond and Lot, 1982; Mossop and Francki, 1978) have reported a study on satRNA in the absence of its helper CMV *in vivo*. Second, we have employed a transient expression method by using agroinfiltration system. It allows a steady supply of satRNA in the infiltrated leaf for several days, which is not possible with mechanical inoculation method that most of the previous studies had employed. Third, the binary vector construct expressing viral suppressor of RNAi (gift from Dr. Show-wei Ding in Plant Pathology Department at UCR) has enabled us to detect satRNA accumulation in the absence of the helper virus. No previous studies have been able to find the existence of the nuclear phase because the methods or tools we have used in this study were either unavailable or unused, and because of the presumption that the co-expression with the helper virus is mandatory for satRNA replication study.

"Nuclear phase" has been implicated before

Many puzzling characteristics of CMV satRNA have been described which probably hinted the existence of the nuclear phase in satRNA replication. Most

notable ones are: (i) CMV satRNAs are unusually resistant to cellular nuclease that it can survive for many days in the host (Jacquemond and Lot, 1982; Mossop and Francki, 1978); (ii) tomato plants harboring CMV with satRNA show significantly milder symptom when infected with PSTVd, and it was shown to be because of the satRNA, not CMV (Montasser, Kaper, and Owens, 1991; Yang, Kang, and Tien, 1996); and (iii) CMV satRNA can be found in the nuclear fraction of the infected cell (Diaz-Ruiz, Avila-Rincon, and Garcia-Luque, 1987).

SatRNAs are predominant RNA species in tobacco plants infected with CMV and SatRNA (Kaper and Tousignant, 1977; Mossop and Francki, 1979). However, in a cell-free system, the CMV replicase purified from tobacco transcribed CMV SatRNA only about half the efficiency of the viral RNAs (Quadt and Jaspars, 1991), probably because this system cannot provide the nuclear phase. Perhaps the following observations also have implicated the involvement of the nuclear phase in satRNA life cycle: (i) SatRNA is more affected by temperature than CMV RNAs (White et al., 1995); and (ii) Sny-strain of CMV replicates equally well in both squash and tobacco, however, it is able to support satRNA only in tobacco, and not in squash (Gal-On, Kaplan, and Palukaitis, 1995). Like viroids, Q-Sat does not encode any protein so its biolological characteristics results from the direct interaction with the components of host plant and its helper virus (Palukaitis and Garcia-Arenal, 2003). One can speculate that a host protein necessary for satRNA nuclear localization is more affected by the temperature or it is only present in certain plants such as tobacco,

but not in others such as squash. CMV satRNAs do not accumulate efficiently in cucurbit hosts (Garcia-Arenal and Palukaitis, 1999). Can this be because they lack the protein that efficiently delivers SatRNA to the nucleus? Whether SatRNA can be localized in the nucleus of cucurbit hosts such as squash remains to be studied.

Nuclear phase is beneficial for QSat RNA

As shown in Chapter 3 of this dissertation, the nuclear phase must be an integral part of QSatRNA replication cycle. The nuclear phase provides several advantages for the survival of satRNA. First, it gives CMV time for replication since satRNA is a better template for CMV replicase (Wu and Kaper, 1995). Even with satRNA going through the nuclear phase, 90% of the dsRNA accumulated in the CMV + satRNA infected leaf is dsSatRNA (Diaz-Ruiz and Kaper, 1977). If satRNA is available immediately as a better template for the CMV replicase, not enough CMV will be made and it would be disadvantageous for long term survival of satRNA. Second, single stranded SatRNA is less stable in the host than dsSat RNA, because it was shown previously that all small RNAs are from ssSatRNA (Du et al., 2007). dsSatRNA does not appear to be a template for dicers (Du et al., 2007), as the HDV RNA replication intermediate is not a template for dicer activity (Chang, Provost, and Taylor, 2003). Third, dsSat RNA maybe a better template for (+)-strand synthesis as previously considered as a possibility (Hayes et al., 1992; Kuroda et al., 1997; Tousch, Jacquemond,

and Tepfer, 1994). Kuroda *et al*., showed the likelihood of absence of single-stranded negative-sense strand of satRNA because the negative strand junction of satellite RNA oligomers were amplified by PCR only when denatured dsRNA samples were used for cDNA synthesis (Kuroda et al., 1997)

Origin of satellite RNA

The heptanucleotide in the junction of the oligomeric form of QSat RNA originated from the host, probably from inside the nucleus. It has been demonstrated that 3'-end deletion of up to 7 nucleotides of CMV satRNA can be repaired in a template independent manner during CMV replication (Burgyan and Garcia-Arenal, 1998). Is the repair done in the host nucleus? Moreover, CMV satellite RNAs exhibit greatest variations in nucleotide sequence among the known SatRNAs of plant viruses (Roossinck, Sleat, and Palukaitis, 1992), and the accumulation of the variant form was highest in tobacco (Kurath and Palukaitis, 1990). Is it possible that all those variations are made in the nucleus? These questions must be answered. However, for now, it is certain that at least a part of QSatRNA, namely the heptanucleotide junction, have originated from the host cell.

Implication of the evolutionary relationship between satellite RNAs, viroids and HDV

The existence of the oligomeric forms of CMV SatRNA (Kuroda et al., 1997; Linthorst and Kaper, 1985; Young, Palukaitis, and Zaitlin, 1987) may imply the CMV SatRNA undergoing rolling circle replication, however, the circular forms of CMV satRNA have not been found (Kuroda et al., 1997; Linthorst and Kaper, 1985). It has been suggested that the oligomers formed in the SatRNA replicated by the CMV is a product of reinitiation (Kuroda et al., 1997). As demonstrated in Chapter 2 of this dissertation, oligomeric forms are also formed in the absence of the helper virus, and there is a distinct difference between the 2 oligomeric forms in the junction that joins two unit-length satRNAs. It is possible that after the addition of the unique heptamer, the oligomeric forms were produced by reinitiation or even self-ligation since self-ligation of ds RNA monomeric satRNA forms has been mentioned previously (Roossinck, Sleat, and Palukaitis, 1992; Sleat and Palukaitis). Viroids and HDV form oligomeric forms as they are replicated (Tabler and Tsagris, 2004; Wang and Lemon, 1993) in rolling-circle mechanism which occurs in the nucleus of the hosts by RNA polymerase II (Macnaughton et al., 2002; Moraleda and Taylor, 2001). Although rolling circle mechanism may not be involved in QSatRNA amplification by the host, the involvement of the host RNA polymerase II cannot be ruled out.

As a subviral pathogen, satRNA has similarity with PSTVd for its small size and having non-coding RNA as its genome. SatRNA also share some

similarity with HDV that they both depend on their helper virus for cell-to-cell and host-to-host movement. Now, these three subviral pathogens have another common feature in their replication cycle that involves the host nucleus for amplification. Because of this similarity, studying satRNA of cucumber mosaic virus may shed more light in the mysteries surrounding the origin and life cycle of viroids and human hepatitis delta virus.

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