STUDIES ON SATELLITE PANICUM MOSAIC VIRUS (SPMV) AND CUCUMBER MOSAIC VIRUS (CMV) SATELLITE RNA (STRAIN Y)

(サテライトパニカムモザイクウイルスおよびキュウリモザイクウイルスサテライトＲＮＡ（Ｙ系統）に関する研究)

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ABBREVIATIONS

PMV, panicum mosaic virus

SPMV, satellite panicum mosaic virus

CMV, cucumber mosaic virus

TNV, tobacco necrosis virus

STNV, satellite tobacco necrosis virus

TMV, tobacco mosaic virus

STMV, satellite tobacco mosaic virus

MWLMV, maize white line mosaic virus

SMWLMV, satellite maize white line mosaic virus

STobRV, satellite tobacco ringspot virus

BMV, brome mosaic virus

SBMV, southern bean mosaic virus

AMV, alfalfa mosaic virus

CpMV, cowpea mosaic virus

TRV, tobacco rattle virus

TBSV, tomato bushy stunt virus

PEMV, pea enation mosaic virus

Mr, molecular weight

INTRODUCTION

Because satellite systems are particularly interesting from the standpoint of viral replication, symptom alteration and evolution, I have chosen two satellites to study, satellite panicum mosaic virus (SPMV) as a satellite virus and CMV satellite RNA as a satellite RNA. I initiated research on the structure of SPMV. My investigation on the satellite virus has the following two main aims: 1) to determine the relationship between panicum mosaic virus (PMV) and its satellite (SPMV) as a prelude to subsequent studies of the interaction between the two; and 2) to elucidate the fine structure of the SPMV RNA genome. In order to initiate this study, I started with characterization of SPMV and PMV to provide background information for genetic studies. This work was done mostly to confirm and extend the previously reported data of Buzen et al. (1984). Subsequently, I cloned SPMV RNA to provide recombinant DNAs suitable for nucleotide sequence analysis. Finally, sequencing was conducted with a variety of RNA and DNA sequencing techniques to determine the structure of the genome of SPMV. The results reported here should now enable us to construct full-length clones in transcription vectors so that infectivity of RNA transcripts can be tested. Achievement of this goal would open up a broad array of experimental opportunities including studies of the function of different parts of the SPMV genome, requirements for encapsidation of RNA, manipulation of the coat protein cistron to

identify domains responsible for encapsidation, and development of genetic engineering vehicles suitable for expression of foreign genes in cereals.

My second objective to elucidation of satellite systems is CMV satellite RNA. Since the length of the satellite is much smaller than SPMV, I was able to construct a full-length cDNA clone of CMV satellite RNA (strain Y) and infectious transcripts from the cDNA without much trouble. The approach as described above for SPMV can be taken towards identification of functional domains in CMV satellite RNA. The results shown in this thesis cotain the information on the nucleotide sequence regions important for biological activity of CMV satellite.

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LITERATURE REVIEW

More than 75% of plant viruses have single-stranded (ss) plus sense RNA as their genetic material, while the plant reoviruses contain dsRNA, and several viruses contain ssDNA (gemini-viruses) or double-stranded (ds)DNA (caulimoviruses). At present, single-stranded RNA viruses are classified into 21 groups based on their shape, size, and composition, but many viruses remain ungrouped (Matthews, 1985). Most plant RNA viruses replicate readily in their host cells, and an RNA-dependent RNA polymerase (replicase) probably mediates the replication of the genomes of plant RNA viruses. However, in addition to virus-specific replicase, the activity of host-encoded RNA-dependent RNA polymerase is often elevated in plants during the course of virus infection. The role of this enzyme for viral replication has been controversial. It seems likely that individual viruses depend on the presence of certain viral-specific enzymes for replication, and that the virus-encoded replicases are distinct from the host-encoded RNA-dependent RNA polymerases (van Kammen, 1985). In contrast to most animal RNA viruses, several groups of plant viruses have two to three genomic species encapsidated in different particles which are individually needed for infection. For example, the genomes of alfalfa mosaic virus (AMV), brome mosaic virus (BMV), and cucumber mosaic virus (CMV) consist of three or four components, whereas those of cowpea mosaic virus (CpMV), tobacco rattle virus (TRV), and

tobacco ringspot virus (TRSV) consist of two components. The frequent occurrence of divided genomes might allow those viruses to overcome the tendency of eukaryotic ribosomes to translate only 5' cistrons and also provides an efficient mechanism for reassortment of viral genomes when mixed infections occur (Davies and Hull, 1982). Although two particles are associated and participate in some infections like divided genome viruses, a different situation exists in the requirement of tobacco necrosis virus (TNV) for replication of its satellite (STNV). These properties will be described in detail below.

The predominant 5' terminal structure of plant RNA viruses is an inverted 7-methyl guanosine triphosphate residue that is commonly called a cap structure. Members of four virus groups; southern bean mosaic virus (SBMV), pea enation mosaic virus (PEMV), CpMV and TRSV contain a small genome-linked protein (VPg) at the 5' termini. Notable exceptions are TNV and its satellite (STNV) because they have neither caps nor VPgs. Although a detailed understanding of the requirement for cap structures is not well developed, it has been suggested that the cap may enhance the stability and translation efficiency of viral RNAs as is thought to be the case with mRNAs. The 5' linked VPg might be involved in initiation of viral RNA synthesis by acting as a primer (van Kammen, 1985), but this has not yet been proven. Other factors apparently must also control efficient translation and replication because both TNV and STNV, which contain neither cap nor VPg structures, are efficient messengers <u>in vitro</u> and multiply to high levels in infected cells. In order to elucidate viral replication and expression mechanisms, the satellite sys-

tems of certain plant RNA viruses are particularly interesting for the following reasons: 1) a satellite is dependent for its replication on the helper virus, and the dependence is quite specific; 2) a satellite does not have a cap or a VPg, which is believed to be very important for replication and translation of many RNA viruses.

A part of the goal of the present thesis is to provide the background necessary to initiate some studies of these properties.

Satellites are regarded as molecular parasites of certain plant RNA viruses. Some common properties of these agents are listed in Table 1. According to the criteria defined by Murant and Mayo (1982), satellites are totally dependent for their replication on the "helper" viruses with which they are associated. Moreover, RNAs of satellites have no appreciable homology with the helper viruses RNAs, but they often affect replication ability of the helper virus and may modify disease symptoms on the host plants. Some satellites can code for their own coat proteins which are serologically distinct from the coat proteins of the helper viruses; they are called satellite viruses. Other satellites are encapsidated by the coat proteins of the helper viruses and do not have their own coat protein cistrons; they are called satellite RNAs (Table 1). Hereafter, as far as I do not note it, I distinguish the following terms, a satellite virus, a satellite RNA and a satellite for any satellite agent. Satellite viruses and satellite RNAs must be distinguished from 1) multipartite virus RNAs, 2) subgenomic RNAs, 3) pseudovirions, and 4) defective interfering (DI) particles. The

Table 1. Properties of satellite viruses and RNAs

Property	Satellite virus	Satellite RNA
1. Helper dependence	Yes	Yes
2. Encapsidation	Specific coat	Helper coat
3. Symptom modification	Yes	Yes
4. Interference with helper replication	Yes	Yes
5. Nucleotide sequence homology with helper	No	No
6. RNA size ($\underline{Mr} \times 10^5$)	3 - 4	1 - 5
7. Stability	High	High

systems listed above are defined as follows: 1) In addition to what is described earlier with multipartite viruses, the largest RNA of bipartite viruses or two RNA species of tripartite viruses can replicate autonomously. However, the smaller RNA species, which carries the cistron for the coat protein, can not replicate in the absence of the larger genome RNA species (Matthews, 1981). 2) Subgenomic RNAs are generated from the genomic RNAs during replication and generally serve as mRNAs for synthesis of viral-specific polypeptides. For example, viruses such as BMV, CMV, AMV, and TMV encapsidate their coat protein mRNAs (Matthews, 1981). 3) Occasionally, host plant RNAs such as ribosomal RNAs and transcripts of chloroplast DNA are encapsidated. Such RNAs are called pseudovirions (Siegel, 1971). Host RNA in TMV rods accounted for up to 2.5 % of TMV preparation (Siegel, 1971; Rochen and Siegel, 1984). 4) Defective interfering particles referred to as DI particles, which were first found in the influenza virus system. Such particles have been identified in a variety of animal virus systems particularly in vesicular stomatitis virus (Huang and Baltimore, 1977; Holland, 1986). A striking feature of DI particles is that they decrease the yield of the wild-type viruses (Huang and Baltimore, 1977). However, no plant virus DI particle was known until tomato bushy stunt virus (TBSV) was found to harbor such agents (B. Hillman, personal communication). Several of these defective particles that attenuate virus symptoms to various extents are derived from the parental viruses by a series of deletions. Because these RNAs are defective, they cannot propagate themselves in the absence of the parental viruses.

The classic satellite relationship between STNV and its dependence on TNV as the helper virus was first discovered by Kassanis (1962). Since then, several other satellite viruses have been reported. These include SPMV (Niblett et al., 1975; Buzen et al., 1984) and satellite tobacco mosaic virus (STMV) (Valverde and Dodds, 1986; Valverde and Dodds, 1987). In addition, a satellite-like particle is known to be associated with maize white line mosaic virus (MWLMV), but its absolute dependence on a helper virus for replication has not been determined, because MWLMV is not mechanically transmissible (Gingery and Louie, 1985). Some properties of these satellite viruses are compared in Table 2. Among them, only STNV has been extensively studied; the properties of this virus, its RNA and coat protein, are well understood (Uyemoto, 1981). The complete nucleotide sequence of STNV RNA is now known (Ysebaert et al., 1980). The STNV RNA has a molecular weight (Mr) of 4×10^5 (1239 nucleotides), accounting for about 20 % of the mass of the virus. A half of the RNA encodes the coat protein subunit; the rest does not code for any known protein. The genome structure of STNV RNA will be described in detail in Chapter I-2 and compared with that of SPMV RNA. Recently, the coat protein of STNV was synthesized in E. coli cells from genetically manipulated cDNA clones (Emmelo et al., 1984). However, TNV has not been extensively studied, we do not have a clear understanding of relatedness of structural features shared between the satellite and the helper virus. The other satellite viruses are practically untouched, and no comparison among the satellite viruses at the molecular level has been reported.

Table 2. Comparison of helper and satellite viruses

Property	Helper virus				Satellite virus			
	TNV	PMV	MWLMV	TMV	STNV	SPMV	SMWLMV	STMV
1. Particle shape	I[a]	I	I	R	I	I	I	I
2. Particle diameter (nm)	30	30	30	18x300	17	17	17	17
3. Coat protein size ($Mr \times 10^3$)	30	31	35	17.5	23	17	24	22
4. RNA size ($Mr \times 10^5$)	14	14.5	12.5-16.5	20	4	2.8	4.4	3
5. RNA length (kb)	4	4	4.5	6.4	1.2	0.8	1.4	1.0

[a]I: Isometric particle; R: Rod-shaped particle.

On the other hand, some satellite RNAs such as tobacco ringspot virus satellite RNA (STobRV) and cucumber mosaic virus satellite RNA have been sequenced, and considerable information concerning their biology and mechanism of replication is emerging. For example, the complete nucleotide sequence of STobRV was recently published (Buzayan et al., 1986a). The monomeric STobRV has a hydroxyl group at the 5' end and a 2', 3'-cyclic phosphate at the 3' end. The available evidence (Buzayan et al., 1986b) suggests that the satellite RNAs replicate by a rolling circle mechanism similar to that proposed for viroids. In the rolling circle model, a circular plus strand is copied into either a multimeric complementary strand or a minus strand of a unit length (Branch et al., 1984). In the former case, multimeric minus strands serve as the template for the production of multimeric plus strands, which are subsequently autolytically cleaved to generate unit length RNA molecules. The linear monomeric strands are again circularized to yield progeny circles (Kiefer et al., 1982; Branch et al., 1984). The features controlling the autolytic processing of satellite RNA are major objectives of research on STobRV.

The molecular properties of CMV satellite RNAs will be described in detail in Capter II. Briefly, the satellite RNAs have a cap structure at the 5' end like the genomic RNAs of CMV, but does not have a polyadenylate sequence at the 3' end. Nothing is known about the origin of the satellites. The biological activities of the satellites RNA is very interesting because they interfere with CMV replication and somehow alter the symptoms caused by CMV infections.

Although no animal satellite agent had been reported until very recently, the association of hepatitis delta virus (HDV) with hepatitis B virus (HBV) resembles the plant satellite system (Bonino et al., 1984; Denniston et al., 1986). HBV has a small circular dsDNA molecule (Mr 1.6×10^6), whereas HDV has a ssRNA molecule (Mr 5.5×10^5) (Tiollais et al., 1985). The replication of HDV is dependent on HBV, and HDV infection enhances severity of liver disease of humans. There is no significant homology between HDV RNA and HBV DNA. HBV consists of a 42 nm particle containing a surface antigen and a core antigen. The HDV particle is 38 nm and has a HDV-specific core surrounded by HBV antigen. This suggests that HDV encodes its core protein and that the HBV-HDV system is somewhat analogous to the plant satellite systems except for a DNA helper virus and a more complex particle morphology.

Although several satellite viruses are known, STNV is the only well-studied member of this group. This system thus provides important information from which we can conduct comparative analyses of the other satellite viruses including SPMV. Therefore, I will briefly describe the biological and physical properties of STNV and TNV. STNV is associated with TNV and is incapable of replicating in the absence of the helper virus. The coat protein of STNV is serologically distinct from that of TNV (Grogan and Uyemoto, 1967). The relationship between STNV isolates and their TNV helper viruses is highly specific. Among 11 isolates of TNV tested in the USA, seven isolates activated satellite serotypes SV-A and SV- B, and four other isolates activated SV-C (Uyemoto et al., 1968). Neither TNV nor STNV is

inactivated at 80-90℃, suggesting that both viruses have reasonably stable capsid structures (Babos and Kassanis, 1963). The dilution end point of TNV is 10^{-6}, which indicates that TNV multiplies in a high concentration in infected plants (Uyemoto, 1981). Structural changes occur between pH 5 and pH 7 (McCarthy *et al.*, 1980). STNV RNA is very stable *in vivo* because it could survive for 10 days in inoculated leaves in the absence of helper virus (Mossop and Francki, 1979). On the other hand, TNV RNA is relatively unstable; its infectivity in leaf sap was lost after 30 min incubation (Mossop and Francki, 1979). Addition of STNV to TNV results in changes in symptoms on infected plants. The number of necrotic lesions and the amount of TNV on bean plants are decreased in inoculum containing STNV (Kassanis, 1962). Both TNV and STNV are transmitted by zoospores of *Olpidium brassicae* and appear to be attached to the surface membranes of the zoospores (Temmink *et al.*, 1970).

TNV is about 30 nm in diameter and has a sedimentation coefficient of 118S (Uyemoto, 1981). The TNV genome consists of a ssRNA molecule of *Mr* 1.4×10^6, and a coat protein of *Mr* 32 K (Lesnaw *et al.*, 1969; Salvato *et al.*, 1977). Two dsRNA species corresponding to subgenomic RNAs present in virus preparations were detected in extracts from tobacco plants infected with TNV alone (Condit and Fraenkel-Conrat, 1979). This suggests that the subgenomic RNAs may replicate via a dsRNA mechanism.

STNV is about 17 nm in diameter and has a sedimentation coefficient of 50S (Uyemoto, 1981). STNV consists of a ssRNA molecule of *Mr* 0.4×10^6 and a coat protein of *Mr* 22 K (Reichmann, 1964). Details of the particle subunit structure of

STNV have been obtained at 3.0 and 2.5 angstrom resolution by X-ray chrystallography studies. Although STNV has an icosahedral structure similar to TBSV and SBMV, the latter have T=3 icosahedral particles with 180 subunits arranged in quasi-equivalent symmetry whereas STNV has a triangulation number of 1 with 60 subunits arranged in exact symmetry (Caspar and Klug, 1962). The results of the 3-dimensional structural determinations of STNV by Liljas et al. (1982), and Jones and Liljas (1984) are summarized below. The amino acid residues 1 to 24 (N-terminus) of the protein subunit include two lysine, four arginine, and one histidine residues. These amino acids form an arm penetrating the RNA region. This hydrophilic arm forms a three-turn alpha helix, and interacts with the RNA. The main part of the protein subunit is built up to two four-stranded antiparallel beta sheets (β strand B-1). Most of the hydrophobic residues are found inside the subunit, while hydrophilic residues are localized in the N-terminal helix and beta strands F and G. The outer surface of the virus contains several polar (asparagine and threonine) and small hydrophobic (alanine) residues, but few charged groups. The negatively charged residues such as glutamic acid and aspartic acid are found mostly in subunit-subunit interfaces where they form salt bridges with lysine and arginine residues. The protein-protein contacts utilize hydrophobic interaction (phenylalanine and isoleucine), and hydrogen bonds. The protein shell appears to be stabilized by calcium ions (Unge et al., 1986).

CAPTER I. STUDIES ON SATELLITE PANICUM MOSAIC VIRUS AND PANICUM MOSAIC VIRUS

I-1. Properties of satellite panicum mosaic virus and panicum mosaic virus

Panicum mosaic virus (PMV) was first found on switch grass in Kansas (Sill and Pickett, 1957), and its associated component was described as a possible satellite virus in 1977 by Buzen et al. PMV can replicate alone, while SPMV needs PMV as a helper virus for replication (Niblett et al., 1975). They have serologically distinct coat proteins, and SPMV is believed to be able to code for its own coat protein like STNV (Buzen et al., 1984). Both the helper and the satellite viruses are mechanically transmissible and infect only gramineous species. Ohio 28 corn and millet are useful indicators and suitable hosts for purification of the virus (Sill and Talens, 1962; Paulsen, 1970; Niblett et al., 1975; Buzen et al., 1984). Neither vector nor seed transmission has been reported. The St. Augustine decline strain of PMV (PMV-SAD) causes serious losses in St. Augustine grass lawns in Texas where lawn and turf grass production is a major enterprise (McCoy et al., 1969). The first symptoms appear as a mild chlorotic mottling of the leaf blades and then stolon growth is retarded. Finally, necrosis of the leaves and stolons occurs. The SAD strain is closely related serologically to the type strain of PMV (Holcomb, 1974). The satellite virus of one PMV strain (type) was activated by the larger particle of a dif-

ferent PMV (SAD strain) (Buzen et al., 1977). On the other hand, two different SPMV serotypes, including SPMV-SAD (LI), were capable of replication in the presence of a single PMV serotype (serotype 5). Therefore, no selective activation as reported for TNV and STNV was observed (Buzen et al., 1984). Six serotypes of PMV and two of SPMV were reported (Holcomb, 1974). PMV and SPMV were extensively characterized by Niblett and Paulsen (1975), and Buzen et al. (1984). These studies revealed the following properties: PMV is about 30 nm in diameter and is an isometric 109 S particle, while SPMV is about 17 nm in diameter and is an isometric 42S particle. The capsid proteins of SPMV and PMV are Mr 16 K and Mr 30 K, respectively, while PMV RNA has a sedimentation coefficient of 28S and SPMV RNA has a sedimentation coefficient of 14S. The dilution end point of the virus is 10^{-5}, the thermal inactivation point (at 10 min) is 85℃, and the longevity in vitro is 14-16 days. Purified PMV appeared to be unstable and degraded during storage (Niblett et al., 1975).

The work described in this section confirms some of the results above, and provides further analyses of the structure of the genome of SPMV RNA and its relationship to PMV. In some cases, the data were slightly different from those of Buzen et al. (1984), but they were reasonably consistent with the most important observations. In this section, the results on characterization of SPMV and PMV are described.

Materials and Methods

Viruses and plant materials. The type strain of PMV was ob-

tained from Dr. S.A. Lommel (Kansas State University) and maintained in pearl millet (Pennisetum glaucum) grown in the greenhouse at 20-28℃. Plants were inoculated 10-14 days after planting by rubbing leaves with sap from infected plants to which celite had been added. Virus cultures were transferred to healthy plants every other week.

Virus purification. The virus was purified by a slight modification of the procedure previously described by Buzen et al. (1984). Infected plants were harvested 7-10 days after inoculation, and immediately processed for purification. The leaves were ground with 3 volumes of 0.1 M potassium phosphate buffer (pH 7.0) (PB) containing 0.1% 2-mercaptoethanol. The tissue was removed by squeezing it through cheesecloth, and the liquid extract was centrifuged for 10 min at 8,000 rpm in the SS-34 rotor (Sorval). The supernatant was recovered, and NaCl and polyethylene glycol 6,000 (PEG) were added to 0.2 M and to 10% (w/v), respectively. After the mixture was stirred at 4℃ for 2 hr, the suspension was again centrifuged at 8,000 rpm in the SS-34 rotor for 10 min. The pellet was resuspended in 0.1 M PB (1 ml per 2 g of tissue) and after Triton X-100 was added to 1%, the suspension was stirred overnight at 4℃. The resulting suspension was mixed with an equal volume of a cold chloroform-butanol (1:1) and the mixture was stirred at 4℃ for 30 min. The emulsion was broken by centrifuging at 5,000 rpm for 10 min in the SS-34 rotor. The aqueous phase was removed and the virus was precipitated by adding NaCl to 0.2 M and PEG to 10%. After centrifugation as described above in the SS-34 rotor, the pellet was resuspended in 0.1 M PB and then layered on sucrose density

gradients (0-30%) in the SW-27 rotor. The sample was centrifuged at 25,000 rpm for 5 hr at 13℃, and the gradients were fractionated using a model D ISCO fractionator. The virus was recovered by PEG precipitation as described earlier, resuspended in 5% ethylene glycol in 0.2 M PB, and then frozen at -20℃.

Extraction of viral RNA. RNAs were isolated from purified virus by the method originally described by Jackson and Brakke (1973), and modified by Hunter et al. (1986). One ml of the virus suspension (1-2 mg/ml) was mixed with 40 μl of 2 mg/ml protease K, and incubated for 30 min on ice. An equal volume of 0.2 M ammonium carbonate buffer (2 mM Na_2EDTA, 2% SDS, 100 μg/ml bentonite, pH 9.0) was added, and the sample was then extracted twice with an equal volume of a phenol and chloroform mixture (40% phenol, 50% chloroform, 0.05% 8-hydroxyquinoline, 5% m-cresol and 5% H_2O). After centrifugation, the aqueous phase was removed and two volumes of absolute ethanol and 1/20 volume of 3 M sodium acetate (pH 4.8) was added. The sample was stored at -20℃ overnight and centrifuged at 8,000 rpm in the SS-34 rotor. The pellet was dissolved in H_2O, and 3 volumes of 4 M sodium acetate (pH 6.0) were added to the RNA. The mixture was stored on ice for 2 hr and centrifuged to precipitate the RNA. The pellet was resuspended in H_2O and precipitated again with 2 volumes of absolute ethanol. The resulting pellet was dissolved in H_2O and stored at -20℃ in small aliquots.

Serology. Antisera against PMV and SPMV were prepared as reported by Hunter et al. (1986). Rabbits were injected intramuscularly three times at 6-7 day intervals with 0.5-1 mg of virus in 1 ml of PBS (15 mM potassium phosphate, pH 7.0, 150 mM

NaCl). For an intramuscular injection, an equal volume of incomplete Freund's adjuvant was used to emulsify the virus. After the rabbits were given a fourth intravenous injection one week later, they were bled and the serum was collected. The antiserum titers were determined against 0.5 mg/ml of homologous antigen in Ouchterlony double diffusion plates (Uyemoto et al., 1968). Antiserum against PMV had a titer of 1:128 and antiserum against SPMV had a titer of 1:256. The antisera were used to identify the existence of satellite particles in a plant throughout this research. Because of slight contamination of SPMV particles in the PMV suspension, non-specific reactions were observed, whereas the anti-SPMV serum had a specific reaction. Thus, the anti-PMV serum was diluted by 2×10^{-1} for the serology tests so that non-specific reactions should not be observed.

Electron microscopy. Purified virus particles were negatively stained by mixing them in a drop of 2% uranyl acetate on formvar-coated copper grids (300 mesh) stabilized with evaporated carbon. After excess liquid was removed by a filter paper, the preparations were examined with a Philips EM-200 electron microscope operating at 60 kilovolts.

Gel electrophoresis of protein. To disrupt coat proteins, two μg of purified virus were incubated in 10 μl of dissociation buffer (35 mM Tris-HCl, pH 8.3, 0.3 M glycine, 7.5% SDS, 0.1% 2-mercaptoethanol) at 50℃ for 1 hr. The proteins were analyzed on a 17.5% SDS polyacrylamide slab gel (acrylamide : bisacrylamide = 75 : 1, 3.75 mM Tris-HCl, pH 8.9, 0.058 mM TEMED, 0.035% ammonium persulfate, 0.075% SDS) as described by Laemmli (1970) and Larkins and Hurkman (1978). The gel was 1.5 mm

thick, with a 9 cm running gel of 17.5% acrylamide overlaid with a 2.5 cm stacking gel of 5% acrylamide. To the sample (10 μl), 5 μl of tracking dye (0.025 M Tris-HCl, pH 8.3, 20% ficoll, 0.1% bromophenol blue, 0.19 M glycine, 1% SDS) and 10 μl of running buffer (0.025 M Tris-HCl, pH 8.3, 1% SDS, 0.1% 2-mercaptoethanol, 0.19 M glycine) were added. After samples were loaded on the gel in the running buffer, electrophoresis was carried out at room temperature at 15 mA through the stacking gel and 25 mA through the running gel until the dye reached the bottom of the gel. The gels were stained for 2 hr in a 0.1% Coomassie blue solution containing 45% methanol and 9% acetic acid and destained overnight in a destaining solution (5% methanol, 10% acetic acid).

Gel electrophoresis of denatured RNA. RNA samples were incubated at 55℃ for 15 min in GFP (1 M glyoxal, 70% formamide, 10 mM sodium phosphate, pH 6.8) as described by Covey and Hull (1981). Tracking dye (20 % ficoll 400, 5 mM EDTA, 0.2 % bromophenol blue, 0.75 % orange G) was added to the sample (1 μl tracking dye per 4 μl of RNA) and the RNA was electrophoresed for 2.5 hr at 7 V/cm in a vertical 1.5 % agarose gel in electrophoresis buffer (25 mM Tris-acetate, pH 7.9, 5 mM sodium acetate, 1mM Na_2EDTA). The gels were stained with 1 μg/ml of ethidium bromide for 30 min, destained in H_2O for 15 min and then photographed with a polaroid camera under ultraviolet light illumination.

Northern blot hybridization. RNAs separated by gel electrophoresis were transferred onto nitrocellulose papers by blotting in the presence of 20 x SSC overnight (Thomas, 1980). The papers were then baked for 2 hr at 80℃ under vacuum.

Hybridization reactions were carried out under the conditions described by Jackson et al. (1983). The RNA samples were prehybridized at 68℃ for 2 hr in a solution containing 3 x SSC, 0.1% each of BSA, PVP, and ficoll and 30 μg/ml of sheared calf thymus DNA. Nick-translated DNA or ^{32}P-labeled cDNA (1.0-1.5 x 10^6 cpm/ml) was added and hybridized at 68℃ overnight. After hybridization, the filters were washed twice at room temperature and twice at 68 ℃ in 2 x SSC and 0.1% SDS. The filters were then autoradiographed at -70 ℃.

cDNA probe synthesis. The reaction mixture (50 μl) contained 50 mM Tris-HCl, pH 7.5, 75 mM KCl, 3 mM $MgCl_2$, 0.1 M DTT, 2.5 mM each of dCTP, dGTP, and dTTP, 125 μM dATP, 25 μCi of [α-^{32}P] dATP, 1-2 μg viral RNA, 50 ng of hexanucleotides as a random primer and 1 μl (200 units) M-MLV reverse transcriptase. After incubation at 37℃ for 1 hr, the mixture was boiled for 2 min to destroy RNA and inactivate the enzyme, cooled on ice, and then centrifuged for 5 min to remove the denatured enzyme. The supernatant was loaded on a Sephadex G-50 column and cDNA was separated from unincorporated isotope by spun centrifuging at 2,000 rpm for 2 min in a clinical centrifuge.

Nick-translation. Nick-translated probes were synthesized as described by Maniatis et al. (1982). The reaction mixture (50 μl) contained 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 2.5 μg BSA, 2.5 mM each of dATP, dGTP, and dTTP, 125 μM dCTP, 50 μCi of [α-^{32}P] dCTP, 1 μg dsDNA, and 30 ng DNase I. After incubation at 37℃ for 10 min, the sample was cooled on ice and DNA polymerase I (10 units) was added, and the mixture was incubated at 15℃ for 2 hr. The reaction was

terminated by adding 5 μl of 0.25 M EDTA and DNA was separated on a column of Sephadex G-50 as described above.

Isolation of dsRNA. Double-stranded (RF) RNAs were extracted from millet tissue infected with PMV and SPMV by cellulose column chromatography essentially as reported by Jackson et al. (1971). Infected leaves (100 g) were frozen in liquid nitrogen and ground to a powder. Two to four volumes of RNA extraction buffer (Rezaian et al., 1983) were added to the powder and mixed well. RNA extraction buffer consists of 50 mM Tris-HCl, pH 8.4, 5% Kirby's phenol (saturated with 25 mM Tris-HCl (pH 8.8) buffer, 10% m-cresol, 1% 8-hydroxyquinoline), 1% triisopropylnaphthalene sulfonate, 6% 4-aminosalycilate, and 1% 2-mercaptoethanol. The resulting slurry was extracted once with an equal volume of phenol-chloroform and then with a half volume of phenol-chloroform, and the aqueous phase was separated by centrifugation at 8,000 rpm for 5 min in the GSA rotor. The RNAs were precipitated by addition of 1/20 volume of 3 M sodium acetate (pH 5.2) and two volumes of absolute ethanol. After storage at -20℃ overnight, the pellet was collected by centrifugation at 8,000 rpm for 25 min and resuspended in STE (50 mM Tris-HCl, pH 6.85, 0.1 M NaCl, 1 mM Na_2EDTA). After another ethanol precipitation, the pellet was dissolved in 20 ml of STE and loaded on a CF-11 cellulose column that had been washed first with STE-20% ethanol, then with STE, and finally again with STE-20% ethanol. After adding the RNA sample, stepwise elution was carried out first with STE-25% ethanol to eliminate mainly DNA and tRNA, then with STE-15% ethanol to eliminate single-stranded (ss) RNA, and finally with STE to collect double-stranded (ds)

RNA. The dsRNA in STE was transferred to a small column and rechromatographed. The recovered nucleic acid was concentrated by ethanol precipitation followed by centrifugation at 10,000 rpm for 10 min in the Sorvall HB-4 rotor. The pellet was dissolved in H_2O and the yield of dsRNA was determined spectrophotometrically (20 A_{260} = 1 mg/ml of ds RNA). Denaturation by boiling and northern hybridizations verified that the dsRNAs obtained by this method were free of ribosomal RNA and were of viral origin.

Results

Purification and electron microscopy. The absorbance profiles of sucrose density gradients loaded with PEG concentrated extracts from virus-infected plants contained two absorbance peaks (Fig. 1). Zone A contained only small isometric particles (Fig. 2B) whereas zone B contained larger isometric particles and some small particles (Fig. 2A). Some degraded particles were also observed in preparations of the larger particles. The shoulder on zone A, which was also observed in the preparations of Niblett and Paulsen (1975), may be dimeric forms of the small particles. The diameters of the small and the large particles were estimated by particle measurement to be about 17 nm and 30 nm, respectively. After the infectivity tests, whose results will be shown below, it was confirmed that the larger particle was PMV and the smaller particle was SPMV. The virus yield by this procedure was about 0.3 mg of each component per gram fresh weight of millet tissue. This was three times as much as that reported by Buzen et al. (1984). Two polyethylene glycol

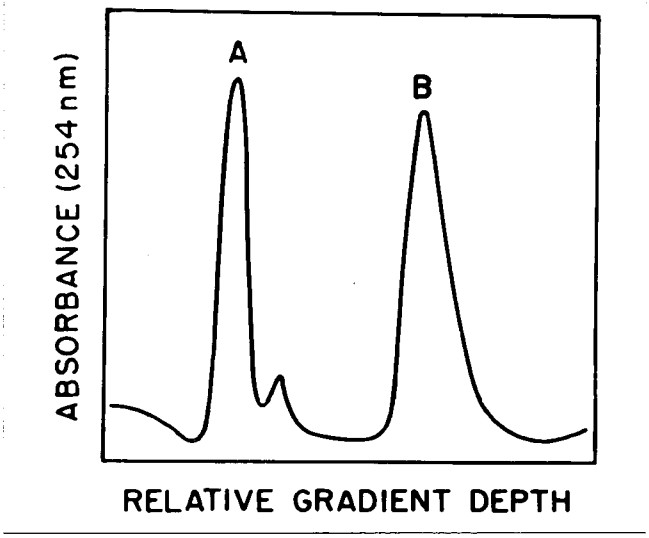

Figure 1. Sucrose density gradient profile of an extract from millet tissue infected with panicum mosaic virus (PMV) and its satellite (SPMV). Samples were centrifuged in a 0-30 % linear sucrose gradient at 25,000 rpm for 5 hr at 13 ℃ in the SW-27 rotor. Zone A and B are SPMV and PMV, respectively. The top of the gradient is to the left.

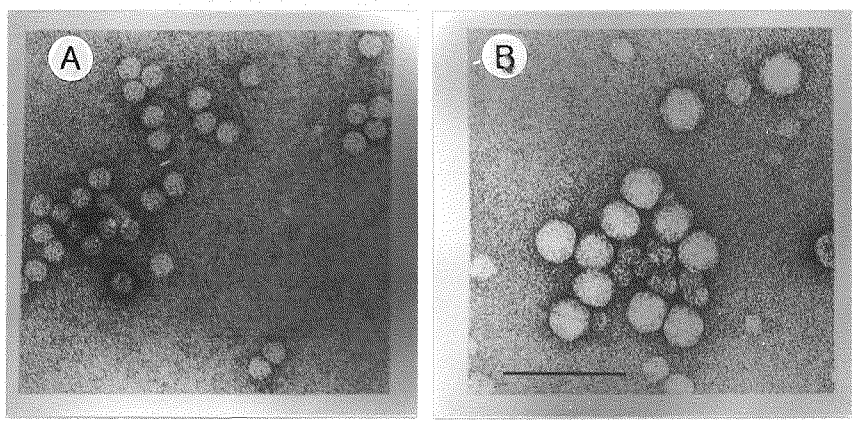

Figure 2. Electron micrographs of virus particles negatively stained with 2 % uranyl acetate. A. satellite panicum mosaic virus (SPMV); B. panicum mosaic virus (PMV). The bar represents 100 nm.

(PEG) concentrations (8 and 10%) were tested at the last PEG precipitation step. As a result, the recovery of SPMV was 30% higher at 10% PEG than at 8% PEG while the recovery of PMV was 15-20% higher at 8% PEG than at 10% PEG.

Symptoms and detection of virus. The large particle, the small particle, and the mixture were inoculated to millet and corn (Ohio 28). Inoculation with only the large particle, which was obtained from Dr. C.L. Niblett, resulted in a mild mosaic symptom, and the infected plants could grow without any significant stunting. However, plants inoculated with the mixture showed severe mosaic symptoms and necrosis (Fig. 3). Inoculation with only the small particle did not elicit symptoms. The existence of each particle in sap from the infected plants was examined by gel double-diffusion assays using antisera which were described in the Materials and Methods section. The antigen against the large particle was detected in the sap from the plants inoculated with the large particle by using a diluted anti-PMV serum. The antigen against the small particle was detected only in the sap from the plants inoculated with both components. In the sap from the plants inoculated with only the small particles, neither the small nor the large particle was serologically detected. These results satisfied the criteria required for a satellite (Murant and Mayo, 1982), eg: that a satellite virus is unable to replicate without the helper virus whereas the helper virus can multiply alone. The third criterion that there be no appreciable sequence homology between the two RNAs, was also met when hybridization tests were conducted (data shown below). Subsequently, I will refer to the small and the

Figure 3. Symptoms induced by PMV and SPMV. Part A. Symptoms on corn leaves (Ohio 28) infected with panicum mosaic virus (PMV) and its satellite (SPMV).

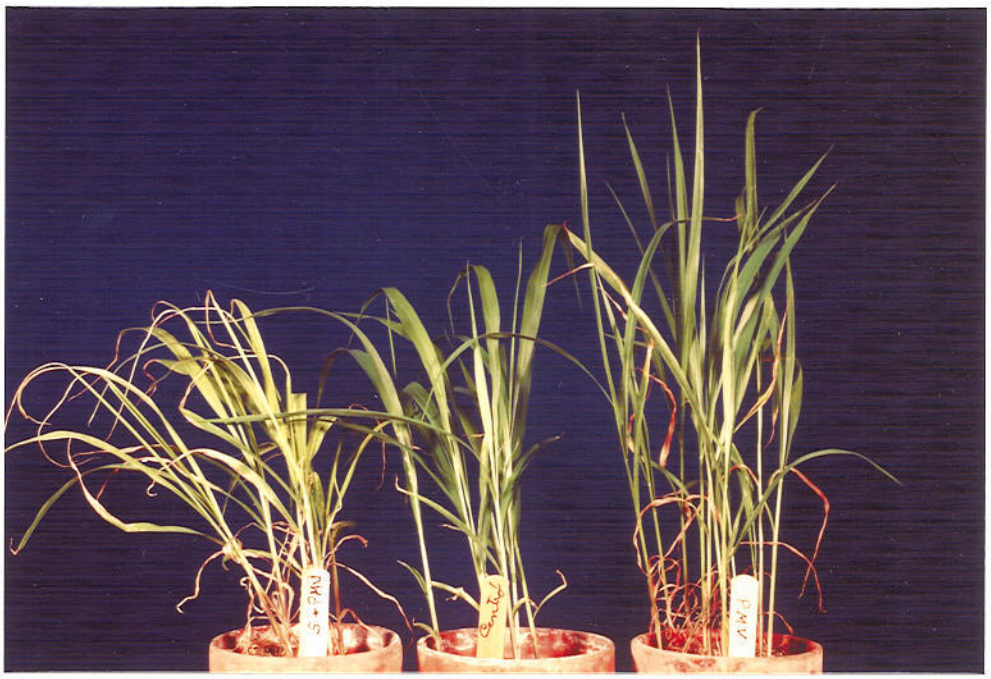

Figure 3 (continued). Part B. Infectivity of PMV and SPMV on millet plants. The plants infected with PMV + SPMV (left) showed more severe symptoms (stunting and die-back) than those infected with PMV alone (right). Healthy plants are shown in the middle.

large particles as SPMV and PMV, respectively.

Coat proteins. The samples containing virus particles reacted only with their homologous antisera in gel diffusion tests. Therefore, PMV and SPMV have serologically distinct coat proteins. Polyacrylamide gel electrophoresis of virus preparations revealed the coat protein polypeptides (Fig. 4). The helper virus coat protein had a size of about Mr 30 K and nearly comigrated with southern bean mosaic virus coat protein. In contrast, the satellite coat protein migrated slightly more rapidly than the TMV coat protein. From these mobilities, the size of the SPMV polypeptide was estimated to be Mr 17 K, which is only slightly different from the Mr 16 K reported by Buzen et al. (1984).

RNAs. The viral RNAs denatured with glyoxal were resolved by electrophoresis in agarose gels (Fig. 5). PMV RNA consisted of at least two distinct components. The major component of PMV had a size of about 4,000 nucleotides, while the minor component was about 1,300 nucleotides in length. The satellite RNA moved slightly more rapidly than BMV RNA 4 which has a size of 876 nucleotides (Ahlquist et al., 1984a). The size of SPMV RNA was estimated to be about 800 nucleotides. This contrasts with the 1,239 residues reported for STNV RNA (Ysebaert et al., 1980). The relatedness of the PMV RNA and SPMV RNA was further examined by nucleic acid hybridizations with radioactive cDNAs from the two viral RNAs. Fig. 6 clearly shows that there is no hybridization of SPMV RNA with a cDNA probe from PMV. Conversely, there is no hybridization of PMV RNA with a cDNA probe from SPMV. These results, therefore, suggest that there is no appreciable

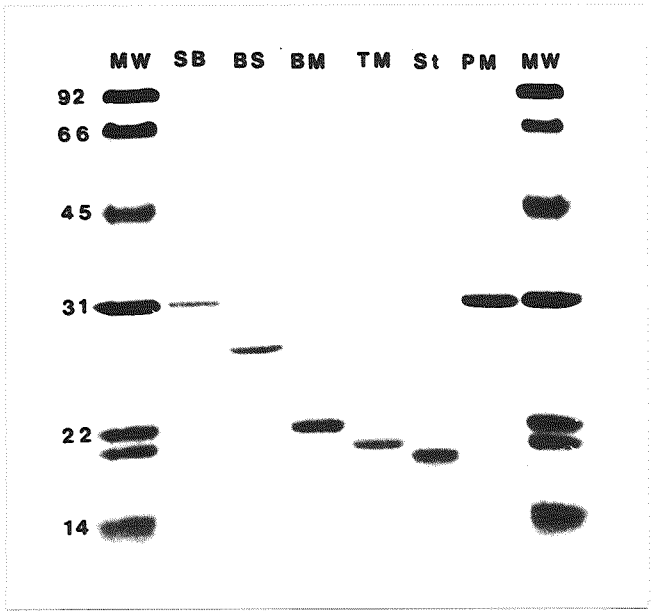

Figure 4. Electrophoretic mobility of panicum mosaic virus (PMV) and its satellite (SPMV) coat proteins in a polyacrylamide gel. As described in the Materials and Methods, two μg of purified virions of PMV (PM) and SPMV (St) were disrupted with 7.5 % SDS and separated in a 17.5 % polyacrylamide gel. The gel was stained with Coomassie blue. Lane SB, BS, BM and TM contain the coat proteins of southern bean mosaic virus, barley stripe mosaic virus, brome mosaic virus and tobacco mosaic virus, respectively. The outer lanes (MW) contain molecular weight standards whose sizes (in kilodaltons) are shown at the left of the gel.

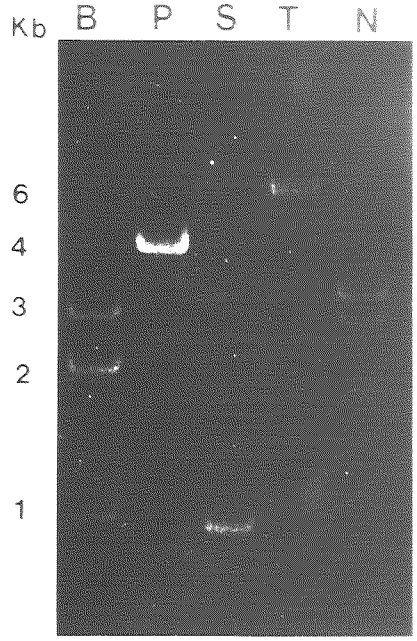

Figure 5. Analysis of RNAs isolated from panicum mosaic virus (PMV) and its satellite (SPMV) by electrophoresis in an agarose gel. Purified RNAs (2 μg) from PMV (P), SPMV (S), brome mosaic virus (B), tobacco mosaic virus (T) and barley stripe mosaic virus (ND18 strain) (N) were denatured with glyoxal and electrophoresed in a 1.5 % agarose gel. The gel was stained with ethidium bromide and visualized under ultraviolet light. Numbers at the left side indicate the expected mobility of different sized RNAs (in kilobases).

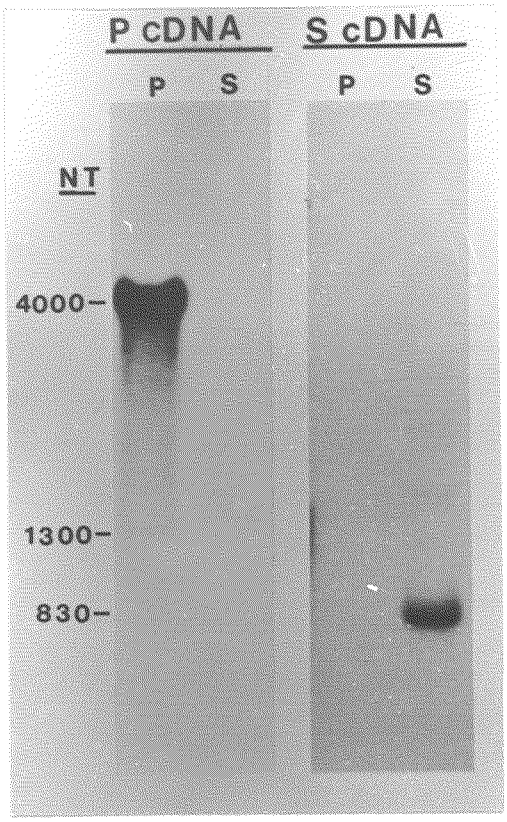

Figure 6. Hybridization of the RNAs of panicum mosaic virus (PMV) and its satellite (SPMV) with cDNA probes from PMV and SPMV. Purified RNAs (1 μg) of PMV (P) and SPMV (S) were denatured with glyoxal, electrophoresed in 1.5 % agarose gels and transferred to nitrocellulose filters in 20 x SSC. The filters were hybridized with [^{32}P]-labeled cDNAs randomly primed from PMV RNA (P) or SPMV RNA (S). The hybridization was conducted at 68℃, overnight. Estimated sizes (in nucleotides) of the viral RNAs are indicated at the left of the gel.

homology between the two RNAs.

Double-stranded RNAs were extracted from infected tissues to verify the existence of replicative form (RF) of the viral RNAs, and to utilize the negative strand of SPMV RNA for sequencing. The dsRNAs were also compared with the single-stranded viral RNAs by gel electrophoresis (Fig. 7). Lanes 1 and 4 of Fig. 7 show PMV RNAs (4,000 and 1,300 nucleotides) and SPMV RNA (800 nucleotides), respectively. The native dsRNAs (lane 2), which contain three major components, migrated significantly more slowly than the three bands released from the dsRNAs melted by boiling for 2 min just before loading on the gel (lane 3). The melted dsRNAs had mobilities identical to the 4,000, 1,300 and 800 nucleotide species representing PMV and SPMV genomic RNAs. The dsRNAs were also denatured and glyoxalated before agarose gel electrophoresis. After blotting onto nitrocellulose papers, these RNAs were hybridized with cDNA probes from PMV and the mixture of PMV and SPMV (Fig. 8). The results suggest the upper two RNA molecules were derived from the dsRNAs of PMV while the fastest RNA molecule was generated from the dsRNA of SPMV. The RNA species with the size of 1,300 nucleotides may be either a subgenomic RNA or possibly part of the genome of a multipartite virus.

The viral RNAs used for synthesis of cDNA probes or kinase-labeled RNA probes were purified in agarose gels using NA-45 membranes in order to assure the purity of the individual RNA species. These probes hybridized specifically to PMV or to SPMV RNA. In addition, a radioactively labeled synthetic primer complementary to the 3' end of SPMV RNA did not hybridize to PMV RNA

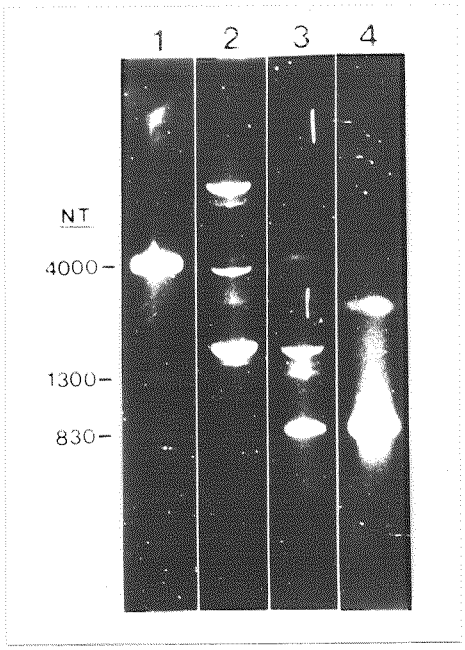

Figure 7. Analysis of dsRNAs isolated from millet tissue infected with panicum mosaic virus (PMV) and its satellite (SPMV). Double-stranded (RF) RNAs (dsRNAs) were isolated by cellulose chromatography as described in the Materials and Methods. Lane 1 and lane 4 are single-stranded RNAs (ssRNAs) of PMV and SPMV, respectively. Lane 2 contains native dsRNAs. Lane 3 contains dsRNAs that were denatured by boiling for 2 min, immediately cooled on ice, and then loaded on a gel. Note that new bands appeared in lane 3, at the positions of PMV RNA and SPMV RNA.

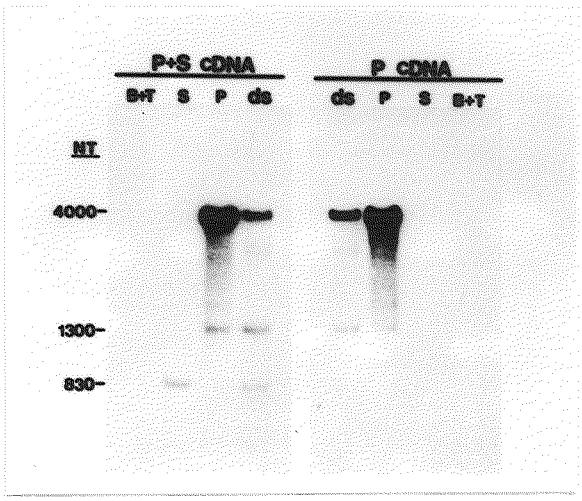

Figure 8. Hybridization of single-stranded viral RNAs and RNAs produced by denaturation of dsRNAs. The cDNA probes were copied from panicum mosaic virus (PMV) and its satellite (SPMV) RNAs. One μg of RNAs from PMV (P), SPMV (S) and brome mosaic virus + tobacco mosaic virus (B + T), and 1 μg of dsRNAs (ds) isolated from infected millet were denatured with glyoxal, electrophoresed in 1.5 % agarose gels and then transferred to nitrocellulose filters in 20 x SSC. The filters were hybridized with [^{32}] P-labeled cDNAs randomly primed from PMV RNA + SPMV RNA (S + P) or PMV RNA (P). The hybridization was conducted at 68℃, overnight. Estimated sizes (in nucleotides) of the viral RNAs are indicated at the left of the gel.

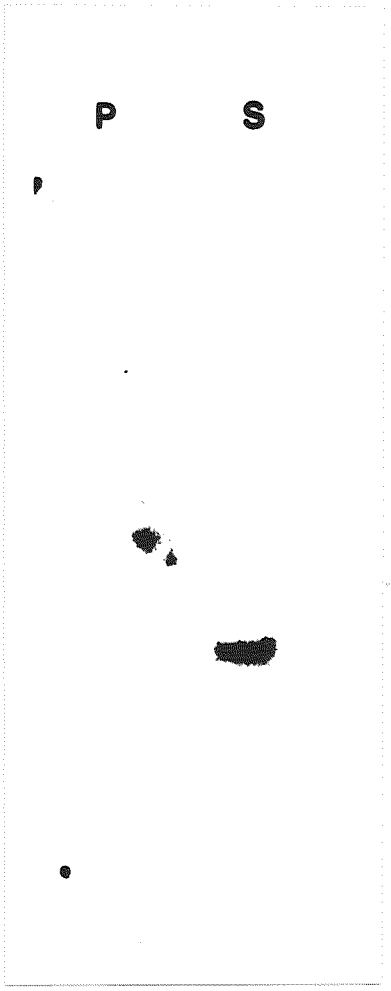

Figure 9. Hybridization of the 3' specific oligonucleotide to satellite panicum mosaic virus (SPMV) RNA. A synthetic oligonucleotide (5'-GTGTCCTAGGAGGGG-3') complementary to the first 15 nucleotides of the 3' end of SPMV RNA was labeled at the 5' end with T4 polynucleotide kinase and [γ -^{32}P] ATP. After free isotope had been removed through a Sep-Pak* (Anspec) column, the [^{32}P] -labeled oligonucleotide was used as a probe.

even though it hybridized well to SPMV RNA (Fig. 9). This result suggests that in contrast with STMV and TMV, there is no significant conserved sequence at the 3' ends of PMV and SPMV RNAs or at least within the first 15 nucleotides of the 3' ends.

Discussion

It is common for satellite agents to interfere with replication of their helpers, and to affect symptom expression in their hosts. Addition of a satellite often results in attenuation or alleviation of symptoms. For example, STNV brings on dramatic changes in symptoms by decreasing the number of necrotic lesions and the diameters of those lesions in certain hosts (Kassanis, 1962). The defective RNA associated with tomato bushy stunt virus (TBSV) while not a satellite, attenuates symptoms, behaving like defective interfering particles associated with animal viruses (Hilman, personal communication). This property of satellites and defective particles from viruses might be useful for protecting plants from disease. In some cases, the presence of a satellite has little effect on symptom expression. One example of this is the satellite RNA of tomato black ring virus (STBRV) which does not affect the replication of its helper and does not change the symptoms induced in the host (Murant et al., 1973). Recently, it was observed that a satellite virus of TMV (STMV) did not induce any visible change in symptoms and did not change the host range of the helper (TMV-U5) on the hosts tested (Valverde and Dodds, 1986). On the other hand, SPMV seems to increase symptom severity at least in millet leaves, and

hence is similar to the effects induced in CMV-infected plants by CMV satellite (CARNA 5). Some CARNA 5 strains induce lethal necrosis in tomato instead of the green mosaic induced by CMVs lacking the satellites. The interactions among satellites, their helpers, and the hosts in symptom expression are of great interest not only from the standpoint of the molecular biology of satellite agents, but also from the standpoint of practical disease control.

My results showed that the coat protein of SPMV is serologically distinct from the coat protein of its helper virus (PMV) and from the coat proteins of other satellite viruses. This appears to be a common feature of satellites. Gingery and Raymond (1985) have also shown that SMWLMV is serologically unrelated to SPMV and STNV, and that MWLMV is serologically unrelated to PMV and TNV. In addition, recently, STMV was also found not to be serologically related to either SPMV or STNV (Valverde and Dodds, 1987). These results indicate that satellite viruses do not share antigenic determinants resulting from similar conformation of amino acids in the proteins (especially near the virus surface).

At the nucleic acid level, Northern blot hybridizations conducted by Valverde and Dodds (1987) revealed that STMV RNA and SPMV RNA have no significant homology. In addition, a computer analysis of the nucleotide sequences of SPMV RNA and STNV RNA (Fig. 16) indicated that there is little appreciable homology between the two viruse RNAs. Therefore, as far as the available data are concerned, satellite viruses seem to have little nucleic acid sequence relatedness, considering that they share a very

unique property, satellitism. This suggests that the known satellite viruses are unlikely to be derived from the same origin although they may possibly have originated by the same mechanism.

SPMV is very similar in particle shape to STNV, which is a T = 1 icosahedral particle with 60 symmetrical subunits (Fig. 2). Assuming that SPMV has also a T = 1 symmetry structure, SPMV consists of 60 identical protein subunits of Mr 17 x 10^3 and one molecule of RNA of Mr 2.8 x 10^5. Calculations from these data suggest that the mass of the RNA accounts for 21 % of the total mass, which is exactly the same as the value calculated for STNV RNA in the particle. The structure of the capsid protein will be discussed in more detail later, based on the amino acid composition and amino acid sequence predictions deduced from the nucleotide sequence of SPMV RNA.

The estimated size of SPMV coat protein (Mr 17 K) agrees reasonably well with the 16 kilodaltons reported by Buzen et al. (1984). Compared to an 11 % polyacrylamide gel for protein analysis used by the above authors, I used a 17.5 % polyacrylamide gel shown in Fig. 4. This resulted in a much better resolution of the viral proteins. In addition, the nucleotide sequence of SPMV verifies that SPMV coat protein has a molecular weight of 16,980 daltons which is in close agreement with my estimate, Mr 17 K. Therefore, the size (Mr 17 K) of SPMV coat protein is significantly smaller than other satellite virus coat proteins (Mr 22-24 K), whereas PMV coat protein (Mr 31 K) is about the same size as TNV and MWLMV coat proteins (30-35 K) (Table 2).

The length of SPMV RNA (800 nucleotides) as estimated from

relative electrophoretic mobility of RNAs was also verified by nucleotide sequence analysis to contain 826 residues. Although Buzen et al. (1984) estimated the size of SPMV RNA to be about 1,200 nucleotides, they did not present any data for visualization. The denatured agarose gel shown in Fig. 5 clearly reveals that SPMV RNA migrated slightly more rapidly than BMV RNA 4, which has 876 nucleotides, and thus, the estimated size of 800 nucleotides agreed well with the value deduced from the sequence data. This RNA size is also considerably smaller than the RNAs of STNV and SMWLMV (1200-1400 nucleotides), but is closer to the size of STMV RNA (1000 nucleotides) (Table 2). As a whole, SPMV has the smallest genome and encodes the smallest coat protein subunit among the four known satellite viruses.

One of the criteria for satellitism is that there is no appreciable sequence homology between the genomes of the satellite and its helper. For example, STNV RNA has less than 2 % homology with TNV RNA (Shoulder et al., 1974). No appreciable nucleotide sequence homology could be found between STMV and its helper (TMV-U5) in Northern blot hybridizations (Valverde and Dodds, 1987). However, they did show that STMV RNA has 3' homology with TMV RNA, because a 3' specific synthetic primer originally prepared for TMV RNA also was suitable for priming STMV RNA for cDNA synthesis. This suggests that STMV and TMV RNAs have some sequence homology at least at the 3' ends, but the homology cannot be detected by hybridization tests. On the other hand, the results of a series of hybridization tests (Fig. 6, 8) show that there is no appreciable homology between SPMV and PMV RNAs.

The results of dsRNA analysis confirmed the existence of

replicative forms of PMV and SPMV RNAs, and hybridization studies also showed that there is no appreciable homology between the two RNAs. These results suggest that both viral RNAs replicate via dsRNA mechanisms rather than by the rolling circle mechanism which has been proposed for satellite RNAs (Buzayan et al., 1986b). The dsRNA of SPMV was as abundant as the RF of PMV suggesting that it does not interfere with replication of PMV to the extent that the yield of the RF of PMV is greatly decreased.

The results described in this section clearly show that SPMV RNA does not have significant homology with PMV RNA. Moreover, the 3' sequence homology, which was observed between TMV RNA and STMV RNA, was not detected by hybridizing PMV RNA with the oligonucleotide complementary to the first 15 nucleotides of the 3' end of SPMV RNA. These observations therefore completely meet the criteria for satellite viruses (Murant and Mayo, 1982).

I-2. Structure of the genome of a satellite of panicum mosaic virus

Little is known about the molecular biology of SPMV and PMV. In this section, I will summarize the molecular aspects of STNV and TNV in order to specify some unusual properties of a satellite virus and determine the relationships between SPMV and PMV at the nucleic acid level.

Previous RNA-RNA hybridization studies showed that STNV and TNV RNAs had less than 2% homology (Shoulder et al., 1974). Subsequently, nucleotide sequence analysis (Ysebaert et al., 1980) revealed that the total nucleotide sequence of STNV RNA consisted of 1239 bases. The open reading frame for the coat protein initiates at residues 30-32 from the 5' end of the RNA, and terminates at residues 618-620. The remaining 622 nucleotides of the 3' end contain a non-coding sequence with a high degree of secondary structure. The coat protein consists of 195 amino acids and has a hydrophobic carboxy-terminus and a basic amino-terminus. The RNA lacks a 3' terminal polyadenylate sequence, and unlike most other virus RNAs and eukaryotic mRNAs, it has neither a M^7Gppp cap nor a genome-linked protein (VPg) at the 5' end. The 5' terminal sequence of both STNV and TNV is ppApGpUp (Wimmer et al., 1968, 1969; Ysebaert et al., 1980). The 3' end region of STNV has a tRNA-like secondary structure, but it is not aminoacylable. Even though the 5' end of STNV does not have a cap, the RNA is a very efficient messenger in

both prokaryotic and eukaryotic *in vitro* translation systems (Klein *et al.*, 1972; Leung *et al.*, 1976). Therefore, the 5' non-coding region must have an important role for replication, expression and possibly regulation of different steps in the replication process. The gene products from TNV RNA have been studied both *in vitro* and *in vivo*. Several proteins have been identified by *in vitro* translation in the wheat germ system. These include proteins of Mr 63, 43, and 26 K as well as the coat protein (Mr 32 K). Since large precursor proteins corresponding to the entire coding capacity of the genome were not observed (Salvato *et al.*, 1977), the identified polypeptides seem to be initiated at individual cistrons and proteolytic processing from a precursor probably is not responsible for appearance of the viral-specific polypeptides.

SPMV appears to have a capsid structure similar to STNV, but the coat protein of SPMV (Mr 17 K) is smaller than that of STNV (Mr 23 K) and the RNA of SPMV with a size estimated to be about 800 nucleotides is significantly smaller than STNV. Like STNV, there is no appreciable sequence relationship between the genomes of SPMV and the helper virus PMV. These similarities and differences between the two satellites prompted me to determine the nucleotide sequence of SPMV so that direct comparison of the relationships between the satellites could be made.

Materials and Methods

Isotopes and enzymes. [α-^{32}P] dATP, [α-^{32}P] dCTP and [5'-^{32}P] pCp were purchased from New England Nuclear (NEN), and

[γ-^{32}P] ATP was purchased from either NEN or ICN Biomedicals. Restriction endonucleases were purchased mostly from Bethesda Research Laboratories (BRL).

Direct sequence analysis of RNA. The 3' end of SPMV RNA was labeled with [5'-^{32}P] pCp by T4 RNA ligase as previously described by England et al. (1980). The reaction mixture (50 μ l) contained 50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 3.3 mM DTT, 6 μM ATP, 10% (v/v) dimethylsulfoxide, 50 pmol [5'-^{32}P] pCp (500-2,000 Ci/mmol), 10 μg of SPMV RNA and 2.0 μg of T4 RNA ligase. The mixture was incubated at 4℃ overnight, and the RNA was separated from unincorporated isotope through a Sephadex G-50 column as described in Chapter I-1 and concentrated by ethanol precipitation. The end-labeled RNA was sequenced by both chemical (Peattie, 1979) and enzymatic (Donis-Keller et al., 1977) methods.

1) Direct chemical sequencing of RNA. Each RNA base was partially and specifically modified by four different base-specific reactions followed an aniline cleavage reaction to generate various lengths of labeled fragments from the modifided bases. The specific chemical reactions are as follows: (A) G reaction. A mixture containing 2 μl of carrier tRNA (10 μg), 5 μl of 3' end-labeled RNA (2.5 μg) and 300 μl of G buffer (50 mM sodium cacodylate, pH 5.5, 1 mM EDTA) was prepared, and after the mixture was chilled on ice 0.5 μl of dimethyl sulfate was added. Subsequently, the reaction was incubated at 90℃ for 1 min and the mixture was chilled on ice. Then, 75 μl of G precipitation solution (1 M Tris-acetate, pH 7.5, 1 M 2-mercaptoethanol, 1.5 M sodium acetate, 0.1 mM EDTA) and 3 volumes

of cold ethanol were added to precipitate the RNA. Ethanol precipitation was carried out by keeping the sample at −70 ℃ for 5 min followed by centrifugation for 5 min in an Eppendorf microfuge. The pellet was resuspended in 10 μl of 1 M Tris-HCl (pH 8.2) and 10 μl of freshly made 0.2 M $NaBH_4$, and incubated on ice in the dark for 30 min. Then, the sample was mixed with 200 μl of $NaBH_4$ stop solution (0.6 M sodium acetate, 0.6M acetic acid, pH 4.5, 25 μg/ml carrier tRNA) and precipitated with ethanol. (B) A>G reaction. A mixture containing 2 μl of carrier tRNA (10 μg), 5 μl of 3' end-labeled RNA (2.5 μg) and 200 μl of A>G buffer (50 mM sodium acetate, pH 4.5; 1 mM EDTA) were prepared. After the mixture was chilled on ice, 1 μl of diethyl pyrocarbonate was added to initiate the reaction, and the resulting mixture was incubated at 90℃ for 10 min. The RNA was chilled on ice and precipitated by addition of 50 μl of 1.5 M sodium acetate and 3 volumes of cold ethanol as described above. (C) C>U reaction. Carrier tRNA (10 μg) and 3' end-labeled RNA (2.5 μg) were dissolved in 10 μl of freshly made anhydrous 3 M NaCl/hydrazine that had been chilled on ice and the resulting mixture was incubated on ice for 30 min. The RNA was precipitated by addition of 1 ml of 80% ethanol that had been stored at −20℃. (D) U reaction. Carrier tRNA (10 μg) and 3' end-labeled RNA (2.5 μg) were dissolved in 10 μl of 50% hydrozine that had been chilled on ice, and the mixture was incubated on ice for 15 min. The RNA was precipitated by addition of 200 μl of 0.3 M sodium acetate/0.1 mM EDTA and 3 volumes of cold ethanol as described earlier. After ethanol precipitation, each of the four chemically modified RNAs was dissolved in 20 μl

of 1 M aniline/acetate buffer (pH 4.5) and incubated in the dark at 60℃ for 20 min. After lyophilization, the samples were resuspended in 3 μl of dye buffer (8 M urea, 20 mM Tris- HCl, pH 7.4, 1 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue). Then, the samples were heated at 90℃ for 30 sec and loaded on a 5% sequencing gel.

2) Enzymatic RNA sequencing. The end labeled RNA was partially digested with base-specific nucleases: T1 (G specific), U2 (A specific), Phy M (A+U specific), B. cereus (U+C specific), and CL 3 (C specific). The digestion mixture (20 μl) for T1 and Phy M contained 2 μl of solution A (0.25 M sodium citrate, pH 5), 14 μl of solution D (10 M urea, 1.5 mM EDTA, 0.05% each of xylene cyanol and bromophenol blue, ^{32}P-labeled RNA (10^5 cpm) and 5 μg of tRNA. For U2, solution B (0.25 M sodium citrate, pH 3.5) replaced solution A in the above mixture. The digestion mixture (20 μl) for CL 3 and B. cereus contained solution C (0.1 M $NaPO_4$, pH 6.5, 0.1 mM EDTA) and solution A, respectively, and 2 x 10^5 cpm ^{32}P-labeled RNA was used. Two units of each enzyme were added to 4 μl of the corresponding digestion mixture and the sample was incubated at 55℃ for T1, Phy M, U2 and B. cereus or at 37℃ for CL 3 for 15 min. CL 3 and B. cereus reactions were terminated by addition of 5 μl of solution D and all the samples were frozen until used. The digested fragments were resolved by electrophoresis through 20% polyacrylamide gels. One hundred ml of a 20% gel consisted of 50 g of urea, 19 g of acrylamide, 1 g of bis-acrylamide and H_2O. The gel was 30 x 40 x 0.04 cm, and electrophoresed at a constant voltage (1500 V) in 1 x TBE (0.1 M Tris-borate, pH 8.3, 2.5 mM EDTA) until the

bromophenol blue moved to the bottom of the gel.

From the sequence analysis of terminally labeled RNA, an oligonucleotide complementary to the first 15 nucleotides of the 3' end of SPMV RNA was synthesized using dimethoxytrityl nucleoside phosphoramidites on a solid support (Mattenci and Caruthers, 1981; Beaucage and Caruthers, 1981). Then, the synthetic oligonucleotide was purified on a 20 % polyacrylamide gel containing 7 M urea and used as a primer for synthesis of cDNA clones.

cDNA cloning. The synthesis of cDNA clones from SPMV RNA was conducted as described by Gubler and Hoffman (1983) with the following modifications. The first cDNA strand was synthesized by priming at the 3' end with the synthetic primer described above and the primer was extended with moloney murine leukemia virus (M-MLV) reverse transcriptase. The first strand reaction mixture (50 μl) included 50 mM Tris-HCl, pH 7.5, 75 mM KCl, 3 mM $MgCl_2$, 100 mM DTT, 1 mM each of dATP, dCTP, dGTP and dTTP, 20 units of RNasin, 2 μg of RNA and 1 μl of M-MLV reverse transcriptase (200 units). The mixture was incubated at 37℃ for 1 hr and the reaction was stopped by adding EDTA to 50 mM. The products were extracted with phenol and precipitated with ethanol in the presence of 2 M ammonium acetate. The second cDNA strand was then synthesized by replacing the template RNA with DNA, using a combination of RNase H and DNA polymerase I. The products from the first strand reaction were dissolved in 50 μl of H_2O and processed for second-strand synthesis in 100 μl of 50 mM Tris-HCl, pH 7.5, 75 mM KCl, 3 mM $MgCl_2$, 100 mM DTT, 1 mM each of dATP, dCTP, dGTP and dTTP, 1 unit of RNase H and 2 units of

DNA polymerase I. The incubations were carried out sequentially at 12℃ for 1 hr and at 22℃ for 1 hr. The reaction products were extracted twice with phenol followed by ethanol precipitation. The double-stranded cDNA was tailed with dGTP under the following conditions: 0.2 M K-cacodylate, pH 6.9, 1 mM $CoCl_2$, 1 mM dGTP, 5 mg/ml BSA, and 20 units of terminal transferase in a total volume of 20 μl. The reaction mixture was incubated at 37℃ for 30 min, because of irrespective of incubation time, the number of G residues added to the ends stops around 20, which is an appropriate length for efficient transformation. Tailing of vectors was carried out by digesting the plasmid pUC 19 with PstI and adding 20 to 30 residues under similar conditions. The length of the C tail was confirmed by a 5% sequencing gel. The annealing of the tailed vector and cDNA was conducted in 100 μl of 10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA, cDNA (200 ng), C-tailed pUC 19 (20 ng). The incubation was for 2 hr at 57℃ in order to obtain an efficient transformation (Peacock et al., 1981). After ligation, the hybrid plasmid was used to transform the JM 105 strain of E. coli. The transformation efficiency of the competent cells made by a calcium procedure (Maniatis et al., 1982) was 1.4×10^7 transformants/μg of DNA. The colonies containing cDNA inserts from SPMV were screened by colony hybridizations, using a kinase-labeled SPMV RNA probe (Rezaian et al., 1983). The cloning efficiency of this procedure was 2,500 to 3,000 clones per μg of viral RNA. In order to size the inserts and further confirm that those inserts are derived from SPMV, the recombinant plasmids were cut with EcoRI and HindIII simultaneously, and subjected to agarose gel electrophoresis and the

subsequent Southern blot hybridization (Maniatis et al., 1982).

Colony hybridization. A piece of nitrocellulose paper was placed on the colonies in a plate, and then the nitrocellulose was transferred to Whatman 3MM filter paper soaked with denaturing buffer (10 g NaOH and 43.8 g NaCl in 500 ml H_2O). After 5 min, the nitrocellulose paper was neutralized on a second filter paper soaked with neutralizing buffer (87.6 g NaCl and 30.3 g Tris-HCl in 500 ml H_2O, pH 6.5). Then, the paper was air-dried and baked at 80℃ for 2 hr. After the paper was washed in washing buffer (3 x SSC, 0.1% SDS) at 65℃ for 4 hr, it was used for hybridizations.

Synthesis of kinase-labeled probe. The viral RNA was degraded into random fragments by mixing 1 μl of viral RNA (1 μg) with 0.5 μl of 0.5 M Tris-HCl (pH 9.5) and 3.5 μl H_2O, and the mixture was incubated at 90℃ for 25 min. Subsequently, 3 μl of 100 mM $MgCl_2$, 0.7 μl of 0.5 M Tris-HCl, pH 9.5, [γ-^{32}P] ATP (30 μCi), and 10 units of T4 polynucleotide kinase were added to the mixture for labeling of the RNA. After incubation at 37℃ for 20 min, the labeled RNA was separated from unincorporated isotope by Sephadex G-50 column chromatography as described earlier.

Rapid isolation of plasmids. Plasmid DNA was isolated by a modification of the alkaline method described by Maniatis et al. (1982). The E. coli cells from a 2 ml miniculture were mixed with 200 μl of solution A (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA) and 2 mg/ml of freshly prepared lysozyme, and the suspension was incubated at room temperature for 5 min. Then, 400 μl of solution B (0.2 M NaOH, 0.1% SDS) were added,

and the mixture was incubated on ice for 10 min. Three hundred μl of 3 M potassium acetate (pH 6.0) were added and stored on ice for 15 min. After addition of 8 μl of diethylpyrocarbonate (DEPC) mixture (5 μl DEPC in 500 μl ethanol), the mixture was incubated at 65℃ for 15 min. Then, after centrifugation for 5 min, the pellet was discarded and plasmid DNA was precipitated by adding 600 μl of isopropanol (-20℃) to the supernatant and subsequently centrifuging for 5 min. The pellet was dissolved in 200 μl H_2O, and plasmid DNA was concentrated by ethanol precipitation.

Southern blotting. After electrophoresis, an agarose gel containing DNA to be analyzed by nucleic acid hybridization was soaked in denaturing buffer (1.5 M NaCl and 0.5 M NaOH) for 15 to 60 min. Then, the gel was neutralized by soaking in neutralizing buffer (1 M Tris-HCl, pH 8.0, 1.5 M NaCl) for the same length of time. The DNA was transferred bidirectionally onto nitrocellulose papers and after washing in 2 x SSC (20 x SSC: 3 M sodium chloride, 0.3 M sodium citrate) and subsequent air-drying, the transferred DNA was immobilized by baking at 80℃ for 2 hr.

Complementary tests (C-tests). In order to determine the orientation of inserts in single-stranded M13 phage, a phage stock containing an insert of known polarity was hybridized with phage containing inserts of unknown orientation. Those phages containing inserts complementary to the standard phage should hybridize to form a duplex of lower mobility in agarose gels than either phage alone. These phage with identical polarity will not form partially double-stranded hybrid DNAs and thus will migrate at the same rate as the unhybridized phage DNA. To initiate

these reactions, 4 μl of hybridization solution (0.2% bromophenol blue, 200 mM EDTA, pH 8.3, and 50% glycerol) were added to 20 μl of the phage DNAs in H_2O to be hybridized. The mixture was then incubated for 1 hr at 65℃ to allow the complementary inserts to anneal and the samples were electrophoresed on a 0.7% agarose gel.

Dideoxynucleotide DNA sequencing. Since GC tails at the ends of the insert caused sequencing discontinuities on the gels, subclones were generated by cutting the recombinant plasmids with restriction enzymes (Figure 12), and recovered on agarose gels by NA-45 DEAE membrane (Schleicher and Schuell). Then, the purified fragments were ligated to the replicative form of bacteriophage M13 (mp18 and mp19) and used to transform E. coli strain JM 101 cells. Bacteriophage forming white plaques were isolated and screened by a complementary hybridization procedure called a C-test that was described earlier (Messing, 1983). The polarity of each cDNA insert was also determined by C-tests. Then, the subclones were sequenced with the Klenow fragment of DNA polymerase I and a synthetic primer (BRL) as originally reported by Sanger et al. (1977). The exact reaction conditions below are described in more detail in the dideoxynucleotide sequence instruction manual obtained from Bethesda Research Laboratories. The concentrations of ddNTPs and dNTPs for each specific nucleotide chain termination reactions are given below: A mixture (60 μM each of dCTP, dGTP and dTTP, and 70 μM ddATP, 1.2mM Tris-HCl, pH 7.5, 0.12 mM MEDTA), C mixture (80 μM each of dGTP and dTTP, 4 μM dCTP and 100 μM ddCTP, 1.6 mM Tris-HCl, pH 7.5, 0.16 mM EDTA), G mixture (80 μM each of dCTP and dTTP, 4

μM dGTP and 75 μM ddGTP, 1.6 mM Tris-HCl, pH 7.5, 0.16 mM EDTA) and T mixture (80 μM each of dCTP and dGTP, 4 μM dTTP and 250 μM ddTTP, 1.6 mM Tris-HCl, pH7.5, 0.16 mM EDTA). Next a separate stock solution containing 7 μl of ssDNA (1 μg) and 1 μl of 10 x HincII buffer (0.6 M NaCl, 70 mM Tris-HCl, pH 7.5, 70 mM 2-mercaptoethanol, 70 mM $MgCl_2$) and 2 μl specific primer (4 μg) was prepared. After the stock solution was boiled for 5 min, the primer was allowed to anneal to the ssDNA during an incubation at 50 ℃ for 45 min. Then, 2 μl of [α-^{32}P] dATP (20 μCi with a specific activity of 3 mCi/nmol), 1 μl of 20 μM dATP and 1.5 units of Klenow DNA polymerase I fragment were added to the stock solution. Then, 3 μl of the stock solution were added to 1 μl of each of the four separate chain termination mixtures described above. The incubations were conducted at 50℃ for 15 min followed by a 15 min chase reaction after addition of 1 μl of the mixture containing each of 0.5 μM dNTP. After the chase step, the reactions were stopped by adding 6 μl of formamide dye mixture (0.1% xylene cyanol, 0.1% bromophenol blue, 10 mM Na_2EDTA, 95% deionized formamide). The samples were immediately heated at 90℃ for 1.5 min, 2.5 μl were applied for each lane and electrophoresed on 0.4 mm thick x 85 cm long 5% (w/v) polyacrylamide gels. Sequence data were analyzed with IBI sequence analysis software on an IBM computer.

Dideoxynucleotide sequencing of RNA. Using available sequences derived from recombinant DNA clones, two synthetic primers (17 nucleotides long oligonucleotide) were made so that one primer was complementary to the positive sense RNA about 80 to 100 residues away from the 5' end of the genomic RNA. The

other primer was constructed so that it hybridized to the negative strand of replicative form RNA about 80 to 100 residues away from the 5' end.

1) 5' sequence analysis. A stock solution (5 μl) containing 0.3 μg of synthetic primer, 2 μg of genomic RNA, and reverse transcriptase buffer (RTB: 10 mM Tris-HCl, pH 8.5, 60 mM KCl, 5 mM $MgCl_2$, 1 mM DTT) was annealed at 70℃ for 10 mim. After cooling to 35℃, 10 μl of a reaction mixture containing dNTPs (0.125 mM each of dCTP, dGTP, dTTP and 62 μM dATP), 20 μCi [α-^{32}P] dATP, 1 x RTB and 200 units of M-MLV reverse transcriptase were added to the hybridization mixture. The individual dideoxynucleotide reactions consisted of 3 μl of the above mixture (15 μl), 1 x RTB and 1 μl each dideoxynucleotide (500 μM). The reaction was conducted at 45℃ for 15 min followed by addition of 1 μl of unlabeled nucleotides (0.5 μM) and subsequent incubation for another 15 min to chase the reaction.

2) 3' sequence analysis. The negative strand was derived from dsRNA and sequenced with the following modification. Three μg of dsRNA were mixed with 1.5 μg of synthetic primer, boiled in the presence of 1 mM EDTA (pH 8.9) for 2 min, and immediately placed in a dry ice - ethanol bath (- 70℃). After addition of 1.2 μl of 4 x RTB, the mixture was incubated at 56℃ for 15 min followed by another 15 min incubation at room temperature.

Determination of the 5' terminal residue of SPMV. Since the 5' end of SPMV RNA was not efficiently labeled in preliminary tests, a dephosphorylation step was included. The conditions were similar to those described by Dillon et al. (1985). The reaction mixture (20 μl) which contained 5 μg of RNA, 50 mM

Tris-HCl, pH 8.9, 0.1 M EDTA, and 5 units of calf intestinal alkaline phosphatase was incubated at 37℃ for 30 min. Afterwards, the sample was extracted twice with phenol/chloroform, the RNA was precipitated with ethanol and the pellet was washed three times with 80% cold ethanol. The dephosphorylated RNA was dissolved in 25 μl of 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA, [γ-^{32}P] ATP (50 μCi), and 5 units of T4 polynucleotide kinase. After incubation at 37℃ for 30 min, the labeled RNA was precipitated with ethanol and washed twice with cold 80 % ethanol. The pellet was resuspended in H_2O, and the sample was subjected to electrophoresis on a 1.5 % agarose gel to separate intact viral RNA from degraded products that may have been labeled. The intact RNA band was collected on NA-45 membranes and eluted in formamide - high salt buffer (55% formamide, 1.8 M sodium acetate, 2 mM EDTA, 0.2% SDS, pH 7.4). The RNA was again precipitated with ethanol and washed three times with cold 80% ethanol. The [5'-^{32}P] labeled RNA was digested exhaustively with nuclease P1 and the radiolabeled nucleoside-5'-phosphate was identified by PEI cellulose thin layer chromatography (Buzayan et al., 1986). The reaction mixture (10 μl) containing 20,000 cpm of 5' end-labeled RNA, 1 μl of 0.2 M sodium acetate (pH 5.5), and 40 μg/ml of P1 nuclease was incubated for 4 hr at 37℃. The digested samples were spotted on a PEI cellulose plate and developed in 1 M LiCl. As standards, 50 μmol of each 5' nucleoside monophosphate (NMP) were run in adjacent lanes. The terminal nucleotide was determined by comparing the migration of the radioactive 5' NMP to the standards which migrated as follows: UMP (Rf: 0.7) > CMP (Rf:

0.6) > AMP (Rf: 0.5 > GMP (Rf: 0.4).

Results

Determination of the 3' terminal sequence of SPMV RNA and oligonucleotide synthesis. The 3' end of purified SPMV RNA was labeled with [5'-^{32}P] pCp by T4 RNA ligase, and the sequence analysis of the terminally labeled RNA was conducted by Dr. Douwe Zaidema, using a combination of chemical and enzymatic sequencing methods (data not shown). Based on the resulting sequence data, an oligonucleotide (5'-GTGTCCTAGGAGGGG-3') complementary to the first 15 nucleotides of the 3' end of SPMV RNA was synthesized as described in Materials and Methods, and the synthetic oligonucleotide was used as a primer for the construction of cDNA clones.

Cloning. The synthetic primer described above was radioactively labeled at the 5' end with polynucleotide kinase, and hybridized to nitrocellulose paper containing PMV RNA and SPMV RNA. As shown in Fig. 9, the hybridization of the primer to SPMV RNA was specific, and there was no cross hybridization to PMV RNA. This synthetic primer was used to construct a cDNA library in the plasmid pUC 19. The colony hybridization procedure outlined in the Materials and Methods section was used to screen 250 transformants with ^{32}P-labeled SPMV RNA. Based on differences in hybridization intensity, 50 clones were chosen to isolate recombinant plasmids. The plasmids were digested simultaneously with EcoRI and HindIII, and electrophoresed in agarose gels. All the plasmids examined contained cDNA inserts which were estimated to

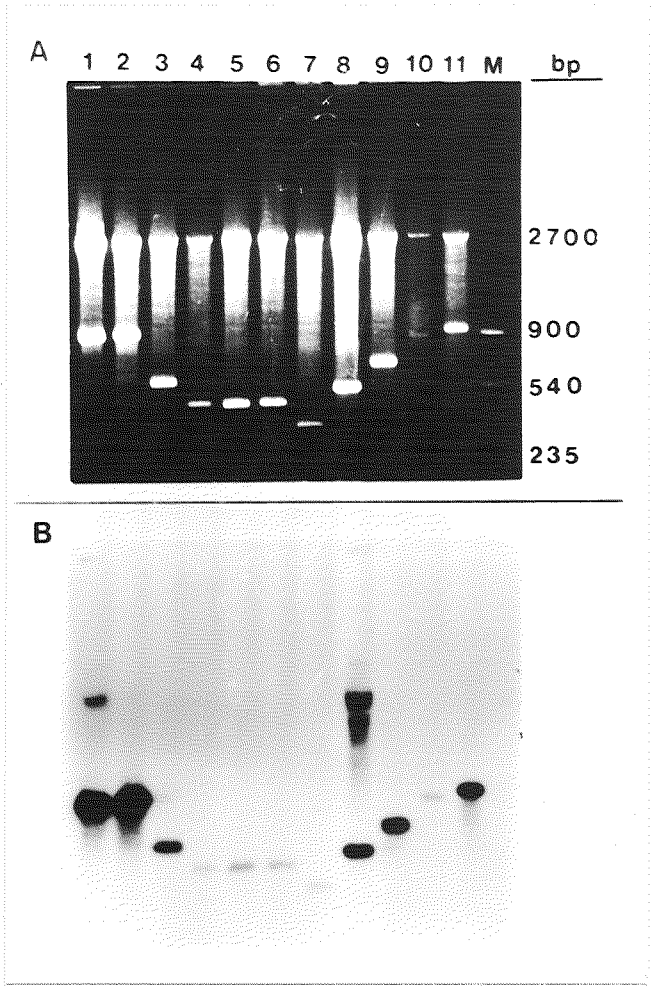

Figure 10. Electrophoretic mobility of recombinant plasmids cut simultaneously with EcoRI and HindIII. A. After double digestion with EcoRI and HindIII, the DNA fragments were separated in 1.5 % agarose gels, and stained with ethidium bromide. The extreme right lane (M) contains marker DNAs whose sizes are indicated in base pairs at the right of the gel. B. After photography under ultraviolet light, the gel was denatured with NaOH and bidirectionally transferred to nitrocellulose filters. The filters were hybridized with a kinase-labeled SPMV RNA probe. The hybridization was conducted at 68℃, overnight.

range in size from 300 bp to 900 bp (Fig. 10A). Four cDNA inserts were estimated to be 900 bp or larger (including homopolymer tails and nucleotides from the multiple cloning site of the vector). Southern blot hybridization of the same clones with kinase-labeled SPMV RNA (Fig. 10B) showed that all the inserts shared extensive sequence homology with SPMV RNA. In addition, nick-translated DNA from these four plasmids hybridized only to SPMV RNA in Northern blot hybridizations (Fig. 11), indicating that those inserts originated from SPMV RNA.

M13 subcloning. Two of the larger inserts were subcloned into the M13 vectors (mp18 or mp19) and sequenced by the dideoxy chain termination method. The sequencing strategy used to analyze the primary structure of the cDNA clones of SPMV RNA is summarized in Fig. 12. In order to overcome ambiguous bands on the sequencing gels when the clones were sequenced beyond the GC tails, nine subclones shown in Fig. 12 were generated after restriction enzyme digestions with SmaI and AccI. These were then sequenced in the directions of those arrows in Fig. 12. Since preliminary digestion of the cDNA clones with several restriction enzymes revealed that the clones contained at least two SmaI sites, seven SmaI fragments (D-1, D-3, D- 5, D-9, D-103, D-106 and D-111) were subcloned into the M13 vector and sequenced. By computer analysis of the restriction sites using the sequence data obtained from the SmaI fragments, an AccI site was found in the clones. The length (about 500 nucleotides) of the AccI fragment (D-203) resolved in the gel agrees well with the value predicted from the computer analysis. In order to confirm that the inserts did not contain two SmaI sites in close

Figure 11. Hybridization of a cDNA insert to the RNA of satellite panicum mosaic virus (SPMV). Purified RNAs (1 μg) from panicum mosaic virus (PMV), SPMV and barley stripe mosaic virus (BSMV) were denatured with glyoxal, electrophoresed in 1.5 % agarose gels, and transferred to nitrocellulose filters. The filters were hybridized with a nick-translated recombinant plasmid (lane 11 in Fig. 10) containing SPMV RNA sequences. The hybridization was conducted at 68℃, overnight.

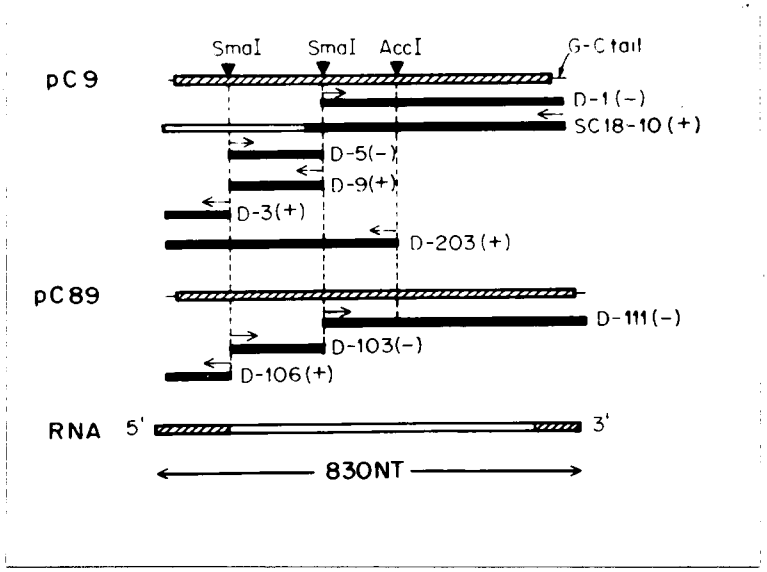

Figure 12. Strategy for nucleotide sequencing of SPMV RNA. The sequence was obtained from analysis of SPMV RNA and its cDNA clones. The cDNA inserts from the recombinant plasmids, pc89 and pc9 were subcloned into the M13 vector. The thick blackened lines of pc9 and pc89 correspond to restriction fragments sequenced by the dideoxynucleotide method. SC18-10, which is a subclone containing the entire insert of pc9 was also sequenced down to the end of the black color. The arrows indicate the directions of the sequence determined. The polarity of the sequences is shown by either (+) or (-). The shadowed parts of the thick line for RNA represent the regions sequenced by extension of the synthetic primers. The primer for the 5' end region of the genomic RNA has the sequence of 5'-GACGATTAGATCGCCTG-3', and the primer for the 5' end region of the negative strand (the 3' end region of the genomic RNA) is 5'-CCGGTGTGTACAACCAAC-3'.

proximity, the sequence across the SmaI sites was determined by sequencing the subclones D-203 and SC18-10. The data revealed no SmaI site in close proximity . These nine subclones were easily ordered by arranging the overlapping sequences. The sequence of each of the subclones was aligned with the sequence of the viral RNA (Fig. 12), based on the orientation of the clones as determined by the C-tests and by Northern hybridizations.

Sequencing from defined region of RNA and DNA clones with synthetic oligonucleotides. In order to verify and extend the terminal sequences of SPMV RNA, two oligonucleotides were synthesized so that they were complementary to regions about 80-100 residues away from the 5' ends of SPMV RNA and its negative complement. One of these primers, which was complementary to the 5' end of the genomic RNA was used to deduce the sequence of the 5' terminal region of the cDNA insert (pc 89) and the homopolymer tail (Fig. 13). This primer (5'- GACGATTAGATCGCCTG-3') was also used to sequence SPMV RNA by the dideoxynucleotide method (Fig. 13). The results show that the 5' end of the largest clone (pc89) represented the entire SPMV genome except for 17 terminal nucleotides.

In another experiment, the dsRNA (RF) of SPMV was used to confirm the 3' terminal region of the viral RNA by the dideoxynucleotide method. The negative-sense RNA released from the melted dsRNA was hybridized with the synthetic oligonucleotide primer (5'-CCGGTGTGTACAACCAAC-3'). The resulting sequencing reaction showed that the 3' end of the cloned viral genome contained not only the specific primer sequence (5'-

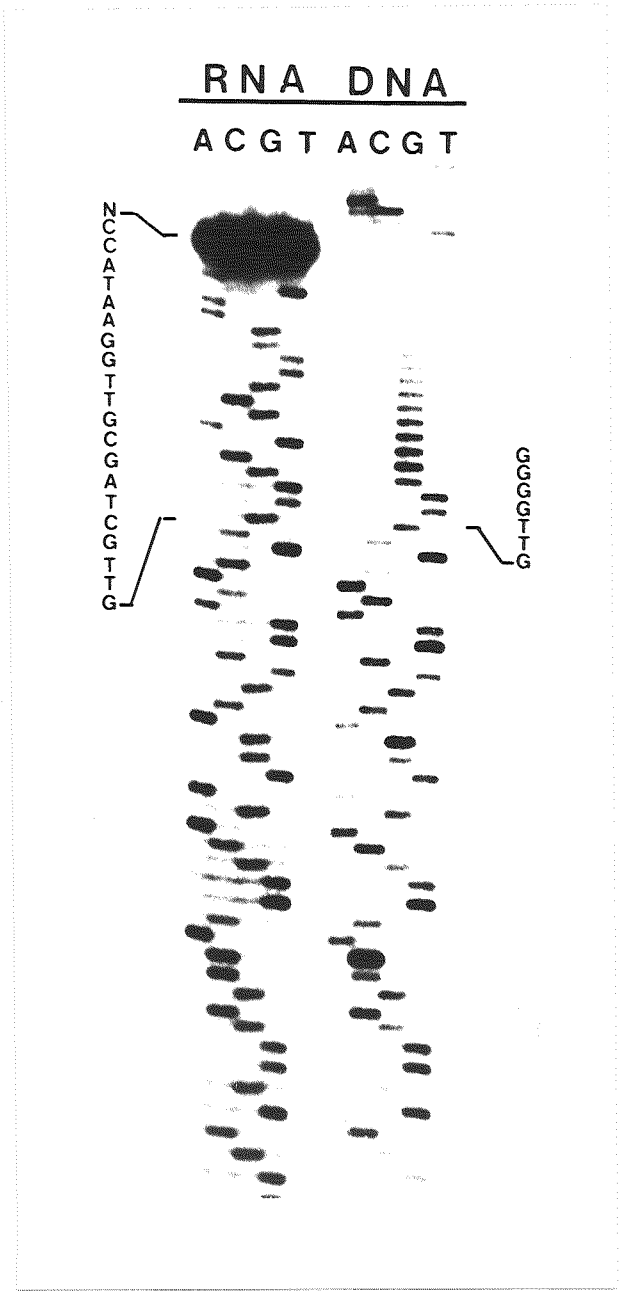

Figure 13. Dideoxynucleotide sequence analysis of the 5' terminal region of SPMV RNA and its cDNA. The sequence presented is complementary to SPMV RNA. The oligo (dG) region in the sequence at the right panel corresponds to the homopolymer linkage between pUC19 DNA and the cDNA insert of SPMV. Note that the sequence derived from the authentic RNA is 17 nucleotides longer than that from the cDNA (pc89 in Fig. 12).

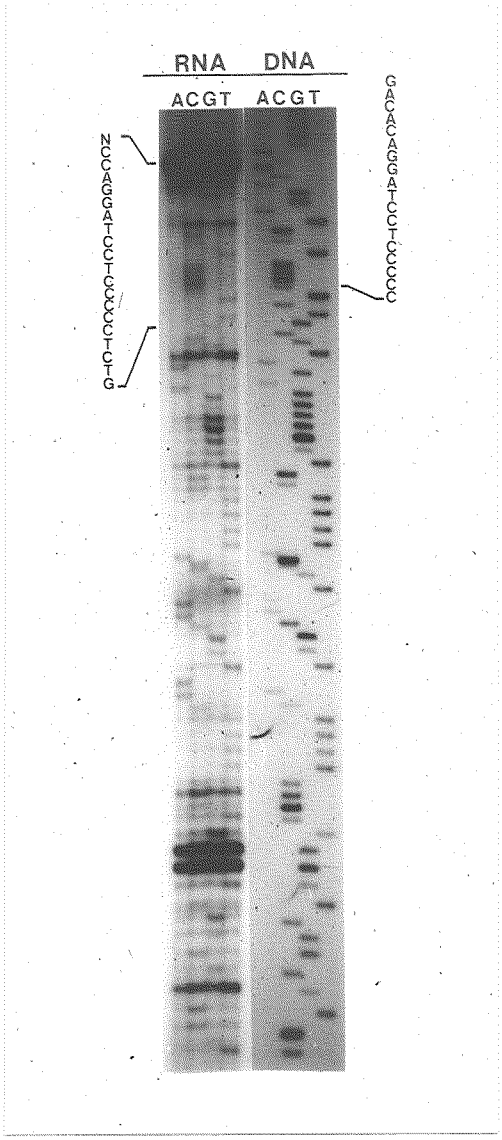

Figure 14. Dideoxynucleotide sequence analysis of the 3' terminal region of SPMV RNA and its cDNA. The sequence is presented in the genomic sense of SPMV RNA. As described in the Materials and Methods, the negative strand was obtained by melting the viral dsRNAs (RF) and sequenced by extension of a synthetic primer which is complementary to a region around 80-100 residues away from the 5' end of the negative strand.

GTGTCCTAGGAGGGG-3') used for the construction of the cDNA clones, but also contained an additional adenosine residue (Fig. 14). The band pattern on the gel for dideoxynucleotide sequencing of RNA shows that the clone (pc89) seems to be one nucleotide longer than the authentic viral RNA. This suggests that one of the first three nucleotides determined by direct RNA sequencing of the 3' end of RNA may be incorrect due to difficulties encountered in reading the 3' end sequence. The sequence of the 3' end region deduced by dideoxynucleotide sequencing of RNA and direct chemical sequencing of RNA is likely to be 5'-CCC-3' rather than 5'-C(A)C-3', but this needs to be verified further.

Determination of the 5' terminal nucleotide. The 5' terminal residue of SPMV RNA was determined by one dimensional thin layer chromatography (Fig. 15). The 5' end-labeled SPMV RNA, which was purified in agarose gels with NA-45 membranes, released predominantly guanosine plus minor traces of adenosine as radioactive ribonucleosides after exhaustive nuclease PI digestion. Unless the 5' terminus is heterogenous with a minor species containing an adenosine residue, guanosine is likely to be the 5' terminal nucleotide because the intensity of the guanosine spot was much higher than the adenosine spot. In addition, when the RNA was isolated in a longer gel with better resolution, the intensity of adenosine spot was greatly reduced. Thus, I believe that the minor spot of labeled adenosine results as a consequence of specific degradation of SPMV RNA at an adenosine site near the 5' end of the molecule. After kinase labeling, this residue would be labeled with ^{32}P, but would migrate close to full-length RNA during gel electrophoresis.

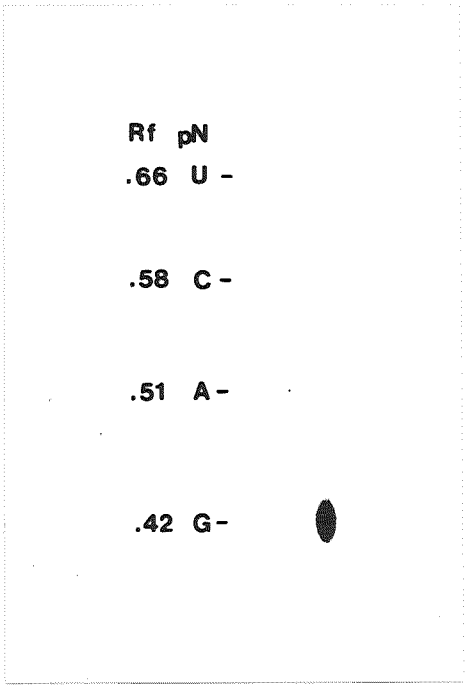

Figure 15. Analysis of the 5' terminus of SPMV RNA. The RNA was first dephosphorylated with alkaline phosphatase and then labeled at the 5' end with T4 polynucleotide kinase and [γ -^{32}P] ATP. The [5'-^{32}P] -labeled RNA was separated from degraded molecules in 1.5 % agarose gels and electrophoresed onto NA-45 membranes. The recovered RNA was exhaustively digested by nuclease P1 and resolved by one-dimensional thin layer chromatography. The Rf values for each 5' ribonucleoside monophosphate are indicated at the left of the panel.

GGGUAUUCCAACGCUAGCAACGAGUGUAAGACGUCCAUCUGCAAGUGGCG 50

CAACAGCAAUUGAACUAGUCUCACGAGGAAUACUCCUGAUGGCUCCUAAG 100

CGUUCCAGGCGAUCUAAUCGUCGGGCGGGCUCCCGGGCUGCUGCCACAUC 150

ACUGGUGUACGAUACGUGCUACGUCACCUUGACGGAGCGCGCUACUACCU 200

CUUUUCAGAGGCAGAGUUUCCCGACCCUCAAGGGGAUGGGGGGACCGUGCA 250

UUCCAGGUUGUCUCGUUUACAAUCCAGGGGGUGUCAGCAGCCCCCCUGAU 300

GUAUAACGCGCGCCUGUAUAACCCGGGCGACACAGACUCUGUCCAUGCCA 350

CCGGGGUACAGUUGAUGGGCACAGUCCCUAGGACCGUUCGGCUCACCCCU 400

AGGGUGGGCCAGAACAACUGGUUCUUUGGUAACACUGAAGAAGCCGAGAC 450

CAUUUUGGCCAUCGACGGACUCGUGUCUACCAAGGGUGCUAACGCCCCCA 500

GCAAUACCGUCAUUGUUACGGGUUGCUUUAGGCUGGUGUUUAGCGAGCUU 550

CAGUCUUCAUAAAGUAUCCCCCUCUUAUGGGGUGUCGCCGUACACACCGU 600

UAGUCCCGCGCCUCUACCGGCUGUCGGACUCCUACCAUGCCCUCCGGUGG 650

AUGUUGAGGAAGUGGGGGAAUCAGGAGGCUACGAAGCCGUCGCAGCAAAG 700

GCGACCGUGUGUACAACCAACCCGGCCAGAAAUAUACCCCGAAAGGGGGG 750

GUCCUGCGGCUGGGUCCCCUUUUCAAUGGCAAUGCCAUUUUCCUGGGGGG 800

AGAUGCGUCUCCCCCUCCUAGGACCC 826

Figure 16. Nucleotide sequnce of SPMV RNA. The sequence is presented in the genomic sense of SPMV. The initiation codon (AUG), the termination codon (UAA) and the purine rich region (ACGAGGAA), which is a possible ribosomal binding site, are underlined. The deduced amino acid sequence of the coat protein is shown in Fig. 17.

From the above data, the primary structure of SPMV RNA is proposed to be that shown in Fig. 16, and thus the total nucleotide sequence of SPMV RNA consists of 826 bases. The sequence as determined from the clones reveals that there are only 6 nucleotide differences between pc89 and pc9. Five of these differences were present in the longest open reading frame, and are transitions from a G (pc89) to an A (pc9) at residue 223, a C (pc89) to a T (pc9) at residues 226, a G (pc89) to an A (pc9) at residue 242, a G (pc89) to an A (pc9) at residue 382, and a T (pc89) to a C (pc9) at residue 490. Four of these five transitions were redundant third position changes and hence induced no amino acid change. An amino acid change of aspartic acid (pc89) to asparagine (pc9) was observed at residue 242 to 244. A sixth transition was found at residue 754 where a C residue in pc89 was read as a T in pc9.

Discussion

Previous studies have shown that an *in vitro* translation of SPMV RNA using a wheat germ system resulted in one polypeptide which comigrated with its authentic coat protein (Buzen *et al.*, 1984). Thus, SPMV RNA resembles STNV RNA in that both act as monocistronic messengers. The complete nucleotide sequence of SPMV RNA (Fig. 16) reveals that the first and largest open reading frame (ORF 1) starts with the first AUG at residues 89 to 91 and terminates at residues 560 to 562 (UAA). The size of the polypeptide encoded by ORF 1 agrees extremely well with the size of the coat protein estimated from gel electrophoresis. More

significantly, the deduced amino acid sequence (Fig. 17) is in good agreement with the result of the previous amino acid composition analysis (Table 3). Comparison of the hydropathy patterns of the coat protein of STNV and the deduced polypeptide of SPMV is shown in Fig. 18. Although neither nucleic acid sequence homology nor amino acid sequence homology was found between the two viruses, the hydropathy patterns have some similarity of the amino- and carboxy-termini. Both polypeptide chains contain a hydrophilic amino-terminal portion and a hydrophobic carboxy-terminal portion. The first 20 amino acids of the amino-terminal part include seven basic amino acids (one lysine and six arginine residues). The coat protein of STNV has a very stable interaction with its RNA, resulting in symmetric arrangement of the protein subunits (T = 1). X-ray chrystallography studies revealed that the amino-terminal part of the coat protein of STNV forms an arm penetrating the RNA region (Liljas et al., 1982). Therefore, it is likely that the coat protein of SPMV interacts with the RNA and constructs the virion in a similar manner to the coat protein and the RNA of STNV.

Several interesting examples of skewed codon usage appear within the open reading frame for the SPMV coat protein. For instance, the dinucleotide CpG is found frequently in the second and third positions of ORF 1 (Table 4). In contrast, vertebrate mRNAs have a low frequency of CpG (Fiers et al., 1978). The third position choice of codons in the SPMV coat protein gene is apparently non-random (U 24.1: C 34.4: G 31.8: A 9.6). C is frequently found in the third position and so is G, but to a less extent, while A is selectively low. These preferences are

Met-Ala-Pro-Lys-Arg-Ser-Arg-Arg-Ser-Asn-Arg-Arg-Ala-Gly-Ser-Arg-Ala-Ala-Ala-Thr- 20

Ser-Leu-Val-Tyr-Asp-Thr-Cys-Tyr-Val-Thr-Leu-Thr-Glu-Arg-Ala-Thr-Thr-Ser-Phe-Gln- 40

Arg-Gln-Ser-Phe-Pro-Thr-Leu-Lys-Gly-Met-Gly-Asp-Arg-Ala-Phe-Gln-Val-Val-Ser-Phe- 60

Thr-Ile-Gln-Gly-Val-Ser-Ala-Ala-Pro-Leu-Met-Tyr-Asn-Ala-Arg-Leu-Tyr-Asn-Pro-Gly- 80

Asp-Thr-Asp-Ser-Val-His-Ala-Thr-Gly-Val-Gln-Leu-Met-Gly-Thr-Val-Pro-Arg-Thr-Val- 100

Arg-Leu-Thr-Pro-Arg-Val-Gly-Gln-Asn-Asn-Trp-Phe-Phe-Gly-Asn-Thr-Glu-Glu-Ala-Glu- 120

Thr-Ile-Leu-Ala-Ile-Asp-Gly-Leu-Val-Ser-Thr-Lys-Gly-Ala-Asn-Ala-Pro-Ser-Asn-Thr- 140

Val-Ile-Val-Thr-Gly-Cys-Phe-Arg-Leu-Ala-Pro-Ser-Glu-Leu-Gln-Ser-Ser 157

Figure 17. Amino acid sequence of the SPMV coat protein deduced from the nucleotide sequence data.

Table 3. Amino acid compositions of SPMV coat protein determined by direct analysis of coat protein and nucleic acid sequence

Amino acid	Amino acid analyzer[a]	Nucleotide sequence
Lys	2.3 %	1.9 %
His	0.8	0.6
Arg	9.2	8.9
Asp (Asn)	8.5	8.3
Thr	10.6	11.4
Ser	9.4	8.9
Glu (Gln)	8.1	7.7
Pro	5.9	5.1
Gly	8.4	7.5
Ala	10.9	10.2
Val	7.4	8.2
Met	1.8	2.5
Ile	2.3	2.6
Leu	7.2	6.9
Thr	2.5	2.6
Phe	4.8	4.4
(Cys)	-	1.3
(Trp)	-	0.6

[a]Data from Buzen *et al*.

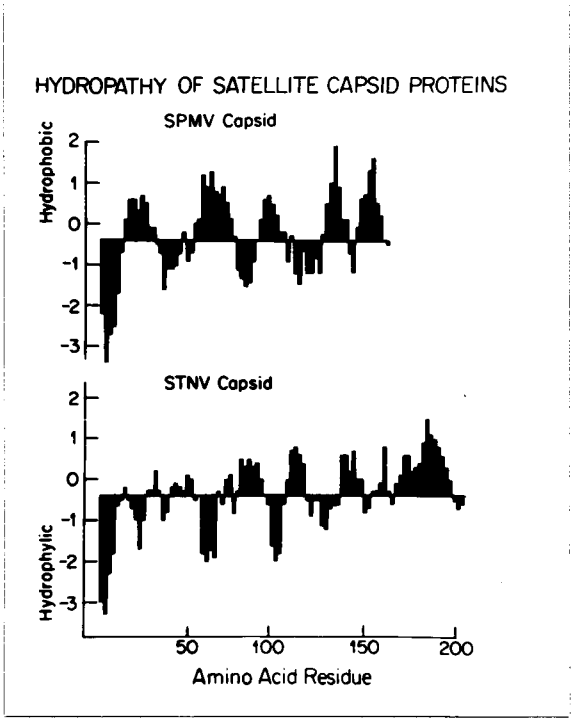

Figure 18. Hydropathy patterns of the capsid protein of satellite tobacco necrosis virus (STNV) and the protein product of SPMV predicted from the nucleotide sequence data. The Y axis shows relative extent of hydrophobicity (+) or hydrophilicity (-) of amino acid residues.

not reflections of the overall base composition of the translated region (U 23.4: C 29.2: G 28.3: A 20.0) and may have arisen by some selection mechanism. The third position redundancies definitely appear to result in discrimination for usage of some codons over others (Table 4). For example, out of 11 leucines, 8 isoleucines, 8 prolines and 7 glutamines, no codon ending with an A is found. Moreover, out of 14 arginines, 13 valines and 12 glycines, each amino acid uses a codon with an A at the third position only once. Such codon preferences may reflect unusual distribution of the coat protein codons or they may indicate peculiarities of the codon usage or availability of host transfer RNAs.

In addition to the largest open reading frame (ORF 1), an additional open reading frame (ORF 2) exists at the 3' end of the genomic RNA (Fig. 19). This ORF starts at residues 651 to 653 with an AUG and terminates at residues 819 to 821 with a TAG triplet. ORF 2 could code for a 56 amino acid residue polypeptide (Mr 6.3 K). The region between ORF 1 and ORF 2 has an 89 nucleotide space that does not contain any purine rich regions related to the Shine-Dalgarno sequence. Therefore, it is not likely that this sequence could be a potential ribosomal binding site on a subgenomic messenger RNA. The sequence surrounding the AUG for ORF 2 contains a U at the -3 position and a U at the +4 position, and therefore it does not satisfy Kozak's rule for favorable translation initiation sites of eukaryotic messenger RNAs. Surprisingly, two additional open reading frames (ORF 3 and ORF 4) exist on the complementary or negative strand of SPMV RNA (Fig. 19). ORF 3 begins at residues 39 to 41 with an AUG

Table 4. Codon usage in the SPMV coat protein cistron

	U	C	A	G	
U	Phe 4[a]	Ser 5	Tyr 2	Cys 0	U
	3	2	2	2	C
	Leu 0	3	1	0	A
	3	1	0	Trp 1	G
C	Leu 1	Pro 4	His 1	Arg 3	U
	3	2	0	2	C
	0	0	Gln 0	1	A
	4	2	7	3	G
A	Ile 2	Thr 2	Asn 2	Ser 1	U
	2	9	6	2	C
	0	4	Lys 0	Arg 0	A
	4	3	3	5	G
G	Val 3	Ala 5	Asp 1	Gly 3	U
	5	6	4	4	C
	1	2	Glu 2	1	A
	4	3	3	4	G

[a]Numbers indicate the number of times each codon was used.

and terminates at residues 225 to 227 with a TAA triplet. This ORF could code for a 62 amino acid residue-polypeptide (Mr 7.1 K). The 5' non-coding region (38 residues) contains a purine rich segment from residues 8 to 19 (5-AGGAGGGGGAGA-3'). This sequence might be a potential ribosomal binding site in a prokaryotic system but it is 20 nucleotides away from the AUG initiation codon. The sequence flanking the AUG obeys Kozak's rule because it has a purine (A) at the -3 position and a G at the + 4 position. ORF 4 starts at residues 314 to 316 with an AUG and terminates at residues 620 to 622 with a TGA, and has the capacity to code for a 102 amino acid residue-polypeptide (Mr 11 K). There is an 87 nucleotide space between ORF 3 and ORF 4, which does not have any purine rich region consisting of AGGA. The sequence surrounding the AUG for ORF 4 contains a purine (A) at the -3 position and an A at the + 4 position instead of a G favorable for Kozak's rule. In the absence of further information from translation studies, it is not possible to speculate on whether ORF's 2, 3 or 4 are translated *in vivo* or not.

SPMV RNA contains an 88 nucleotide non-coding region at the 5' side of the largest open reading frame. This contrasts with the presence of a relatively short 29 nucleotide leader sequence in STNV. Interestingly, there is a purine rich region (ACGAGGAA) 9 nucleotides before the AUG in the SPMV coat protein gene. This 8 nucleotide segment contains the sequence AGGA, which is an essential part of the Shine-Dalgarno sequence (AGGAGGU) thought to be important in ribosomal binding to RNAs (Stryer, 1981). In prokaryotes, the signal sequence (AGGA) usually is present 7-8 bases to the 5' side of the initiation

AUG. The Shine-Dalgarno sequence is believed to direct proper alignment of ribosomes on RNA by base pairing with a complementary sequence at the 3' end of 16 S rRNA in prokaryotes. A Shine-Dalgarno sequence was also found in the 5' non-translated region of STNV RNA, and this sequence might promote translation of STNV RNA in a prokaryotic translation system. Since a previous study of the in vitro translation of SPMV RNA was carried out in a wheat germ system (Buzen et al., 1984), it would be very interesting to examine the translation efficiency of SPMV RNA in a prokaryotic system.

The 5' non-coding regions of mRNA also influence the efficiency of initiation of translation in eukaryotic systems and the importance of some of these sequences is slowly being deciphered. According to the rules proposed by Kozak (1986a), the 5' non-coding regions of eukaryotic mRNAs tend to be low in G, but purines are preferred at position -3 and a G is present at position +4 surrounding the first AUG from the 5' terminus. Many plant viral RNAs appear to follow Kozak's rule. For example, STNV RNA has the sequence of AACAUGG and fits Kozak's rule well. However, plant virus RNAs also have several exceptions to the rule. The initiation codon for TMV coat protein, which is efficiently translated, has a purine (A) at position -3, but lacks a G at position +4 (Goelet et al., 1982). BMV RNA 3 does not fit the rule at all, having a C at position -3 and a U at position +4 (Ahlquist et al., 1979). In addition, even among the satellites which are able to code for some protein, a satellite RNA of tomato black ring virus (STBRV) has the initiation codon for a 48 K protein with a C at position -3 and an A at

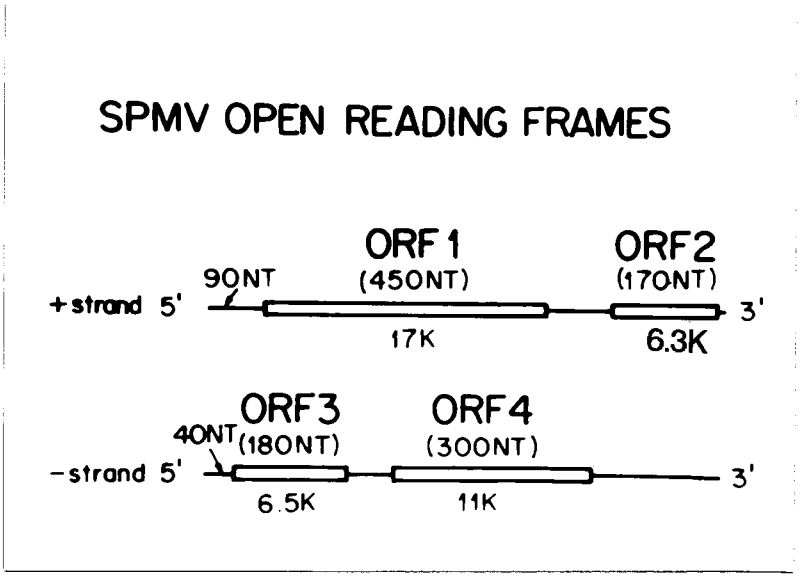

Figure 19. Open reading frames in the SPMV genome and its complement (negative strand). Only ORF 1 has the capacity to code for a polypeptide of the size of the SPMV coat protein.

position +4 (Meyer et al., 1984). The complete sequence of STBRV (Mayer et al., 1984) revealed that the leader sequence had only 13 nucleotides and that a VPg was linked to the 5' terminus. From this very short non-coding region, there is little information to predict how secondary structure will facilitate the translation of the gene. There is no evidence that a VPg can enhance translation efficiency and in fact, mRNAs of poliovirus are devoid of the VPg (Spector and Baltimore, 1975). Therefore, there must be other factors which regulate the production of the Mr 47 K polypeptide. The only exception to Kozak's rule at the 5' terminus of SPMV RNA is that the RNA has a pyrimidine (C) at position -3 instead of a purine. There is a G residue at position +4, following Kozak's rule. Interestingly, the base composition of the 5' non-coding region is low in G and C (U 20.5: C 25.0: G 25.0: A 30.0) compared to the values in the whole nucleic acid (Table 5) or the translated region. In addition, it is noteworthy that the number of A residues is greatly increased in the leader sequence whereas the 3' non-coding region is very low in A (U 20.2: C 30.7: G 29.2: A 12.4). Since SPMV has a long leader sequence (88 residues), it is possible that some secondary or tertiary structure may affect the translation efficiency of the coat protein gene. In Fig. 20, one possible folding of the 5' end region is shown. Two adjacent hairpin loops could exist just before the initiation codon with the stabilities of - 0.28 Kcal/mole nucleotide for the loop next to the AUG, and 0 Kcal/mol nucleotide for the distant loop (those free energies were calculated as described by Tinoco (1973) and Salser (1978)). Since those loops are weak, initiation by

ribosomes should not be inhibited by the secondary structure. A highly stable stem- and loop-structure inhibits ribosome binding and subsequent translation (Kozak, 1986b). The region downstream of the initiation AUG may have a tertiary base-pairing interaction with a segment from the 5' end of the genome so that the 5' end region and the initiation codon can be adjacent to each other. In BMV RNA 3, which also has a long leader sequence (91 residues), the 7-methyl guanosine cap structure could be positioned very close to the initiation codon by folding (Ahlquist et al., 1979). As the hairpin loop at the very 5' end of STNV RNA is believed to play an important role in the formation of a translation initiation complex behaving like a cap structure, those secondary structures proposed in SPMV RNA may also substitute for several functions usually provided by a cap structure at 5' end of eukaryotic mRNAs.

If we assume that SPMV RNA is a monocistronic messenger for its coat protein and ignore the short ORF 2, the 3' non-coding region would have 267 residues, which accounts for about 33 % of the whole nucleic acid. This portion of the genome has a high degree of potential base pairing regions, and several secondary structure models can be envisioned. One possible folding of the 3' end is shown in Fig. 20, and it is quite possible that this part of the genome is stabilized by stable secondary or tertiary structure. The free energies required for the formation of those three hairpin loops (Fig. 20)are to the 5' side - 0.74 Kcal/mol nucleotide, - 0.7 Kcal/mol nucleotide and - 1.02 Kcal/mol nucleotide, respectively. Such a secondary structure could be involved in specific binding of the coat protein and the

Table 5. Base composition of SPMV RNA determined by HPLC analysis and nucleic acid sequence

Nucleoside	HPLC[a]	Nucleic acid sequence
Uridine	20.7 %	21.5 %
Cytidine	32.1	29.5
Guanosine	28.4	28.1
Adenosine	18.9	20.8

[a]Data from Buzen et al.

viral RNA polymerase as demonstrated in AMV RNAs (Koper-Zwarthoff and Bol, 1980) and BMV RNAs (Dreher et al., 1984). Among the viruses which are known to have the capacity to accept an amino acid, the 3' end of turnip yellow mosaic virus (TYMV) RNA can give a folding similar to the cloverleaf structure of tRNAs and that of BMV is less similar, and that of TMV RNA is not at all (Guilley et al., 1979). In spite of the fact that TMV RNA does not have the anticodon for histidine, it can be aminoacylated with histidine. On the other hand, the 3' end of STNV RNA can also form a cloverleaf structure (Ysebaert et al., 1980), but no binding capability for an amino acid has been detected. Although the 3' end of SPMV RNA shown in Fig. 20 did not reveal a typical tRNA-like structure, this RNA may be able to accept an amino acid (possibly proline), because the most distant loop from the 3' end bears a proline anticodon.

We have no direct information concerning the origin of SPMV or other satellites. However, several speculative hypotheses have been proposed. Two possible origins of CMV satellite RNA have been discussed in detail (Kaper and Waterworth, 1981). One is that satellite RNA is derived from a specific region of host DNA. The other is that satellite RNA could arise de novo as a consequence of random non-template generation of RNA molecules. Such events are not without precedent as highly purified Q beta replicase can synthesize 6 S RNA in vitro from nucleotides in the absence of an exogenous template (Biebricher et al., 1981). In this system where the products are sequentially transferred to fresh replicase and nucleotides, a large number of RNA species, quite variable in length and sequence appear during the early

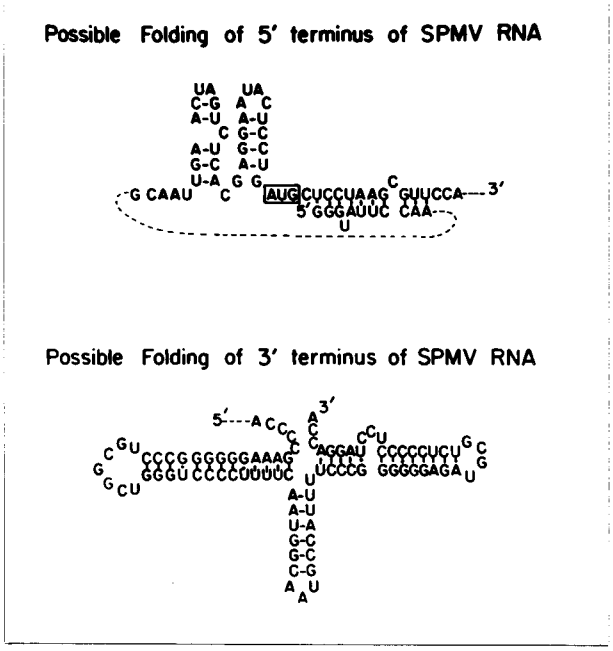

Figure 20. Possible secondary structures of 5' and 3' termini of SPMV RNA. An initiation codon (AUG) is boxed and the 5' end of the molecule could hybridize to the region in close proximity to the AUG. Two hairpin loops may exist just before the AUG. The estimated values of thermostability are −0.28 kcal/mol nucleotide for the loop next to the AUG, and 0 kcal/mol nucleotide for the distant loop. The 3' end could have a high degree of secondary structures. The free energies for formation of each loop are to the 5' side, −0.74, −0.7, and −1.02 kcal/mol nucleotide, respectively. Although the structure is not similar to known tRNAs, the third loop may have an anticodon for proline (CGG).

stages of the *in vitro* reaction. The self-replicating RNAs evolve rapidly in size and sequence during the early stages of replication, and after many successive generations of replication, long chain products (up to 220 residues) evolve as a consequence of natural selection of the initial small template molecules. The sequence of one of the 6S RNAs appears to have originated from only two building blocks and their complements, i.e. CCC(C), UUUG and GGG(G), CGAA. This sequence probably comes from selection of those oligomers by the enzyme through its inherent binding affinity to these patterns. However, such patterns as the repeat of some building blocks of oligomers are not evident in the sequence of SPMV RNA. Thus, it seems that random generation is an unlikely hypothesis for the origin of SPMV RNA.

Generation of satellite RNA from the host genome has also been proposed, but no evidence exists for this hypothesis with SPMV. The possibility that such events could occur infrequently from low abundance RNAs, which might evolve rapidly, makes the likelihood of a successful search remote. Although there was a claim that the satellite RNA of turnip crinkle virus (TCV) shares nucleotide sequences with the genome of turnips (Altenbach and Howell, 1984), serious doubts exist about the reliability of the published data. On the other hand, some researchers believe that TNV and STNV may have evolved from an RNA phage because STNV RNAs resemble phage RNAs in lacking both a cap and poly (A) (Francki, 1985). This theory helps explain the presence of the Shine-Dalgarno sequence (purine rich region) upstream of the first AUG as well as the terminal structures

since the Shine-Dalgarno sequence is the binding site for the 30 S ribosomal subunit of phages infecting prokaryotes. Since SPMV RNA also contains a purine rich region 10 nucleotides before the initiation codon, the sequence might occur universally among RNAs of satellite viruses. However, other than these superficial similarities of the genomes of satellite viruses and RNA phages, little definitive data such as nucleotide sequences is available to screen for close relationships between satellite viruses and phages.

On considering that the RNAs of satellite viruses encode a coat protein but are defective in replication, it is possible to imagine that a subgenomic RNA species derived from an unrelated conventional plant virus could somehow parasitize another plant virus if it were able to recognize the replicase of that virus. During the course of evolution, the parasitic RNA would probably evolve rapidly to enable more efficient replication by the new host and to become more efficiently encapsidated by the coat protein encoded by the parasitic RNA. Such alterations might even involve removing a peptide domain to improve the efficiency of encapsidation. Such a reduction in the size of the genome might also increase the rate of replication of the parasitic molecule. Unfortunately none of these conjectures has supporting evidence, and until additional data become available from a wide variety of sources, we will be unable to conduct comprehensive analysis to test these possibilities.

CHAPTER II. STUDIES ON CUCUMBER MOSAIC VIRUS (CMV) SATELLITE RNA (STRAIN Y)

The virus particle of cucumber mosaic virus (CMV) contains three single-stranded genomic RNA molecules and a subgenomic RNA, designated RNA 1-4 in decreasing order of molecular weight; RNA 4 is actually the mRNA for CMV coat protein (Peden and Symons, 1973; Schwinghamer and Symons, 1975; Takanami et al., 1977). In addition to these four RNA molecules, some CMV isolates have a small single-stranded linear RNA encapsidated into CMV virions. This fifth RNA species, referred to as a satellite RNA, depends on CMV for its replication and shares no significant nucleotide sequence homology with CMV genomic RNAs (Diaz-Ruiz and Kaper, 1977; Gould et al., 1978).

CMV satellite RNAs have been extensively characterized in many laboratories because disease modulation by the RNA is quite dramatic in certain host plants (Waterworth et al., 1979; Takanami, 1981; Gonsalves et al., 1982; Kaper and Tousignant, 1984). Many strains of these satellite RNAs have already been sequenced (Richards et al., 1978; Godon and Symons, 1983; Collmer et al., 1983; Hidaka et al., 1984; Avila-Rincon et al., 1986; Garcia-Arenal et al., 1987; Kaper et al., 1988). The sequence data revealed that the CMV satellite RNA ranges in size approximately from 330 to 340 nucleotides except for our satellite (369 nucleotides) and the 386-nucleotide satellite (Hidaka et

al., 1988), and has a cap structure at 5' end.

The satellite RNAs often attenuate the symptoms induced by CMV in certain host plants. Although the effect of satellite RNA on CMV disease severity depends on the strains of satellite RNA, symptoms are usually unchanged or even attenuated (Takanami, 1981; Mossop and Francki, 1979; Gonsalves et al., 1982). A certain strain of the satellite RNA occasionally induces lethal necrosis on tomato, resulting in more severe symptoms (Kaper and Waterworth, 1977). The efforts to use satellite-mediated disease attenuation for biological control against CMV infection have been made over the last few years. For instance, preinfection with CMV containing a certain isolate of satelite RNA successfully protected tomato plants from the infection with virulent CMV, mimicking cross protection effects between a mild viral strain and a virulent one (Yoshida et al., 1985; Tien et al., 1987). Moreover, it was demonstrated that cDNA clones of the satellite RNA were integrated into tobacco plants by means of Ti plasmids, and the transgenic plants were able to produce biologiaclly active satellite RNA when inoculated with CMV and actually acquired virus resistance (Baulcombe et al., 1986; Harrison et al., 1987).

In this chapter, I will describe the data at molecular level on CMV satellite RNA (strain Y) originally isolated by Takanami (1981). My research on the satellite consists of the following information : 1) disease modulation on several plants by the satellite, 2) the nucleotide sequence domains involved in the biological activity and the symptom induction, 3) construction of transcription vectors for production of infectious

transcripts, 4) expression of the satellite RNA in transgenic tobacco plants. The goal of the studies described in this chapter is to elucidate mainly the mechanism(s) of the symptom alteration by the satellite, the sequence domains responsible for replication and encapsidation, and hopefully molecular aspects of the relationships between CMV and the satellite RNA.

II-1. Disease modulation on several plants by CMV satellite RNA (strain Y)

In this section, I initiated investigations on the disease modulation on several plants infected with CMV by the addition of satellite RNA (strain Y). A satellite RNA maintained in our laboratory is particularly interesting as it induces dramatic yellow symptoms on the leaves of tobacco plants infected with CMV (Takanami, 1981). In order to identify the nucleotide sequence region responsible for induction of the yellow symptoms, we constructed a full-length cDNA clone of the satellite. The nucleotide sequence of these clones necessitated minor revision in the nucleotide sequence reported previously (Hidaka et al., 1984). We successfully produced biologically active RNA transcripts from cDNA of the satellite RNA using RNase H treatment (Masuta et al., 1987).

Materials and Methods

Isolation of satellite RNA and satellite-free preparation of CMV. Satellite RNA (strain Y) was propagated in tobacco plants (Nicotiana tabacum L. cv. Xanthi-nc) in the presence of CMV-Y. CMV virions were purified from the infected leaves by differential centrifugation, and satellite RNA was isolated from purified preparations as previously described (Takanami, 1981). CMV ordinary strain (CMV-O) (Hidaka and Tomaru, 1960) and yellow strain

(CMV-Y) (Tomaru and Hidaka, 1960), which were used for coinfection with RNA transcript tested for its infectivity, were obtained by two cycles of single lesion transfer to avoid possible contamination of satellite RNA (Takanami, 1981). CMV-Y induces yellowish green mosaic which is more severe than CMV-O does. When twenty tobacco plants were inoculated with the CMVs, all the plants infected showed the typical green mosaic symptoms, indicating that the viruses were free from satellite RNA. The crops used for the symptom observations are tobacco (Xanthi nc), tomato (Best of All and Rutgers), spinach (King of Denmark), maize (Golden Cross-Bantam T-51), lettuce (Great Lake), melon (Prince), petunia (Rose) and red pepper (Takanotsume). The leaves of each assay plant were dusted with carborundum and inoculated with CMV containing CMV satellite RNA (strain Y) (10 μ g/ml) by fingers. Infected plants were grown in a green house at 23-28℃. The construction of cDNA clones of the satellite RNA and *in vitro* transcription from the cDNA will be described in Capter II-2.

Results

Disease modulation by CMV satellite RNA (strain Y). I investigated the disease modulation on several plants infected with CMV by the addition of satellite RNA (Y strain), which causes unique bright yellow symptoms on tobacco plants.

In the previous publication by Takanami (1981), it was shown that the satellite RNA caused a unique brilliant yellow symptom on tobaccos(*N. tabacum*), *N. glutinosa* and *N. sylvestris* while

Table 6. Symptoms of tomato induced by satellite RNA (Y strain)

Inocula		Tomato cultivars[a]	Symptoms[b]
Helper	Satellite		
CMV-Y	-	Best of All	SM, st
	natural		M, st, LN (10/10)[d]
	transcript[c]		M, st, LN (10/10)
	-	Rutgers	SM, st
	natural		M, st, LN (8/10)
	transcript		M, st, LN (7/10)
CMV-O	-	Best of All	M, st
	natural		M, st
	-	Rutgers	M, st
	natural		M, st

[a] Young seedlings were inoculated with CMV (10μg/ml) and satellite RNA (1μg/ml).
[b] Symptoms were evaluated 14 days after inoculation. M : mosaic, SM : severe mosaic, st : stunting, LN : lethal necrosis.
[c] The satellite RNA synthesized in vitro. See text.
[d] Number of dead plants/total number of inoculated plants.

lethal necrosis was observed on tomato. In addition to the yellow symptoms by the satellite RNA, I also observed somewhat attenuated symptoms on tobacco. On the other hand, Kaper et al. reported that the satellite RNA was incapable of inducing lethal necrosis in tomato and necrosis-inducing property of CMV-Y was due to a minor contaminated satellite molecule designated Yn-CARNA 5 in their paper (1986). However, We could not detect any minor satellite RNA like Yn-CARNA 5 in the CMV-Y preparation because all the plants inoculated with CMV-Y containing no satellite showed only green mosaic although the inocula were prepared after passaging the virus in tobacco. In order to eliminate the possibility of contamination of a necrogenic satellite RNA in the satellite preparation, we synthesized infectious transcripts from a cDNA of the satellite (Masuta et al., 1987). As shown in Table 6 and Plate I, after the infectivity assays, it turned out that both natural satellite RNA and the transcript elicited lethal necrosis on tomato coinoculated with CMV-Y. Best of All tomato was more sensitive to the satellite than Rutgers tomato in respect of inducing lethal necrosis. However, the tomato plants infected with CMV-O together with the satellite RNA were not killed by lethal necrosis and showed green mosaic symptom with some disease attenuatation.

Some disease attenuation effects by the satellite RNA were observed on several plants other than tobacco infected with CMV-O. Most of the plants inoculated with CMV-O and the satellite RNA showed better growth than control plants infected only with CMV-O (Plate II). Especially on spinach and corn, the attenuation effects were dramatic (Plate II-2, 3). The satellite-

induced bright yellow mosaic on tobacco, which discriminates the satellite isolate from the others, was shown in Plate Ⅱ-1. The CMV symptoms on lettuce, eggplant and melon were also effectively suppressed by the presence of the satellite RNA (Plate Ⅱ-4～6). On petunia, the addition of the satellite resulted in increase of the number of flowers although the flower discoloration by CMV infection was not altered (Plate Ⅱ-7). Interestingly, pepper showed systemic bright yellow symptoms like tobacco (Plate Ⅱ-8).

Discussion

The results showed that CMV satellite RNA (strain Y) was capable of inducing lethal necrosis on tomato in combination with CMV-Y but not with CMV-O. Moreover, the satellite RNA could induce some symptom attenuation on several plants infected with CMV-O except that it hastened the death of the tomato infected with CMV-Y. Since we still do not know where Yn-CARNA5 found by Kaper et al. (1986) came from, whether our satellite can induce lethal necrosis on tomato may remain unresolved until we successfully synthesize infectious RNA transcripts from cDNAs of CMV RNAs. The yellow symptom induction by the satellite RNA was so far observed only on some solanums such as tobacco and pepper while egg plant and tomato do not produce yellow mosaic. Even in Nicotiana plants, N. glutinosa and N. sylvestris develop the yellow symptoms but N. clevelandii does not (Takanami, 1981). The importance of these observations is not clear.

Two hypotheses currently are favored for explaining interference of CMV satellite RNA with its helper (CMV). These are:

1) that both the satellite RNA and the helper virus compete for a replicase molecule (Kaper, 1982) or 2) that the satellite RNA hybridizes *in vivo* to the domain of the coat protein cistron of the helper virus RNA and inhibits the translation of the coat protein gene of CMV or replication of CMV RNA3. (Rezaian and Symons, 1986).

Although our satellite may not be suitable for applying to practical field-control of CMV disease, because of some problems, such as bright yellow symptoms on tobacco and pepper, and lethal necrosis on tomato infected with CMV-Y, the studies at molecular level may enable us to overcome these problems.

II-2. Effects of 5' extentions on the biological activity of CMV satellite RNA (strain Y)

Janda et al. (1987) reported that addition of 7 or 16 bases to the 5' ends of brome mosaic virus (BMV) RNA 3 transcripts dramatically suppressed their infectivity. With respect to CMV satellite RNA, Kurath and Palukaitis (1987) synthesized infectious transcripts from cDNA of (n)CARNA 5 (or D-CARNA 5) and showed deleterious effects of 3' extra bases on transcript infectivity although they did not quantify specific activity of their transcripts. In this section, I mainly describe the effects of 5' extension on transcript infectivity, using the RNase H method for successive trimming of the 5' extra bases.

Materials and Methods

Oligonucleotide synthesis. Oligonucleotides were synthesized on a solid phase column support in an Applied Biosystems Model 381A DNA synthesizer with (2-cyanoethyl)-phosphoramidites (Eadie and Davidson, 1987). After synthesis, the products were purified by C_{18} reverse phase high-performance liquid chromatography (RP-HPLC).

Synthesis of full-length cDNA. The synthesis of cDNA clones was conducted as essentially described by Gubler and Hoffman (1983) except that a synthetic oligonucleotide was used as a primer for the second cDNA strand synthesis. The isolated satellite RNA was annealed with an excess amount of the synthetic

oligonucleotide (5'-GGGTCCTGTAGAGGAAT-3') complementary to the 3'end of the satellite RNA, which was prepared based on the published sequence data (Hidaka et al., 1984). The first cDNA strand was synthesized in a 50 μl reaction mixture containing 50 mM Tris-HCl, pH 7.5, 75 mM KCl, 3 mM $MgCl_2$, 100 mM DTT, 1 mM each of dATP, dCTP, dGTP and dTTP, 20 units of RNasin (ProMega), 3 μg of annealed RNA and 200 units of moloney murine leukemia virus (M-MLV) reverse transcriptase (BRL). The mixture was incubated at 42℃ for 1.5 hr and then the products were extracted with phenol and precipitated with ethanol. The DNA-RNA hybrid was digested with RNase H (Pharmacia) at 37℃ for 20 min in the presence of an excess amount of the synthetic oligonucleotide (5'-GTTTTGTTTGATGGAGA-3') which is complementary to the 3'end of the negative strand. After boiling for 2 min followed by incubation at 42℃ for 20 min, the second cDNA strand was then synthesized in 50 μl of 50 mM Tris-HCl, pH 7.5, 75 mM KCl, 3 mM $MgCl_2$, 100 mM DTT, 1 mM each of dATP, dCTP, dGTP and dTTP, 3 units of DNA polymerase I (Takara Shuzo, Japan). The incubation was carried out at 42℃ for 1.5 hr. The full-length double-stranded cDNA was precipitated with ethanol after phenol extraction and then ligated into pUC 13 previously cleaved with SmaI to create a recombinant plasmid, pC 3 (Fig. 21). The resulting plasmid was used to transform E. coli JM 109, and the transformed cells were screened on a medium containing ampicillin and X-gal. The cDNA insert was subcloned into the M 13 phage (mp 18 and 19) and sequenced by the dideoxy chain termination method originally described by Sanger et al. (1977).

Construction of pIBI 31-MC . As illustrated in Fig. 21, the

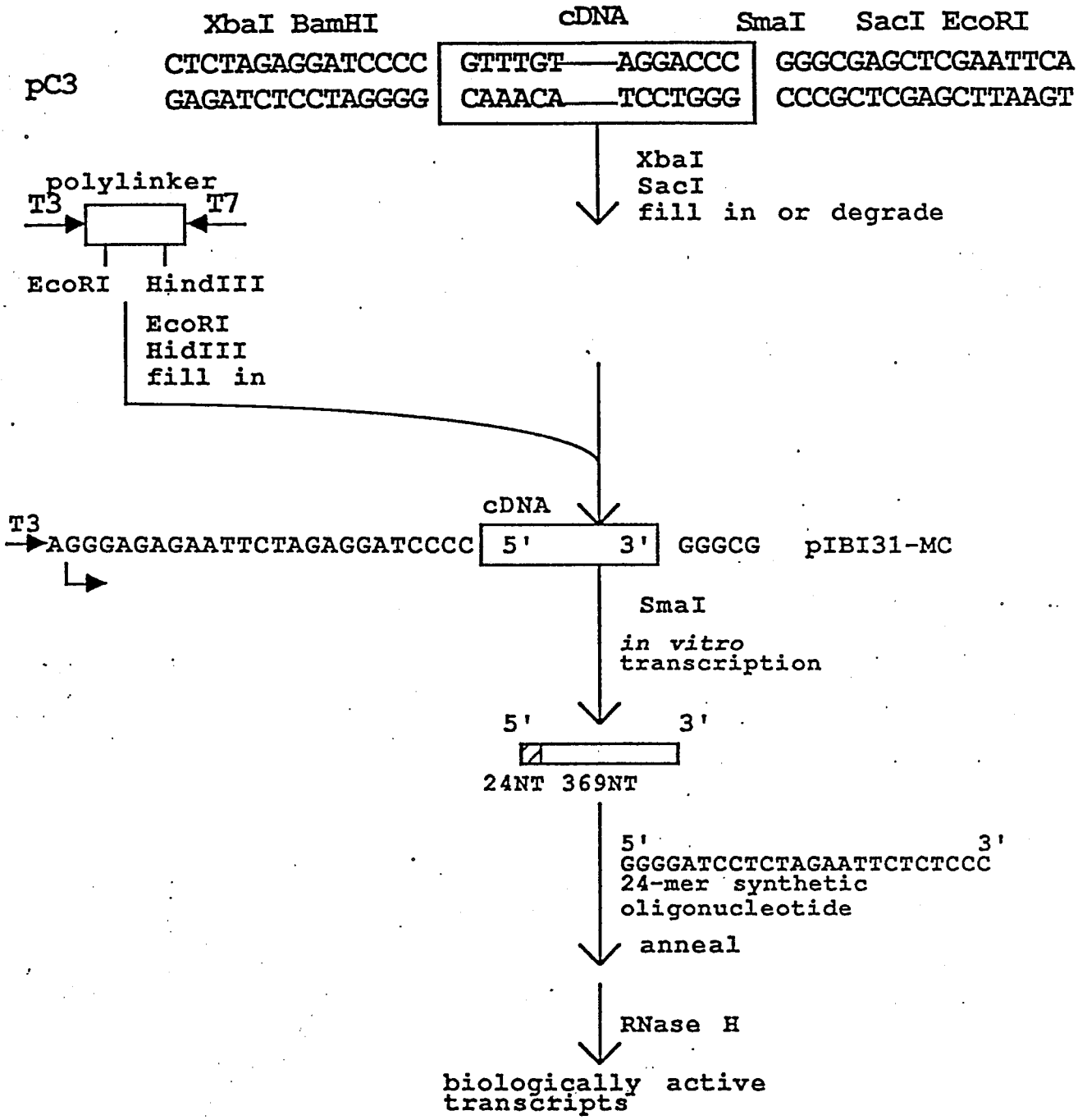

Fig. 21. Schematic representation of the synthesis of infectious RNA from full-length cDNA of satellite RNA (strain Y). The satellite cDNA is boxed and indicated as cDNA. Treatment with Klenow fragment and T4 DNA polymerase is abbreviated to fill in and degrade, respectively. Arrows(↑) show the restriction enzyme sites used in the construction of the recombinant DNA or the cleavage before in vitro transcription; the closed arrow denotes the transcription initiation site. T3 and T7 designate T3 promoter and T7 promoter, respectively.

400 bp fragment containing a full-length cDNA of satellite RNA was obtained by digesting pC 3 with XbaI and SacI, and the overhanging nucleotides were removed with Klenow fragment and T4 DNA polymerase. The plasmid pIBI 31-MC was constructed by "blunt end" ligating the 400 bp fragment into EcoRI/HindIII-cut pIBI 31, a transcription vector purchased from IBI, whose 5'overhanging nucleotides had been filled in with Klenow fragment. The junction between the T3 promoter and the cDNA insert was confirmed by sequencing.

In vitro transcription. Two to three micrograms of plasmid DNA were digested with SmaI to generate a 3'terminus identical to that of the authentic satellite. The reaction mixture (20 μl) contained 40 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 2 mM spermidine-HCl, 10 mM NaCl, 10 mM DTT, 1 mM each of ATP, CTP, UTP, GTP, 20 units of RNasin and 10 units of T3 RNA polymerase (IBI). The mixture was incubated at 37℃ for 1 hr and the reaction was stopped by adding EDTA to 25 mM. After phenol extraction followed by ethanol precipitation, the nucleic acid was dissolved in 25 μl of 50 mM Tris-HCl, pH 7.5, 75 mM KCl, 3 mM $MgCl_2$ and 100 mM DTT. The non-viral sequence at the 5'end of the transcripts was removed by the method of Masuta et al. (1987). Briefly, the mixture was left at room temperature for 15 min to anneal the nucleic acid to the synthetic oligonucleotide (5'-GGGGATCCTCTAGAATTCTCTCCC-3') that is complementary to the non-viral sequence of the 5'ends of the transcripts. In the analysis of 5' more limited sequence, 20-mer (5'-ATCCTCTAGAATTCTCTCCC-3'), 15-mer (5'-CTAGAATTCTCTCCC-3') and 10-mer (5'-ATTCTCTCCC-3') oligonucleotides were used. Then RNase H (1 unit) was added and

the mixture was incubated for another 20 min. The transcribed RNA was purified by phenol extraction and ethanol precipitation.

Infectivity assay. Infectivity of the in vitro transcribed RNAs was assayed using about one month old tobacco plants (N. tabacum L. cv. Xanthi nc) which were previously dusted with carborundum. Purified CMV-O or CMV-Y used as a helper virus was diluted to 10 μg/ml in 0.1 M phosphate buffer (Na_2HPO_4-KH_2PO_4), pH 7.0. RNA transcribed from 1-1.5 μg of the plasmid DNA was dissolved in 60-100 μl of water. Equal volumes of virus and RNA solutions were mixed to make an inoculum, and about 40-50 μl inoculum per plant was inoculated with a forefinger. Seven to ten days after inoculation, bright yellow symptoms appeared indicating the transcribed RNAs were biologically active.

Dot blot hybridization test. RNA was extracted from the infected leaf tissue (0.1 g). The leaf tissue was homogenized in a mixture of 0.5 ml water-saturated phenol, 0.5 ml 25 mM Tris-HCl, pH 7.5, 25 mM $MgCl_2$, 25 mM KCl, 1 % SDS by a mortar and pestle. After centrifugation to separate the phases, the aqueous phase was extracted twice with phenol-chloroform (1:1), and nucleic acids were precipitated with 3 volumes of ethanol. Pellets were resuspended in 1 ml H_2O. Dot blot hybridization was performed according to the method described by Meinkoth and Wahl (1984). Ten μl of the suspension was spotted onto nitrocellulose membrane, and 10^7 cpm of nick-translated pIBI 31-MC was used as a probe.

Analysis of the 5' ends of transcripts. The transcripts from pIBI31-MC and RNase H-treated transcripts were annealed to a synthetic oligonucleotide that is complementary to residues 100-

116 of the authentic satellite, and reverse transcriptase was used to extend the annealed primer. The primer extension experiment was carried out in a 20 μl reaction mixture containing 50 mM Tris-HCl, pH 7.5, 75mM KCl, 3mM $MgCl_2$, 100mM DTT, 250 μM each of dATP, dGTP, dTTP, 12.5 μM dCTP, 10 μCi of [α-^{32}P] dCTP (3,000 Ci/mmol), 1 μg of annealed RNA and 200 units of M-MLV reverse transcriptase. The reaction mixture was incubated at 42° C for 30 min, and 1/16 amount of the products were analyzed in a 5 % polyacrylamide sequencing gel.

Determination of the 5' terminal sequence of progeny satellite RNA. The 5' terminal sequence of progeny satellite RNA was determined by using a synthetic oligodeoxyribonucleotide (5'-GCCTCCAGTCCGAGATA-3'), complementary to nucleotides 100-116, to prime reverse transcription in the presence of dideoxynucleotides (Gustafson et al., 1987).

Results

Revision of the sequence of satellite RNA (strain Y). The dideoxy nucleotide sequence of two cDNA inserts of the satellite RNA revealed that the published sequence (Hidaka et al., 1984) missed a C residue at position 234 and therefore, satellite RNA (strain Y) was 369 nucleotides in length (Fig. 22). This insertion revealed the presence of second open reading frame (ORF) containing 60 amino acid residues on almost half of the entire RNA, in addition to a smaller ORF beginning at position 11. Comparison of the amino acid sequence encoded by the second ORF showed a great deal of homology between the putative polypeptide

GUUUUGUUUGAUGGAGAAUUGCGUAGAGGGGUUAUAUCUGCGUGAGGAUC 50

CAUCACUCGGCGGUGUGGGAUACCUCCCUGCUAAGGCGGGUUGAGAGUGU 100

AUCUCGGACUGGAGGCGGGAUGUCUGCGGGUGUUCCGUCUGCUGCCCACG 150

AUGGUGGGAGUCACCCAAGGGGUGACUUUUUCAGCUCUGCAUUUCUCAUU 200

UGAGCCCCCGCUCAGUUUGCUAGCAAAACCCGGCACAUGGUUCGCCGUUA 250

CUAUGGAUUUCGAAAGAAACACUCUGUUAGGUGGUAUCGUGGAUGACGCA 300

CGCAGGGAGAAGCUAAGGCUUAUGCUAUGCUGAUCUCCGUGAAUGUCUAU 350

ACAUUCCUCUACAGGACCC 369

Fig. 22. Nucleotide sequence of CMV satellite RNA (strain Y). Revised nucleotides are underlined.

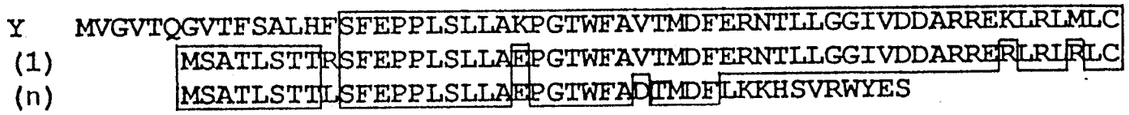

Fig. 23. Amino acid sequences encoded by the second open reading frames of satellite RNAs. Y represents CMV satellite RNA (strain Y) whose sequence was originally reported by Hidaka et al. (1983) and revised in this paper. (1) and (n) are a non-necrogenic strain, (1)CARNA 5 sequenced by Collmer et al. (1983) and a necrogenic strain, (n)CARNA 5 sequenced by Richards et al. (1978), respectively. The putative polypeptides start at the second AUG triplets: position 135-137 for (1) and (n), position 151-153 for Y. Homologous regions are boxed.

of the Y strain satellite RNA and that of (1)CARNA 5 (Fig. 23). Moreover, the Y strain satellite RNA and (n)CARNA 5 share 20 homologous amino acids. Three other differences in the nucleotide sequence of the Y satellite RNA previously observed by Kaper et al. (1986) were also confirmed by sequencing the cDNA clones. These consisted of transitions from a C to a U at residue 161 (Ala to Val), a C to an A at residue 167 (Pro to Gln), and a C to a U at residue 173 (Ala to Val).

Cloning of cDNA of the satellite RNA into a transcription vector. Because full-length cDNAs of the satellite RNA were originally cloned into the SmaI site in pUC 13 by blunt-end ligation, the cDNA insert could not be excised without non-viral nucleotides derived from the vector. However, since the 3'end of the cDNA ends with -CCC, a SmaI site was restored by the cDNA insertion and so run-off transcription after SmaI cleavage of the recombinant plasmid should result in an RNA transcript whose 3'end is coterminal with that of authentic satellite RNA. In order to excise the shortest fragment carrying the entire satellite sequence, XbaI and SacI sites in the multiple cloning polylinker were used to isolate the insert. This produced a fragment with 13 nucleotides from the vector at the 5'end of the cDNA. BamHI could not be used because a BamHI site existed inside of the cDNA. The resulting cDNA fragment was then cloned in the appropriate orientation 11 nucleotides downstream of T3 promoter in pIBI 31. Thus, the transcribed RNA was expected to have 24 extra residues at its 5'end (Fig. 21). In order to quantify the amount of RNA, we first isolated the transcript from a 2 % agarose gel after in vitro transcription followed by DNase I treatment. The

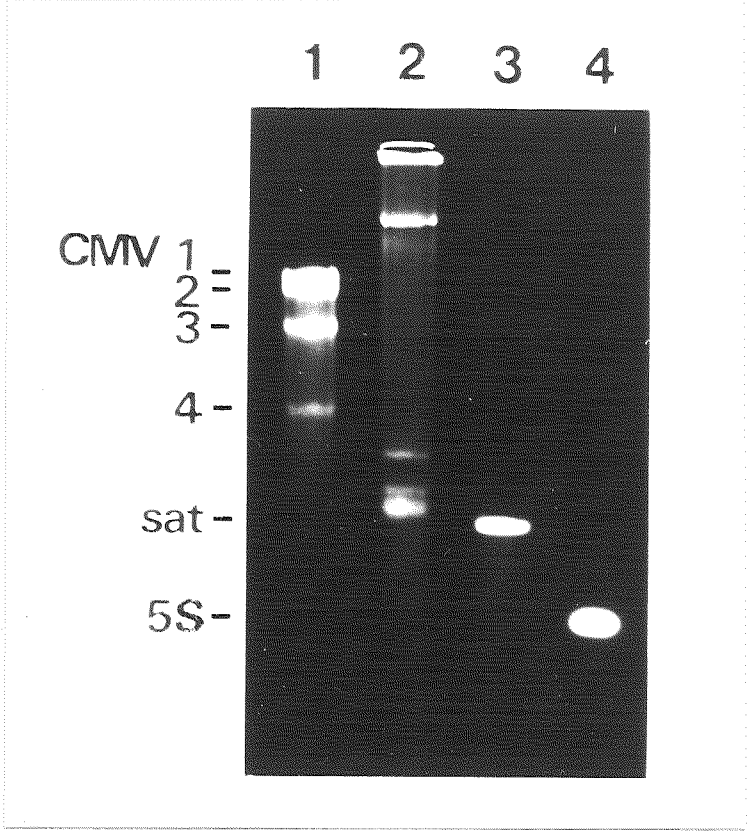

Fig. 24. In vitro transcript from SmaI-cut pIBI31-MC cDNA clone of CMV satellite RNA (strain Y). Lane 1, 3 and 4 contain CMV RNAs (1:3,389 NT, 2:3,035 NT, 3:2,193 NT, 4:1,027 NT), authentic satellite RNA (369 NT) and 5S ribosomal RNA (120 NT), respectively. Lane 2 contains 1/20 amount of the total transcripts. RNAs were electrophoresed in a 2% agarose gel under non-denaturing conditions and visualized by ethidium bromide staining. NT, nucleotides.

quantity of the transcript was spectrophotometrically determined, and an infectivity assay was conducted. In the result, the RNA of the major band on lane 2 in Fig. 24 was found to be biologically active and appeared to be the expected run-off transcript from SmaI-cut pIBI 31-MC. The in vitro transcription with T3 RNA polymerase normally produced about 1-2 μg of transcripts from 1 μg of template plasmid. Those RNA molecules migrated a little more slowly than the authentic RNA in a 2% agarose gel (Fig. 24).

In the several experiments, we roughly estimated the quantity of the transcript by comparing the intensity of the ethidium bromide fluorescence of the sample with that of RNA standard (natural satellite RNA). The estimation by this simple method agrees well with spectrophotometric determination because the transcript and natural satellite RNA should have similar sequences. Although some bands of high molecular weight, which did not always appear, were also observed, they are possibly the products from uncut plasmid templates. Since the infectivity of the RNA transcripts having 24 additional nucleotides at their 5'ends was very low (see below), the 5'extra sequence was removed as described in "Materials and Methods".

Infectivity assay of in vitro transcripts. SmaI-cut plasmids containing full-length cDNA inserts were used as templates for in vitro transcription. The transcribed RNA was coinoculated onto tobacco plants with a helper virus (CMV-O or CMV-Y). The results of the infectivity assay are summarized in Table 7 and Table 8.

Although transcripts from pIBI 31-MC showed very low infectivity, they became 10^3-10^4 fold more infectious after RNase H

Table 7. Infectivity of transcripts from cDNA of satellite RNA

inocula[a]	Plants showing yellow symptoms/ inoculated plants					
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Total
pIBI31-MC + CMV-Y	1/6	0/6				1/12
pIBI31-MC + CMV-O			0/4	0/4		0/8
pIBI31-MC /RNase H[b] + CMV-O				8/8	12/12	20/20
CMV-Y[c]	0/6	0/6				0/12
CMV-O[c]			0/4	0/11	0/12	0/25
satellite + CMV-Y	6/6	6/6				12/12
satellite + CMV-O			4/4	4/4	12/12	20/20

[a] Concentrations of RNA transcripts, natural satellite RNA and helper viruses are 0.1-0.3 μg/ml, 1 μg/ml and 10 μg/ml, respectively. [b] /RNase H denotes the removal of the non-viral bases at the 5'ends of the transcripts with 24-mer oligonucleotide and RNase H after *in vitro* transcription. [c] The symptoms induced by the satellite RNA were not observed.

Table 8. Dilution end-point assay for yellow symptoms induced by transcripts from pIBI31-MC

Concentraion of satellite or Transcripts (μg/ml)	Inoculum consisted of CMV-O[a] and None (Control)	Natural satellite	Transcripts	RNase H-treated transcripts[b]
10^0		5/5	3/5	5/5
10^{-1}		5/5	2/5	5/5
10^{-2}		5/5	0/5	5/5
10^{-3}		5/5	0/5	5/5
10^{-4}		4/5	0/5	2/5
0	0/10			

[a] Final concentration of CMV-O in inoculum was 5 μg/ml. [b] Transcripts were hybridized with 24-mer oligonucleotide and then treated with RNase H.

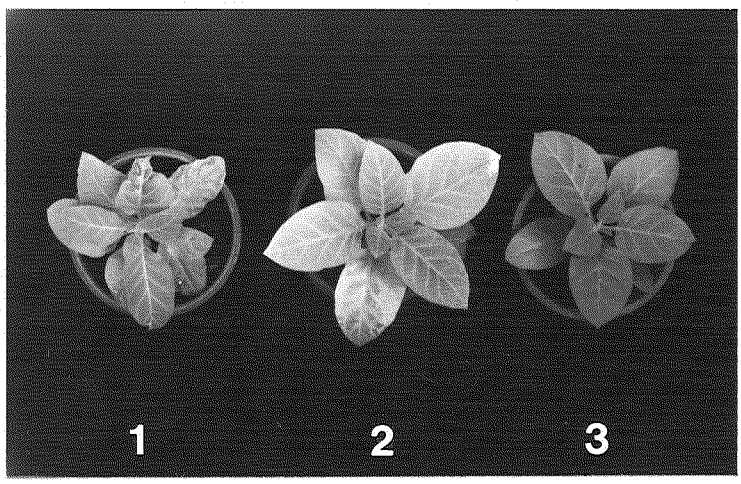

Fig. 25. Symptoms on tobacco plant (var. Xanthi nc) infected with CMV-O alone(1) and RNA transcripts from pIBI31-MC in the presence of CMV-O(2). Note that bright yellow symptom and some attenuation in disease severity are observed on the middle plant. 3:healthy plant.

treatment. If satellite RNA (from CMV strain Y) was inoculated together with a helper virus CMV, the infected tobacco plants developed the bright yellow symptoms (Takanami, 1981). The transcribed RNA also induced the same symptoms on the plants as the authentic satellite RNA did (Fig. 25). As expected, none of the tobacco plants inoculated with the helper virus alone showed the satellite-inducing symptoms. When 20 tobacco plants were inoculated with the template DNA (pIBI 31-MC) and a helper virus (CMV), all the plants showed typical green mosaic symptoms induced by CMV, suggesting that the template DNA is not infectious.

In order to check the replication of the transcripts, total nucleic acids were extracted from an upper systemically infected leaf, and dot blot hybridization analyses were performed by using nick translated pIBI31-MC probe. Replication of cloned CMV satellite RNA was judged by comparing the hybridization signal of the samples with that of a series of dilutions (from 1 μg to 10 pg) of natural satellite RNA after autoradiography. In the result, the intensity of the hybridization signals from all the plants showing yellow symptoms were between 100 ng and 1 μg of natural satellite RNA, whereas no hybridization signal was detected in the leaves lacking the yellow symptoms.

Effects of 5' extensions on transcript infectivity. The transcripts that have 5' non-viral bases at the 5' terminus were annealed with 3 oligonucleotides (10-, 15- and 20-mer), and then the RNA-DNA hybrid portions were degraded with RNase H. As a result, we could obtain 3 populations of transcripts with 16-18, 11-14 and 6-9 extra residues at 5' end (Fig. 26). The strong band on the 11th position, which were observed in all the lanes,

Table 9. Effects of the number of 5' additional bases on the infectivity of the satellite

Concentration of transcripts (g/ml)	Oligonucleotides used for triming 5' extra bases			
	20-mer (6-9)[a]	15-mer (11-14)	10-mer (16-18)	none (24)
10	4/4	4/4	4/4	4/4
10^0	4/4	3/4	2/4	2/4
10^{-1}	4/4	3/5	2/4	1/5
10^{-2}	2/4	1/4	0/5	0/4
10^{-3}	1/5	0/5	0/5	

[a] Extra 5' non-viral bases after RNase H treatment.

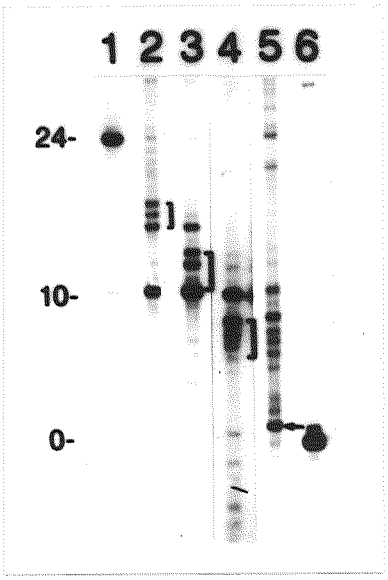

Fig. 26. Autoradiograph of a primer extension experiment of transcripts after successive trimming of 5' extra bases with RNase H. A 17-base oligodeoxynucleotide primer, annealing to 100-116 bases from the satellite 5' end, was extended with reverse transcriptase, and the products were analyzed on a 5 % polyacrylamide sequencing gel. Lane 1 is the transcript containing 5'extra 24 bases. The transcript shown in lane 1 was annealed with 10-mer (lane 2), 15-mer (lane 3), 20-mer (lane 4) and 24-mer (lane 5) oligodeoxynucleotide to generate various lengths of 5' extra bases, and RNA-DNA hybrid portions were removed with RNase H. Lane 6 contains the natural satellite RNA. The remaining 5' extra bases of transcripts are indicated on the left. A sequencing reaction was performed to generate a ladder on the same gel (not shown in this figure). (↑)and ( ]): the bands generated by RNase H treatment.

is perhaps due to some particular RNA structure where reverse transcriptase may stop (lane 1-3) or RNase H preferably cleaves (lane 5, 6). When hybridized to 24-mer oligonucleotide, the RNase H-treated transcripts gave one main band one nucleotide above the natural 5' end as we previously reported (Masuta et al., 1987). The results of infectivity assay of these transcripts are summarized in Table 9, suggesting that addition of at least some non-viral bases at 5' end inhibited the infectivity of the satellite, and that a longer 5' extension gave rise to considerable decrease of the biological activity.

Discussion

It was reported that RNase H was successfully used to remove the poly(A) sequences from mRNA in the presence of oligo(dT) (Sippel et al., 1974; Vournakis et al., 1975) and even to cleave RNA at specific sites desired (Miller et al., 1986; Minshull and Hunt, 1986; Shibahara et al., 1987). Therefore, the 5'end of the synthetic transcripts similar to that of the authentic satellite is likely to be produced by cleavage at DNA-RNA hybrid portion with RNase H. In my previous publication (Masuta et al., 1987), I have already demonstrated successful use of RNase H treatment to generate infectious transcripts, analyzing the 5'termini of the transcripts from pIBI31-MC, RNase H-treated transcripts and progeny satellite. The progeny satellite RNA appeared to be coterminal with the authentic satellite RNA.

Although Kurath & Palukaitis (1987) suggested that the 57 non-viral bases at the 5'end of their satellite transcripts did

not inhibit replication and therefore, a natural 5'end was not essential for replication, our results imply the importance of a natural 5'end for the biological activity of the satellite RNA because after removal of 5'non-viral sequence with RNase H, the resulting transcripts became 10^3-10^4 fold more infectious than non-treated transcripts (Table 8). We think that because they used an extremely high concentration (200 μg/ml) of transcripts, the relatively high infectivity was obtained, masking deleterious effects of extra 5' bases (also considerable 5' leader sequence) on the infectivity.

In order to determine how many bases can be added to the 5' end of the satellite RNA without losing its infectivity, I analyzed the effects of more limited 5' additional sequence on transcript infectivity. Although I can not suggest the threshold number of extra 5' bases at which the biological activity of transcripts should be dramatically enhanced, if there is one, I conclude that extension from a natural 5' end should be minimized to maintain high infectivity of the satellite. Since I decided to focus on the specific activity of the transcripts with various lengths of extra 5' bases, I made little attempt to sequence the progeny satellite RNAs. However, some progeny satellite RNAs were shown to be coterminal at 5' end with natural satellite RNA by primer extension experiments. In general, any extra non-viral bases at 5' terminus considerably reduce infectivity of the satellite RNA, and as shown in Table 9, the extent of inhibition of satellite RNA infectivity was not necessarily proportional to length of the extra bases.

The fashion of site-directed cleavage with RNase H and

chimeric oligonucleotides containing deoxyribonucleotides and 2'-O-methylribonucleotides was investigated in detail by Shibahara et al. (1987). If RNA-DNA duplex contains more than 5 base-pairs, the enzyme appears to cleave the duplex region at the 3' end and mainly at the penultimate position. In my results shown in Fig. 26, some additional bands above the penultimate site were also observed on each lane. This might be due to incomplete digestion with RNase H. To control the numbers of 5' extra bases of transcripts from a viral cDNA, if cleavage at a strict site is not required, the RNase H method will be quite useful.

As shown in Fig. 27, the 5' terminal nucleotide sequence of the progeny satellite RNA derived from the transcript with 5' non-viral bases was found to be identical to that of natural satellite RNA, suggesting that the extra nucleotides at the 5' terminus of the transcripts were lost during the replication process. I do not know the exact mechanism (s) of the loss of terminal, non-viral sequences in the progeny satellite RNAs. Two routes can be considered: (1) the extra 5' bases were mechanically or enzymatically trimmed in the plant cells, resulting in the generation of the natural 5' end, and (2) the viral replicase could identify the initiation site for minus-strand synthesis although the 5' ends of the transcripts have some extra non-viral bases. My results showed that as the 5' non-viral sequence was trimmed down, the specific activity of the transcript was elevated. Both mechanisms seem to prefer a small number of additional 5' non-viral bases to produce the natural 5' end.

Inoculation of satellite RNA (strain Y) on tobacco plants along with CMV elicits unique bright yellow mosaic symptoms

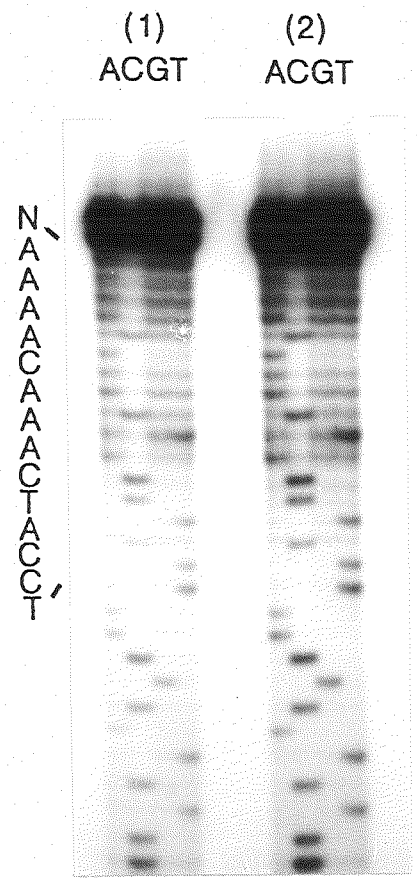

Fig. 27. Dideoxynucleotide sequence analysis of the 5' terminal region of progeny satellite RNA. RNA samples: (1) progeny satellite RNA isoleted from the tobacco plants inoculated with the transcript containing 24 extra 5' non-viral bases and CMV; (2) natural satellite RNA (strain Y). The first residue (N) could not be determined from the X-ray film, and the first four A bands are obscure in the photograph. However, these four A residues could be identified in the original X-ray film. Note that the sequence derived from the progeny satellite is identical to that from natural satellite RNA and that the sequence is presented complementary to satellite RNA.

(Takanami, 1981). Compared with the sequences of the other strains (Gordon and Symons, 1983; Avila-Rincon *et al.*, 1986), satellite RNA seems to have an unique domain around the region from nucleotides 100 to 200. In the revised sequence shown in Fig. 22, a long open reading frame (ORF), which starts at residue 151 and terminates at residue 333 with UAG, was revealed. It is of great interest to know whether the putative polypeptide from the ORF could be responsible for the yellow symptoms or not. My preliminary results using *in vitro* translation of the satellite RNA in a rabbit reticulocyte lysate system suggested that this ORF is not translated, so it may not be expressed *in vivo*.

II-3. Construction of transcription vectors for production of infectious transcripts

In this section, I describe the construction of new transcription vectors that are a tool indispensable for the studies based on infectious transcripts from viral cDNAs.

Using recombinant DNA technology, we can now manipulate viral genomes to analyze structural and functional features of plant virus RNAs. Several infectious transcripts from viral cDNAs were successfully synthesized in many laboratories. Since commercially available transcription vectors add at least several nucleotides to the 5' end of transcripts, resulting in considerable decrease in the infectivity of the transcripts, efforts to construct a transcription vector, which can synthesize transcripts with the 5' ends identical to the natural 5' ends, have been made over a few years. Transcripts from E. coli RNA polymerase transcription vector, pPM1 made by Ahlquist and Janda (1984), contain no 5' extension and seven 3' non-vial bases. SP6 RNA polymerase transcription vector made by Bujarski and Kaesberg (1987) can synthesize RNAs having two additional G residues at 5' end and produce a good yield of transcript. Although some cDNA-based experiments such as development of BMV RNA3 and TMV as RNA vectors (French et al., 1986; Takamatsu et al., 1987), and analysis of genomic structures of these viruses have been successfully conducted using pPM1, relatively low-transcription efficiency of the vector could not afford to use the commercially available E. coli RNA polymerase activity. Thus, Janda et al. (1987) reported

high efficiency T7 polymerase synthesis of infectious RNAs from cDNAs of BMV. Encouraged by the demonstration, we have successfully constructed cloning vectors, pUT118 and pUT118GG which contain T7 promoter consensus sequence upstream of the cloning sites (NsiI for pUT118, KpnI for pUT118GG). pUT118 can initiate transcription at the same 5' position as natural viral RNAs but unless the first two nucleotides are G's, the transcription efficiency was very low. Depending on the first two nucleotides of the cDNA insert, pUT118 might be replaced by pUT118GG from which transcripts containing two extra G's at 5' end can be synthesized.

Materials and Methods

Oligonucleotide synthesis. Oligonucleotides used for the construction of transcription vectors were synthesized as described in Chapter II-2.

Construction of pUT118 and pUT118GG. According to consensus promoter sequence for the RNA polymerase of phage T7 (Watson et al., 1987), a T7 promoter was synthesized by annealing two 31-oligonucleotides (Fig. 28). The T7 promoter contained a HindIII overhang at 5' end, a XbaI overhang at 3' end and a unique NsiI site at residue +4 relative to the transcription start site. The HindIII-XbaI fragment containing T7 promoter sequence was then subcloned between HindIII and XbaI site of pUC118 purchased from Takara Shuzo, Japan. The recombinant plasmid obtained was designated pUT118. pUT118GG was constructed from pUT118 as illustrated in Fig. 29 by deletion of NsiI-SmaI fragment. The 3'

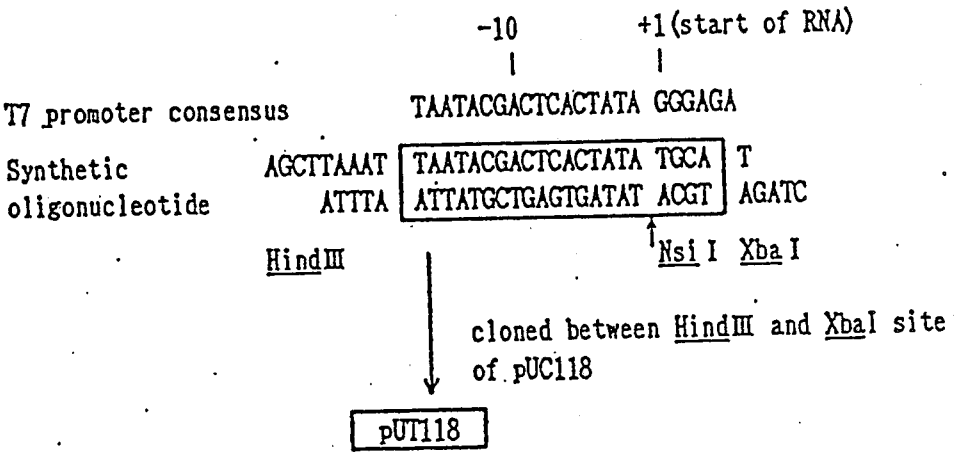

Fig. 28. Construction of pUT118. T7 promoter consensus was altered by creating a unique NsiI site (indicated by an arrow) at position +1 relative to the transcription initiation site.

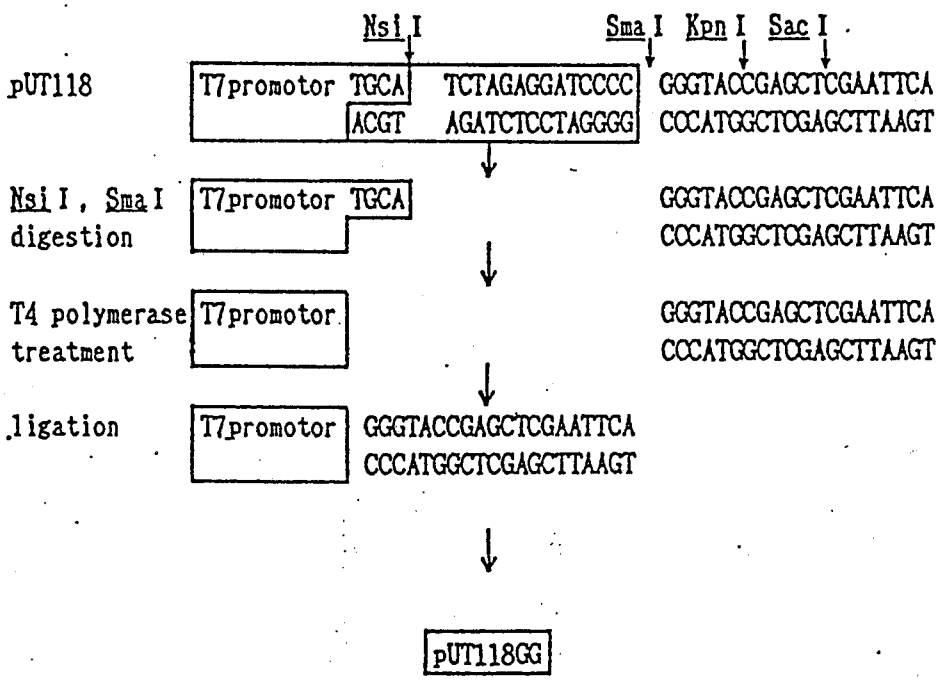

Fig. 29. Construction of pUT118GG from pUT118. The NsiI-SmaI fragment was deleted to recreate the T7 promoter consensus surrounding the transcription initiation site.

overhang after NsiI digestion was trimmed by T4 DNA polymerase, and self-ligation of the resultant plasmid created pUT118GG.

Synthesis of full-length cDNA of the satellite RNA. cDNA cloning was similar to that described by Masuta et al. (1987, 1988) and described in detail in Chapter II-2. The full-length double-stranded cDNA was ligated into pUT118 and pUT118GG, as described in Results creating pUT118-S and pUT118GG-S.

In vitro transcription. Transcription mixture (20 μl) contained 2-3 μg of Xba I-cut pUT118-S or Eco RI-cut pUT118GG-S, 40 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 2 mM spermidine-HCl, 10 mM NaCl, 10 mM DTT, 1 mM each of ATP, CTP, GTP, UTP, 20 units of RNasin and 10 units of T7 RNA polymerase (IBI) and was incubated at 37℃ for 1 hr.

Biological activity test. One and half month-old tobacco plants were inoculated with CMV-O (5 μg/ml) and transcripts from XbaI-cut pUT118-S or EcoRI-cut pUT118GG-S, and scored for yellow symptoms 2 weeks later.

Results

Construction of pUT118 and pUT118GG. The sequences flanking the transcription initiation sites in pUT118 and pUT118GG are shown in Fig. 28 and Fig. 29, respectively. The T7 promoter of pUT118 can initiate transcription at the first nucleotide of any DNA fragment inserted at a unique NsiI site whose 3' overhang had been degraded by T4 DNA polymerase before cloning. Since alteration of the promoter consensus sequence considerably reduced promoter activity as described below, we constructed pUT118GG

that can be used for efficient *in vitro* synthesis of transcripts with two extra G's, which satisfy the promoter consensus sequence.

Cloning of full-length cDNA of the satellite RNA into pUT118 and pUT118GG. cDNA of the satellite RNA was synthesized by using synthetic oligonucleotides to prime first- and second-strand. These primers provided cDNA exactly coterminal with the satellite RNA. The resultant double-stranded cDNA was then blunt-end ligated at the previous *Nsi*I site of pUT118 and *Kpn*I site of pUT118GG (Figs. 30, 31). After transformation into *E. coli* JM101 cells, transformants were selected on media containing ampicillin. Restriction analysis was conducted to check the orientation of cDNA inserts and to confirm the completeness of the nucleotide sequence.

Biological activity test on the in vitro transcripts. *Xba*I-cut pUT118-S and *Sac*I- or *Eco*RI-cut pUT118GG-S were used as templates for *in vitro* transcription. Only 10-20 ng of transcripts were obtained from 1 μg of *Xba*I-cut pUT118-S. As shown in Fig. 32, however, 1-2 μg of transcripts were synthesized from 1 μg of *Eco*RI-cut pUT118GG-S. Because of the low yield of transcripts, we could not verify the 5'-terminal sequence of the transcript from pUT118-S. 5' two additional nucleotides of the transcript from pUT118GG-S was confirmed by a primer extension experiment (Fig. 33). Since addition of a cap is not necessary for biological activity of the satellite (Collmer and Kaper, 1986; Kurath and Palukaitis, 1987), uncapped transcripts were used for biological activity tests summarized in Table 10. The symptom-inducing property of the transcripts

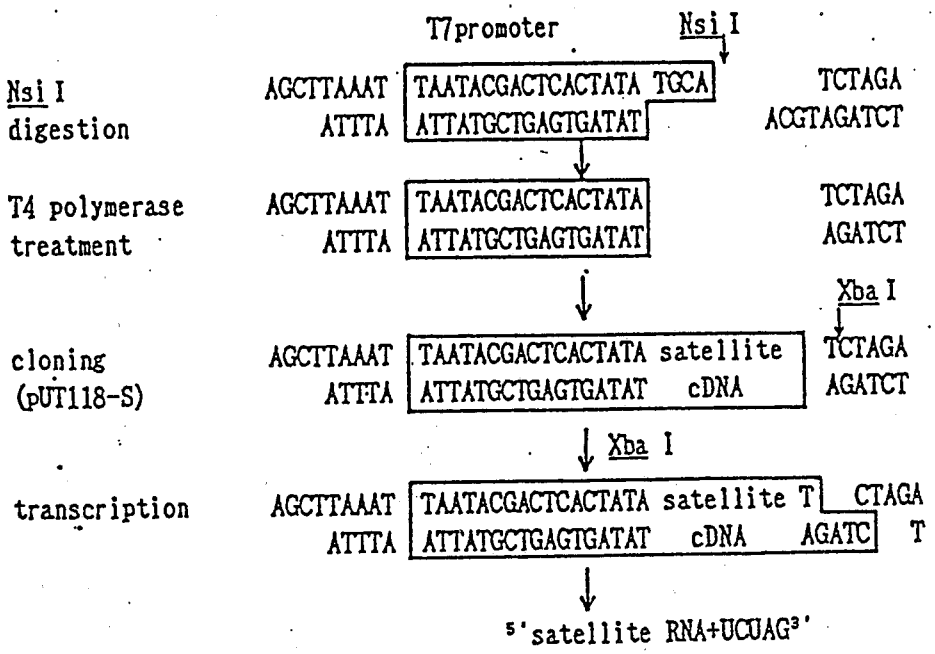

Fig. 30. Schematic diagram of process of transcription from a cDNA cloned in pUT118. Run-off transcripts from XbaI-cut pUT118-S contained no extra 5' nucleotide and five extra 3' nucleotides.

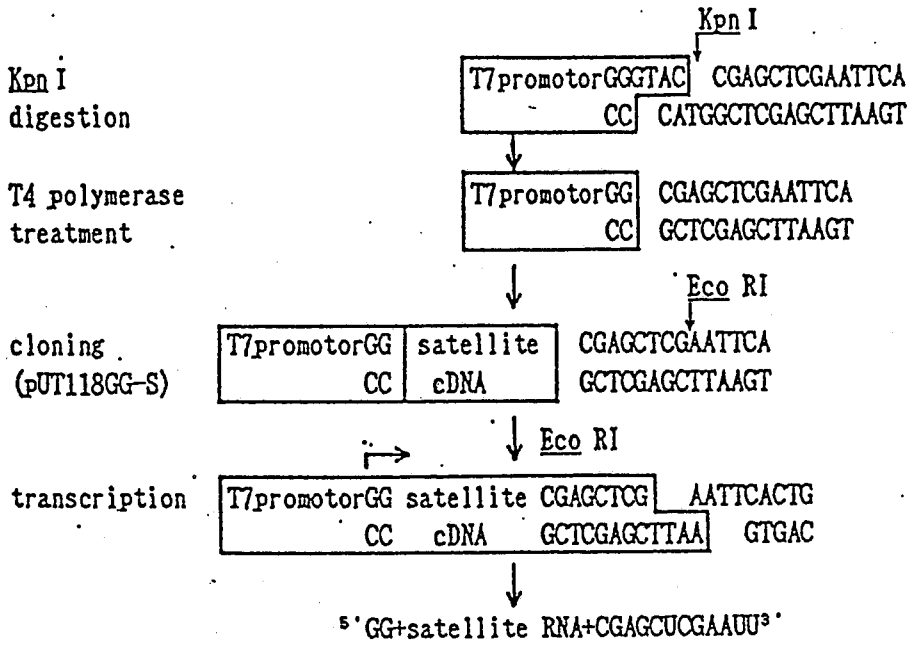

Fig. 31. Schematic diagram of process of transcription from a cDNA cloned in pUT118GG. Run-off transcripts from EcoRI-cut pUT118GG-S contained two extra nucleotides at 5' end and twelve extra nucleotides at 3' end. The transcription start site is indicated by the bent arrow.

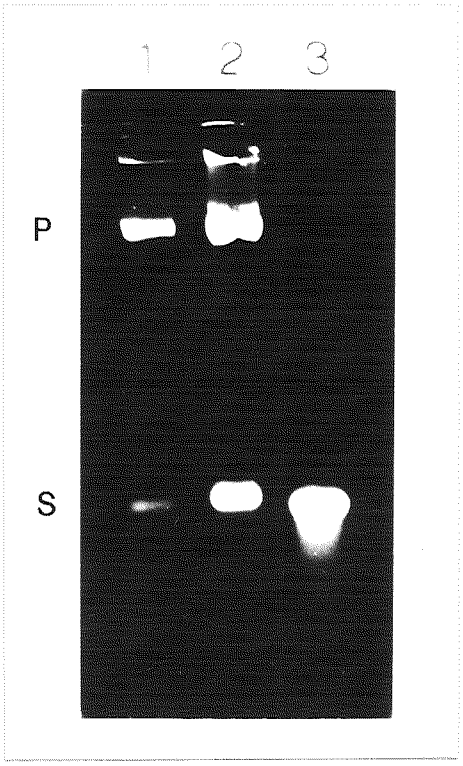

Fig. 32. Electrophoretic mobility of *in vitro* transcripts from *Eco* RI-cut pUT118GG-S. RNAs were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. Lane 1 and 2 contain 1/20 and 1/5 amount of the total transcripts, respectively. Lane 3 is the natural satellite RNA. P: template plasmid, S: satellite RNA.

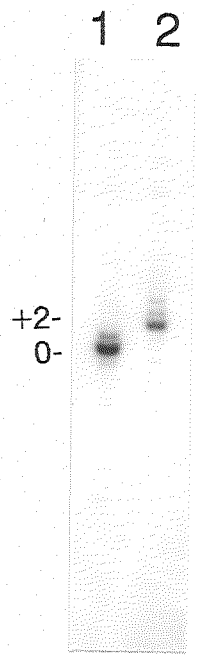

Fig. 33. Autoradiogram of a primer extention experiment of transcripts from pUT118GG-S. A 17-mer oligonucleotide primer, annealing to 100-116 bases from the 5' end of the satellite, was extended with reverse transcriptase. The primer extension experiment was performed in a 20 μl reaction mixture containing 50 mM Tris-HCl, pH 7.5, 75mM KCl, 3mM $MgCl_2$, 100 mM DTT, 250 μM each of dATP, dGTP, dTTP, 12.5 μM dCTP, 10 μCi of α-^{32}P-dCTP (3,000 Ci/mmol), 1 μg of annealed RNA and 200 units of M-MLV reverse transcriptase. The reaction mixture was incubated at 42 ℃ for 30 min, and the products were separated on a 5% polyacrylamide sequencing gel. Lane 1 is the natural satellite RNA and lane 2 is the transcripts with two extra 5' G residues.

Table 10. Biological activity tests[a] of transcripts from pUT118-S and pUT118GG-S

Plasmids or Satellite	Concentrations of inocula (μg/ml)	Plants showing yellow symptoms/Inoculated plants	
Xba I -cut	10^{-1}	7/8	4/4
pUT118-S[b]	10^{-2}	2/8	1/5
EcoR I -cut	10^{0}	3/4	3/3
pUT118GG-S[b]	10^{-1}	1/4	1/3
	10^{-2}	0/4	1/4
Natural satellite	10^{-2}	5/5	
	10^{-3}	5/5	3/3
	10^{-4}	4/5	1/3

[a]RNAs were coinoculated with CMV-O(5μg/ml) and the satellite-induced yelllow symptoms were judged 2 weeks after inoculation.

[b]Run-off transcripts synthesized from pUT118-S and pUT-118GG-S contained 5 and 12 3'non-viral bases, respectively.

suggests successful use of these plasmid vectors for production of biologically active transcripts.

Discussion

I have described the construction of transcription vectors, pUT118 and pUT118GG which were designed to facilitate synthesis of biologically active (or infectious) transcripts from viral cDNAs. Addition of extra 5' or 3' residues is known to reduce the infectivity of transcripts from cloned viral cDNAs (Dasmahapatra et al., 1986). Thus, Ahlquist and Janda (1984) previously showed production of infectious BMV RNAs, using pPM1 with the modified P_R promoter of λ DNA (designated P_M), which starts transcription at the first nucleotide of the inserted double-stranded cDNA. However, they suffered from relatively low yield of transcripts with the pPM1 system (sometimes 0.3 copies or less per plasmid), and so fusion of BMV cDNA to T7 promoter was tested for possible replacement of P_M with modified phage promoter (Janda et al., 1987). In contrast to P_R promoter, T7 promoter consensus sequence requires several bases (at least two 5' G's) beyond the initiation site. In the case of pUT118, I have perhaps broken constraints governing the promoter function by modifying the promoter sequence. In fact, the promoter activity was very low (10-20 ng/μg plasmid), suggesting that two more G's should be added to the 5'end of the transcripts. As I expected, pUT118GG could produce a much higher yield of transcript (1-2μg/μg plasmid) than did pUT118 or pPM1. If a full-length viral cDNA starts with G's, pUT118 will enable us to

easily conduct experiment depending on biologically active (or infectious) transcripts.

II-4. Expression of the satellite RNA in transgenic tobacco plants

In the last section, I will describe the production of transgenic tobacco plants with cDNA copies of satellite RNA (strain Y), expression of the satellite sequences in the transgenic plants and the genetic analysis of their progeny plants. Recently developed plant genetic engineering now enables us to produce transgenic plants containing virus-related nucleotide sequences in their chromosomal DNAs. For example, Abel et al. (1986) reported that the plants expressing TMV coat protein were delayed in disease development following inoculation with TMV. With another approach, Harrison et al. (1987) demonstrated that CMV replication and symptom development were greatly decreased in the transgenic plants producing a CMV satellite RNA. It is of great interest to transform tobacco plants with the sequence of CMV satellite RNA (strain Y) to elucidate the satellite-mediated disease modulation. The main objective in this experiment is to check if the satellite sequences expressed to a certain amount in plant cells can cause any yellow symptoms without CMV infection and to approach the mechanism(s) for the yellow symptom induction by the satellite RNA.

Materials and Methods

Cloning of satellite RNA (strain Y) before inserting into plant exression vector. Full-length cDNA of the satellite was

synthesized using synthetic oligonucleotide primers both for first and second strand synthesis as described by Masuta et al. (1988) and Chapter II-2. The full-length double-stranded cDNA was then inserted into the SmaI site of pUC 13 to create a recombinant plasmid pC3. We also constructed pT31-TC containing a head-to-tail concatemer satellite cDNA molecule in the same SmaI site of pUC 13.

Subcloning cDNA of the satellite into plant expression vector. The satellite cDNA was excised from pC3 as a BamHI-EcoRI fragment having 7 and 14 non-viral bases at 5' and 3' ends, respectively. The excised fragment was ligated between unique BamHI and EcoRI sites of plant expression vector pLGV 2382 (Herrera-Estrella et al., 1983). The resultant plasmid is referred to as pLGV-S1. To subclone a dimeric copy of the cDNA in a head-to-tail arrangement into pLGV 2382, pLGV-S2 was constructed from pLGV-S1 by replacing the BamHI fragment with a 369 bp BamHI fragment from pT31-TC because the satellite cDNA has a unique BamHI site at position 46. pLGV-S1 and pLGV-S2 were linearized at the HindIII sites and inserted into the HindIII sites of binary vectors pGA 492 and pGA 472 to give pTOK 136 and pTOK 101, respectively. pTOK 136 and pTOK 101 were introduced into Agrobacterium tumefaciens LBA4404 (Hoekema et al., 1983) by the freeze-thaw method described by An et al. (1988).

Transformation. Tobacco leaves were surface-sterilized, and 6-mm (in diameter) disks were prepared using a paper punch. The disks were incubated with approximately 10^8 cells of LBA4404 (pTOK 101) or LBA4404 (pTOK 136) in 2 ml of liquid medium that consisted of Linsmaier and Skoog (1965) salts and 30 g/l sucrose

(LS-R medium). After 48 hours of co-cultivation, the bacteria were washed off and the disks were tranferred to LS-S medium (LS-R medium plus 0.3 mg/l indole-3-acetic acid and 10 mg/l isopentenyl adenine) supplemented with 0.9% agar, 250 mg/l cefotaxim (Chugai Pharmaceutical Co., LTD), and 100 mg/l kanamycin. Root induction of drug-resistant regenerates was achieved on LS-R agar (0.9%) medium. The plants were then transferred to soil and grown in a greenhouse. Induction of calli from the explants of the transformants was performed on an agar (0.9%) medium that consisted of Linsmaier and Skoog salts, 30 g/l sucrose, 3 mg/l indole-3-acetic acid, 3 mg/l naphthaleneacetic acid, and 0.1 mg/l kinetin (LS-C medium). The medium used for germination of the seeds obtained from the transformants was the same as the medium used for induction of roots. When appropriate, 100 mg/l kanamycin was added to the media for germination and callus induction.

Isolation of DNA and Southern blot hybridization. DNA was isolated from leaf tissue frozen in liquid nitrogen and ground in a morter. The ground tissues were suspended in an extraction buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5 % SDS, 0.75 M NaCl and 0.25 % 2-mercaptoethanol, and then extracted with a 1:1 mixture of phenol and chloroform. DNA was precipitated by the addition of 1/2 vol. of 7.5 M ammonium acetate and 1 vol. of isopropanol. After centrifugation, the pelleted DNA was resuspended in TE buffer containing 10 mM Tris-HCl, pH 7.5 and 1 mM EDTA. DNA (10 μg) was digested with HindIII, separated on 1 % agarose gels, transferred to nitrocellulose, and hybridized with nick-translated pIBI31-MC probes. Southern blot hybridiza-

tion was carried out according to the methods described by Maniatis et al. (1982). Since the genome size of tobacco is 9.67 x 10^9 base pairs per nucleus, 10 μg of the DNA sample contain about one picogram of the dimeric copy (~1000 bases) of the cDNA of the satellite RNA. Ten picograms of the DNA fragment containing the dimeric cDNA copy equivalent to 10 copies in a transgenic plant was used as a control.

Northern blot hybridization. Total RNA was extracted from leaves and analyzed by electrophoresis on a 1.5% agarose gel containing 6% formaldehyde as described by Gerlach et al. (1988). The RNA was transferred onto Gene Screen Plus membrane (DUPONT) and hybridized with randomly primed probes (Boehringer Mannheim). Hybridization and washing conditions were according to the protocols supplied by the manufacturers.

Inoculation of CMV. The transgenic plants and their self-fertilized progeny were analyzed for the ability to produce biologically active satellite RNA. Leaves were dusted with carborundum and inoculated with purified CMV-O (10 μg/ml), followed by rinsing the leaves with water. Symptom development in the individual plants was observed for two weeks. Total RNA from the infected leaf tissue was analyzed on a 1.5% agarose gel under non-denaturing conditions. To identify the satellite bands, RNAs from gel pieces excised were inoculated on tobacco together with CMV-O and tested for yellow symptom induction.

Results

Construction of plant expression vectors containing the satellite cDNA copies and transformation. Monomeric and dimeric copies of the cDNA of the satellite RNA (strain Y) were connected to the regulatory sequences of the nopaline synthase gene (Herrera-Estrella et al., 1983), and the gene cassettes were then inserted into Agrobacterium-based binary vectors, as described in "Materials and Methods". The resultant plasmids were designated as pTOK 101 and pTOK 136 (Fig. 34). Tobacco leaf disks were transformed with A. tumefaciens that carried pTOK 101 or pTOK 136 by using the kanamycin-resistance gene in the vectors as the selection marker, and kanamycin-resistant plants were regenerated and grown in a greenhouse. Before further analyses, explants from those plants were tested for the ability to form calli on the medium containing kanamycin. We have obtained 18 independent transgenic plants containing the dimeric cDNA copies of the satellite RNA (TS-1～TS-10 and BS-1～BS-8) and 10 independent transgenic plants containing the monomeric cDNA copies (CS-1～CS-10). All of the transformants were morphologically indistinguishable from non-transgenic plants and had no yellow symptom that is induced by the combination of CMV and satellite RNA (strain Y) although those plants were expected to have small amount of transcribed satellite RNA.

Detection of the satellite sequences. The Southern blot hybridization tests showed that the nuclear DNA of the transformed tobacco plant (TS-1) contained 1-2 copies of the satellite

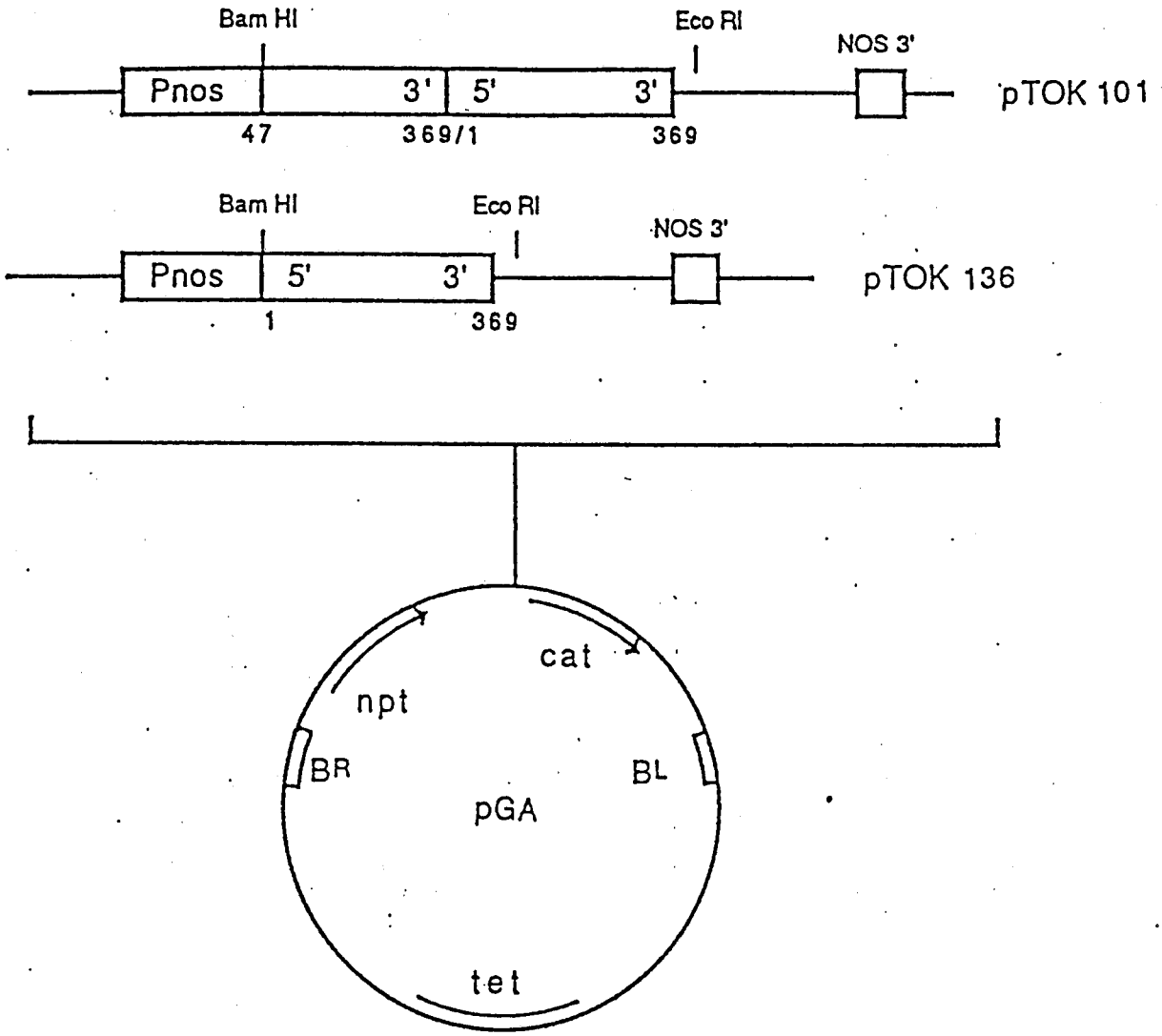

Fig. 34. Schematic representation of the gene expression cassettes containing the satellite cDNA copies. The promoter and the terminator are the 5' upstream sequence and the polyadenylation signal of 3' end sequence of the nopaline synthase (nos) gene, respectively. In the plasmid pTOK 101, the 323 nucleotides satellite sequence extended from position 47 to the 3' end (position 369) was immediately ligated to the 5' end of a complete satellite unit. Transcripts derived from the gene cassette should contain 32 nucleotides (NT) at the 5' ends and ~550 NT at the 3' ends. The expected sizes of RNA transcripts from pTOK101 and pTOK136 are about 1.5 kb and 1.0 kb, respectively. npt: kanamycin resistance in plant cells, cat: chloramphenicol acetyltransferase gene in pGA 492; pGA 472 does not contain the cat gene, B_R and B_L:T-DNA right and left borders, respectively, tet: tetracycline resistance in E. coli cells.

cDNA. The Northern blot hybridization tests revealed the presence of the transcripts from cDNA of the satellite in the transgenic plants. As shown in Fig. 35, transcripts of ~1.0 (from a monomeric copy) and ~1.5 kb (from a dimeric copy), which are the expected sizes for the mRNAs predicted from the original plasmid construction (Fig. 34), were clearly identified in the transformed plants.

Infection of transgenic tobacco plants with CMV. After post-inoculation with CMV, the transgenic plants showed the satellite-inducing yellow symptoms (Fig. 36, Table 11), resulting from the production of large amounts of unit-length satellite RNA, which was detected by electrophoresis (Fig. 37). This is consistent with replication of the satellite sequences supported by CMV. The infected plants also showed CMV disease attenuation; appearance of abnormally-shaped leaves was suppressed.

Inheritance of the production of biologically active satellite RNA. The S_1 progeny from a transgenic plant TS-1, which contains the dimeric copy of the satellite RNA, were assayed for the kanamycin resistance and the expression of biologically active satellite RNA when inoculated with CMV. It turned out that the production of the satellite RNA was tightly linked to the kanamycin resistance in the S_1 progeny. However, as shown in Table 12, the segregation ratios of those traits were not 3:1 (+:-).

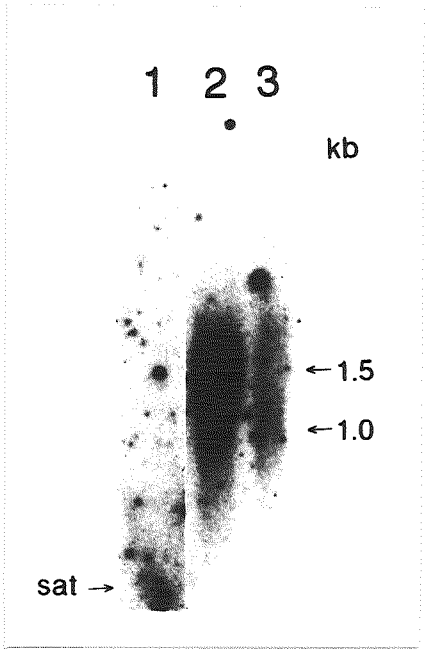

Fig. 35. Northern blot hybridizations from transgenic tobacco plants. Total RNA isolated from leaves of transgenic plants was separated on a 1.5% agarose gel containing 6% formaldehyde, blotted onto Gene Screen Plus and hybridized with randomly primed probes. Lane 1, a non-transgenic plant; lane 2, the plant transformed with pTOK 101; lane 3, the plants transformed with pTOK 136. Although the band with 1.0 kb in lane 3 is vague in this picture, a faint band was resolved in the original X-ray film.

Fig. 36. Symptoms on the transgenic plants inoculated with CMV. Note the bright yellow symptoms on the transgenic plant BS-1 (right) compared to a healthy non-transgenic plant (left). When a non-transgenic plant is infected with CMV, it does not show yellow symptoms but green mosaic. Photographs were taken 7 days after inoculation.

Table 11. Expression[a] of CMV satellite RNA (strain Y) in the transgenic tobacco plants

Transgenic plants	Arrangement of cDNA copies	No. of plants expressing satellite/No. of transformants
TS	tandem	10/10
BS	tandem	8/8
CS	single	10/10

[a] Biological activity of the transcripts from cDNA copies of the satellite integrated into tobacco plants was judged by appearance of yellow symptoms 14 days after inoculation with CMV-O. Tobacco varieties of the transgenic plants are Tsukuba 1 (TS), Bright Yellow 4 (BS) and Consolation 402 (CS).

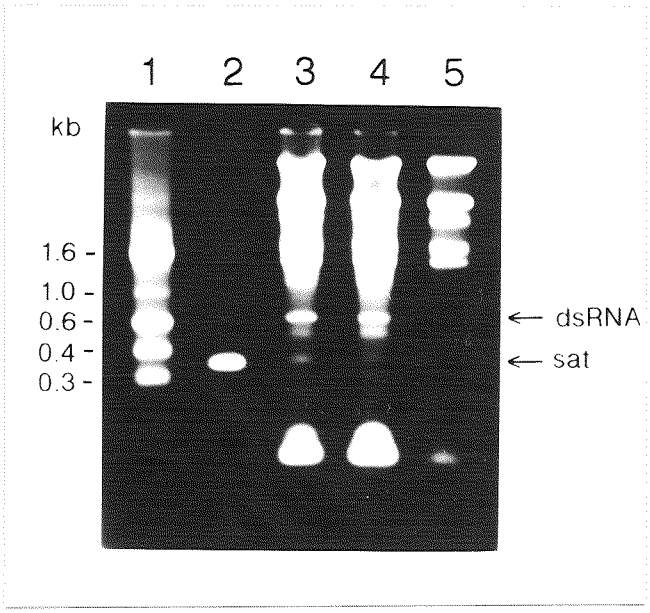

Fig. 37. Electrophoretic mobility of the progeny satellite RNAs produced in the transgenic tobacco plants after inoculation with CMV. Lane 1 is marker RNA species. Lane 2 is authentic satellite RNA (369 NT). Lane 3, 4 and 5 contain the total RNAs from the tobacco plants CS-1, TS-1 and non-transgenic plant 10 days after inoculation with CMV, respectively. dsRNA: double-stranded RNA of the satellite, sat: single stranded satellite RNA. RNAs were electrophoresed in a 2% agarose gel under non-denaturing conditions.

Table 12. Genetic analysis of the S_1 progeny of the transgenic tobacco plant (TS-1)

Phenotype	Number of seedlings (+/−)[a]	χ^2 (3:1)
Kanamycine resistance	53/37	12.46[b]
Satellite expression	61/37	8.50[b]

[a] +/−: No. of the plants that express the phenotype/ No. of the the plants that do not express the phenotype.
[b] Significant at the 0.01 probability level.

Discussion

To determine the molecular basis of disease modulation (especially yellow symptoms) by satellite RNA (strain Y), I have transformed tobacco plants with cDNA copies of the satellite RNA. Previously, Baulcombe et al. (1986) successfully produced transgenic tobacco plants producing biologically active satellite RNA, which was originally isolated from CMV strain $I_{17}N$ and different from our strain. They used cDNA copies of 1.3 and 2.3 units of the satellite RNA in a head-to-tail arrangement downstream of the promoter to mimic multimeric satellite RNAs which they believe as replication intermediates. However, there is no reason why CMV satellite RNA can not replicate through simple double-stranded (ds) RNA mechanism. In fact, a large amount of dsRNA of unit-length satellite RNA accumulated in tobacco protoplasts or plants inoculated with CMV and its satellite (Takanami et al., 1977; Habili and Kaper, 1981). Thus, I constructed a plasmid pTOK 136 having a single cDNA copy of the satellite and transformed tobacco plants. The resultant transformant (CS) was able to produce biologically active satellite RNA when post-inoculated with CMV. Since there was no delay of the yellow symptom appearance compared to the transformed plants containing concatemer satellite cDNA copies, we have concluded that there was no difference in the ability to produce biologically active transcripts between the monomeric and the dimeric constructs.

All the transgenic plants obtained had neither discrete symptoms nor abnormalities. As those plants have always expressed transcribed satellite RNA, we first expected some leaf-

color alteration mimicking yellow symptoms induced by satellite RNA (strain Y). However, only when the transformed plants were inoculated with CMV, the yellow symptoms appeared. Therefore, the yellow symptoms by the satellite are attributed to amplification of the satellite sequence by CMV infection and/or more complex interaction among CMV, satellite RNA and the host plants.

The cDNA copies of the satellite RNA in a transgenic plant were transmitted to the progeny, indicating that the DNA sequences were maintained in the tobacco genome. Usually, foreign DNA sequences introduced to plants by transformation behave as single mendelian genes. In this case, however, the segregation ratios of the cDNA copies and the kanamycin resistance were not the expected values of 3:1. The reason is not clear from the current data and whether this phenomenon is associated with the satellite RNA sequences remains to be elucidated.

SUMMARY AND CONCLUSION

In this thesis, I describe the properties of panicum mosaic virus (PMV) and its satellite (SPMV), and the nucleotide sequence analysis of the genome of SPMV in Chapter I, and the studies based on the cDNAs of cucumber mosaic virus (CMV) satellite RNA (strain Y) in Chapter II. The results in Chapter I reveal that SPMV differs from the other satellite viruses including satellite tobacco necrosis virus (STNV) in many aspects. For example, although SPMV is similar to the other satellite viruses in particle shape and in particle size, it contains the smallest genome and coat protein subunit among the four known satellite viruses. On the other hand, the coat proteins and the RNAs of PMV and tobacco necrosis virus (TNV) are difficult to separate on gels. In contrast with STNV and many other satellite agents, SPMV enhances the severity of symptoms induced by its helper PMV. No serological relationship exists between SPMV and the other satellite viruses or between PMV and other helper viruses (Gingery and Lorrie, 1985; Valverde and Dodds, 1986b). Nucleic acid hybridization tests indicate that there is no appreciable homology between STNV RNA and SPMV RNA (Valverde and Dodds, 1986b). In addition, comparison of the nucleotide sequence of SPMV RNA and STNV RNA by computer analysis also reveals that they do not share any conserved sequence, suggesting that there is no significant homology between the two RNAs. In spite of those differences, there is little doubt that SPMV

shares a unique property, satellitism, i.e.: the ability to be replicated by a helper virus, with the other three satellite viruses. Two interesting questions arise: How have satellite viruses evolved and what makes them satellite viruses? This answer will be provided only by nucleotide sequence comparison of the genome of the satellite viruses and their helpers.

Several common features are observed between the primary structure of SPMV RNA and STNV RNA. Like STNV RNA, neither a 7-methyl guanosine cap structure nor a genome-linked protein (VPg) is found at the 5' end of SPMV RNA, and the 3' terminus is not polyadenylated. The 5' non-coding region of SPMV and STNV RNAs have a Shine-Dalgarno sequence (Stryer, 1981), which is a ribosomal binding site in prokaryotic mRNAs. Moreover, as observed in STNV RNA, the 3' terminus of SPMV RNA has a high degree of secondary structure or perhaps tertiary structure whose role may be important for the replication and encapsidation of the RNA. Nevertheless, there are some noteworthy differences between the two genomes of the two satellites. First, SPMV has an 88 nucleotide long 5' non-coding region (leader sequence) while the leader sequence of STNV is relatively short (29 residues). Secondly, the amino acid sequence of SPMV has no homology with STNV in spite of the fact that the hydropathy patterns of the coat proteins of STNV and SPMV are somewhat similar at amino- and carboxy-termini. In order to elucidate the role of the helper viruses, an analysis of the genome organization of the helper RNAs must be conducted. This will enable a better understanding of the relationship between the satellite and its helper. In addition, this kind of research will also

enable a more general understanding of the mechanisms of virus replication and encapsidation.

Other than the coat protein genes, potentially translatable open reading frames exist both in SPMV RNA and STNV RNA. Although STNV RNA is believed to be a monocistronic messenger, there is no direct evidence to determine whether or not the other possible open reading frames are expressed. Whether those potential polypeptides occur *in vivo* and have an important role in establishing the relationship between the satellite and its helper is one of the main objectives for subsequent research. An analysis of fusion proteins synthesized in an expression vector will enable us to induce antibodies against the chimeric proteins, and then to judge the viral-specific products *in vivo*.

In addition to satisfying intrinsic interest in basic biology of satellite viruses, practical use of a satellite agent in agriculture is now within our reach. For example, it was suggested that cDNA clones of a satellite RNA (CARNA 5) can be utilized to attenuate the symptoms induced by CMV (Baulcombe *et al.*, 1986). In addition, recent advances in genetic engineering should enable us to genetically manipulate cDNA clones of SPMV RNA, and use it to develop a system for understanding genetics of the virus. In order to achieve this, full-length cDNA clones must first be constructed and then, using a transcription vector such as pPM 1 (Ahlquist *et al.*, 1984b), infectious transcripts must be generated from the cDNA clones of SPMV RNA.

The results shown in Chapter II were mainly obtained from the experiments based on cDNA clones of CMV satellite RNA (Y strain). In order to manipulate the nucleotide sequence and

specify the domains responsible for replication, encapsidation and symptom induction, I first established *in vitro* transcription system by which biologically active transcripts were synthesized from a cDNA clone of the satellite. Since *in vitro* transcription products from the recombinant plasmid containing the satellite cDNA habored 24 non-viral bases at their 5' ends and had very low infectivity when coinoculated with CMV. After removal of the 5' extra sequence of the transcripts with RNase H, the infectivity of the transcripts increased markedly. The analysis of more limited 5' additional sequence confirmed importance of natural 5' ends for biological activity of the satellite. The progeny satellite RNA did not retain the 24 non-viral bases at the 5' end.

Meanwhile, I constructed a novel transcription vector pUT118, which starts transcription to be directed to the natural 5' end of viral cDNA insert, to facilitate the production of biologically active transcripts. However, due to the modified T7 promoter in pUT118, the transcription efficiency of the vector was very low. Therefore, pUT118GG was also created from pUT118 to add two extra G residues to the 5' end of the transcripts, allowing high efficiency of transcription initiation of the cDNA insert.

After *in vitro* trancription systems for production of infectious transcripts were set up, I proceeded to the studies of symptom modulation by the satellite. The *in vitro* transcripts from a cDNA clone of the satellite were capable of inducing not only bright yellow mosaic on tobacco but also lethal necrosis on tomato in combination with CMV-Y. The results agree with the ob-

servation by Takanami (1981) although Kaper et al. (1986) denied the lethal necrosis on tomato by the satellite RNA.

To approach the mechanism(s) for the yellow symptom induction by the satellite, monomeric and dimeric copies of cDNA of the satellite RNA were connected to a promoter that can function in plants. Tobacco plants transformed with either type of the constructs did not show any symptoms or abnormalities, but produced biologically active satellite RNA when inoculated with CMV. Transcribed satellite RNA sequences were confirmed to be of the expected sizes by the Northern blot hybridization tests. Inheritance of the satellite RNA sequences was analyzed in the progeny.

In this thesis, I dealt with two kinds of satellite agent, SPMV and CMV satellite RNA (Y strain). While they share many apparent properties as satellites, striking homology is also revealed at molecular level. CMV satellite RNAs sequenced so far have a 3'-terminal octanucleotide (5'-$CAGGACCC_{OH}$) which is identical to seven of the last eight nucleotides (5'-$UAGGACCC_{OH}$) of SPMV. Although the significance of the region is obscure and may be due to random occurrence, I would like to point out the possibility of some relevant function in satellite replication.

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Plate I. Symptoms on tomato plants, photographed about one month after inoculation.

1. Rutgers tomato infected with CMV-Y and satellite RNA (Y strain). Note the lethal necrosis on the plants inoculated with the natural satellite (S) or transcripts (T).
2. Best of All tomato infected with CMV-O and satellite RNA (Y stain).

Abbreviations: H, healthy plant; V, inoculated with CMV; S, inoculated with CMV and natural satellite RNA; T, inoculated with CMV and transcript RNA.

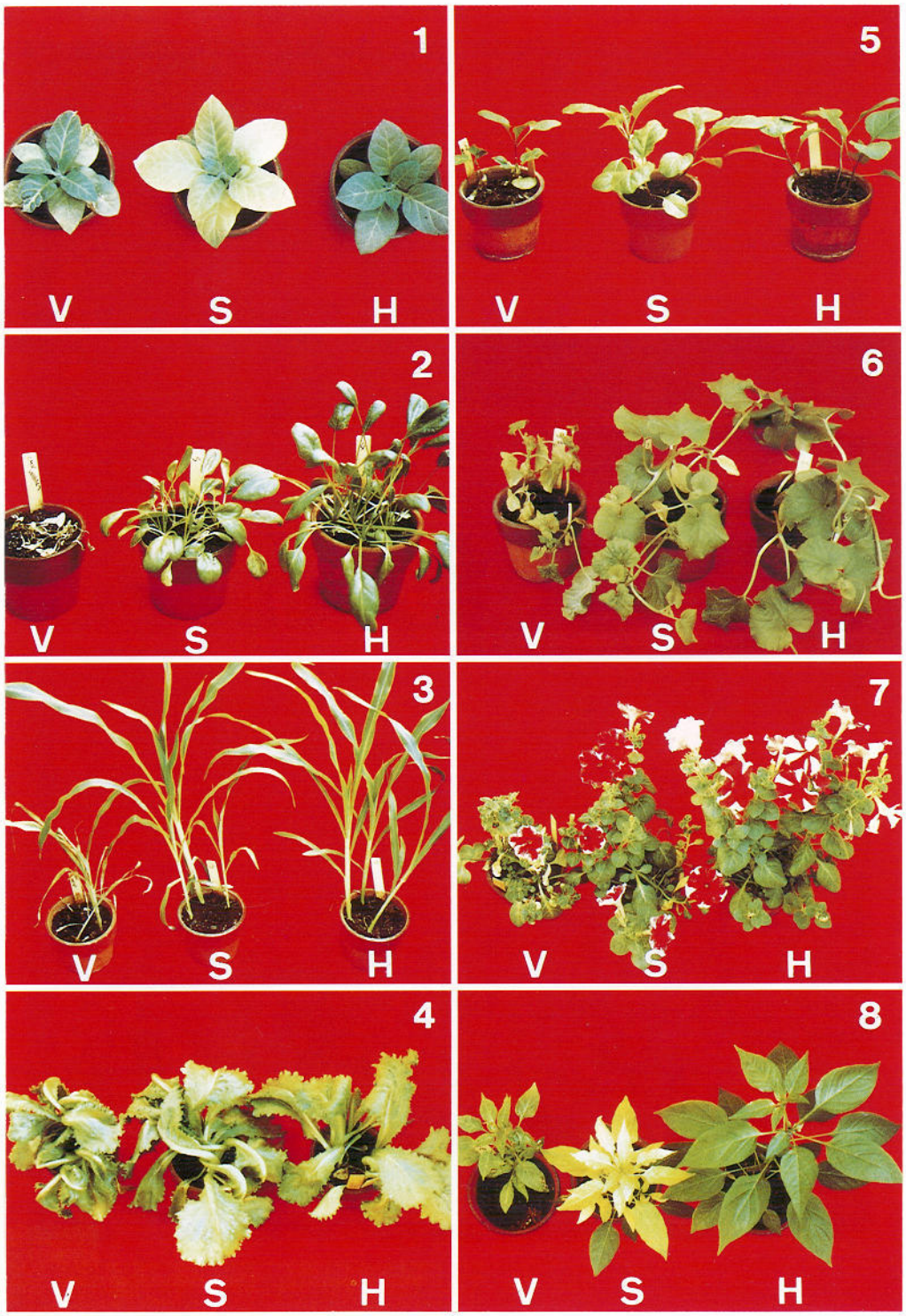

Plate II. Symptoms on several plants infected with CMV-O and satellite RNA (Y strain), photographed about one month after inoculation.

1. Tobacco (N. tabacum L. cv. Xanthi nc)
2. Spinach (Spinacia oleracea L.)
3. Maize (Zea mays L.)
4. Lettuce (Lactuca sativa L.)
5. Eggplant (Solanum melongena L.)
6. Melon (Cucumis melo L.)
7. Petunia (Petunia hybrida Viln.)
8. Red pepper (Capsicum annuum L.)