Formation of multimers of cucumber mosaic virus satellite RNA

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Double-stranded RNA multimers of cucumber mosaic virus (CMV) satellite RNA were detected in CMV-infected plants. RT–PCR showed that plus-sense and minus-sense monomers and plus-sense multimers of satellite RNA were present. Multimeric minus-sense RNA was not present except in the form of multimeric dsRNA. Sequence analysis of 52 cloned junction regions in head-to-tail repeats of unit-length satellite RNA indicated that about 35% of the junction sequences were precise fusions of monomer units, 56% lacked sequence of the 5’ component, and 10% lacked sequence of both 3’ and 5’ components. No junction contained additional nucleotides. Deletions at the junction regions may have accumulated during CMV multiplication in inoculated plants. These data suggest that replicase is not released from the template during synthesis of multimeric molecules of satellite RNA.

Introduction

Cucumber mosaic cucumovirus (CMV) is a small spherical plant virus with a tripartite, single-stranded (ss) RNA genome. The two larger segments, RNA 1 and RNA 2, encode replication-related proteins, and RNA 3 encodes movement and coat proteins. RNA 4 is a subgenomic RNA generated from RNA 3, and is the messenger RNA for the virus coat protein. In addition, several isolates contain a fifth RNA component, referred to as a satellite RNA, which depends on a helper virus for its replication but is not required for the replication of the virus. Like other plant virus satellites (Mayo et al., 1995), the satellite RNAs of CMV have little sequence homology with the helper virus genomic RNAs (Palukaitis et al., 1992; Roossinck et al., 1992).

The replication of some satellite RNAs is thought to occur by a rolling circle mechanism (Kiefer et al., 1982; Forster & Symons, 1987; Mayo et al., 1995). Although multimeric forms of satellite RNA of CMV, peanut stunt cucumovirus (PSV), turnip crinkle carmovirus (TCV) and cymbidium ringspot tombusvirus (CyRSV) have been detected, the mechanisms of replication of these satellites are unclear (Roossinck et al., 1992). Recently, Carpenter et al. (1991) proposed that multimers of TCV linear subviral RNAs are not formed by a rolling circle mechanism, but are generated by a replicase-driven copy-choice mechanism (Cascone et al., 1990). In the case of CyRSV, concatemeric forms of satellite RNAs were present in infected plants (Burgyan & Russo, 1988). However, Dalmay & Rubino (1994) suggested that satellite RNAs of longer than unit-length were constituted by aggregates of double-stranded (ds) monomers because they could not detect ss satellite RNA oligomers. Multimeric forms of CMV satellite RNAs have also been detected in CMV-infected tissue (Roosinck et al., 1992; Wang et al., 1990) but circular forms were not found. Since large quantities of CMV satellite dsRNA often accumulate, it has been suggested that the multimeric forms may be produced by the autocatalytic reaction of dsRNA during satellite RNA replication (Linthorst & Kaper, 1984; Roosinck et al., 1992).

In this paper, we show that the multimers of ds and plus-sense of ss CMV satellite RNAs are present in infected plants, but that the minus-sense of the ss form is absent. The presence, origin and role of multimers of CMV satellite RNA are discussed.

Methods

- **Virus propagation and purification.** CMV-UD13 (originally containing UD13 satellite RNA) was isolated from a fodder turnip (*Brassica campestris*) and the virus with or without satellite RNA was propagated in *Nicotiana tabacum* (cv. KY-57) or *N. glutinosa*. Virus particles were purified from inoculated tobacco plants as described by Francki et al., 1979).

- **RNA extraction, electrophoresis and Northern blot hybridization.** Encapsidated ssRNA was isolated from purified virus particles by SDS–phenol–chloroform extraction and collected by ethanol precipitation (Peden & Symons, 1973). DsRNAs were purified from CMV-infected plant tissue essentially as described by Dodds & Bar-Joseph...
**Construction of infectious cDNA clones.** Infectious cDNA clones of monomer and dimer UD13 satellite RNA were created to investigate the biological activities of the satellite RNA dimer. The EcoRI–HindIII fragment of pBI121 (Clontech), containing the cauliflower mosaic virus (CaMV) 3SS RNA promoter and nopaline synthase polyadenylation signal sequence, were cloned into pUC18 to create pUC35S. The cDNAs downstream of the CaMV 35S RNA promoter were transcribed in *in vivo* and infection was achieved (see review by Boyer & Haenni, 1994). Small–Xbal-cut pUC18 was ligated with the RT–PCR product of the full-length cDNA of UD13 satellite RNA (treated with Xbal and T4 polynucleotide kinase) to create plasmid pD13sat. This plasmid was digested with Xbal and Saci, and then the fragment was inserted between the Xbal–Saci sites of pUC35S, downstream of the CaMV 35S RNA promoter, to create pD13–35S.

pD13sat and the RT–PCR product of the junction sequence of a complete 3'–terminal region joined to the exact 5' end were used to create pD13dim containing a complete dimer of the cDNA of UD13 satellite RNA. The Xbal–Saci fragment of pD13dim was ligated into pUC35S to make pD13d-35S.

**Results**

**Detection of the multimeric forms of CMV satellite RNA**

DsRNA extracted from CMV-UD13-infected tobacco plants and analysed by 5% PAGE formed five major bands which corresponded to RNA 1, RNA 2, RNA 3, RNA 4 and satellite RNA (Fig. 2a). Several minor bands were also detected between dsRNA 4 and the ds satellite RNA. In Northern blot hybridization of dsRNAs, these minor bands and unit-length satellite RNA were also detected in total RNA extracts, multimeric forms of minus-sense ss RNA were not detected (Fig. 2b).

Several tests were carried out to determine whether the multimers of ds satellite RNA could have arisen as an artifact of phenol–chloroform extraction. DsRNAs from CMV-UD13-infected tobacco plants were purified by binding to a CF-11 cellulose column followed by one, two or no phenol–chloroform extractions. The number of phenol–chloroform extractions did not affect the electrophoretic migration of multimeric forms of ds satellite RNA. Although the multimeric forms of plus-sense ss satellite RNA were also detected in total RNA extracts, multimeric forms of minus-sense ss RNA were not detected (Fig. 2b).

**PCR amplification of the junction region between monomers**

Total RNA samples were extracted from *N. glutinosa* plants 1 week after inoculation, and mixed with primer 1, 2, 3 or 4 for...
Multimers of CMV satellite RNA

Fig. 2. Northern blot hybridization of dsRNA (a) and total RNA (b). (a) DsRNA extracted from CMV-UD13-infected plants was electrophoresed in 5% polyacrylamide gel, stained with silver (lane A), or transferred to a nylon membrane which was subjected to hybridization with photobiotin-labelled ds cDNA of UD13 satellite RNA probe (lane B). Arrows indicate the bands longer than monomer satellite RNA. The positions of the CMV RNAs (1 to 4) and satellite RNA (5) are marked on the left-hand side. (b) Total RNA samples, prepared from N. glutinosa 2 weeks after inoculation, were electrophoresed in formaldehyde–1.2% agarose gel and transferred to nylon membranes. The 32P-labelled DNA probes specific to the minus (lane 1) or plus (lane 2) strand of satellite RNA were used. Positions of the monomer and oligomers of satellite RNA \( \times 1, \times 2, \times 4 \) are indicated to the right of lane 2.

Fig. 3. Electrophoresis of dsRNAs extracted from CMV-UD13-infected tobacco plant by different procedures. DsRNAs were purified on a CF-11 cellulose column followed by one cycle of phenol–chloroform extraction (lane 1), two cycles of phenol–chloroform extraction (lane 2) or no phenol–chloroform extraction (lane 3). The positions of the CMV genomic or subgenomic RNAs (1–4) and satellite RNA (5) are marked on the right-hand side. Arrows indicate the position of bands longer than the ds satellite RNA monomer. Lane M, dsRNA of rice dwarf virus genomic RNA.

cDNA synthesis. Primers 1 and 3 were used to synthesize cDNA from the minus-strand ssRNA of UD13 satellite RNA, while primers 2 and 4 were used to synthesize cDNA from the plus strand (Fig. 1). The first-strand cDNA preparations were subjected to PCR to amplify cDNA of unit-length satellite RNA or to amplify cDNA of the junction region between the monomers using primers 1 and 4, or 2 and 3, respectively. These RT–PCR products were subjected to 5% PAGE (Fig. 4) and the plus and minus-strand ssRNAs of UD13 satellite RNA were detected as a 382 nt DNA band (Fig. 4, lanes 1 and 2). The plus-strand junction region was detected (Fig. 4, lane 4), and the PCR products were about 180, 220 and 450 nt long. However, the 450 bp band arose as a heteroduplex between the 180 and 220 bp products. If satellite RNA 3' and 5' ends were joined together, the PCR products would be 224 nt long. These results suggested that the plus-strand multimer existed in plants. The minus-strands of the junction region were detected by RT–PCR using the heat-denatured purified dsRNA (Fig. 4, lane 5), but not when the PCR template was obtained from total RNA samples which were not denatured (Fig. 4, lane 3). These results indicate that in CMV-infected plants there are free plus/minus ssRNA and dsRNA of unit-length satellite RNA, multimeric forms of plus ssRNA and dsRNA, but no multimeric form of minus ssRNA. Furthermore, using RNA samples extracted from purified CMV-UD13 preparations, only unit-length plus-strand satellite RNA was detected by RT–PCR, suggesting that the plus-stranded multimers and the unit-length minus-strand of satellite RNA may not be encapsidated in CMV particles.

Sequence analysis of the junction region between monomers

In addition to the 224 bp DNA which was expected from the satellite RNA sequence, a DNA band of about 180 bp was
detected by RT–PCR (Fig. 4, lane 4). To investigate the sequence of the junction region non-denatured total RNA, extracted from systemic leaves of *N. glutinosa* 1 week after inoculation, was subjected to RT–PCR using primers 2 and 3. The PCR products were cloned into Smal-cut pUC18 and 52 clones were sequenced (Fig. 5). One-third of the junctions of satellite RNA multimers had a complete 3′-terminal -GTTTT- sequence joined to the exact 5′ end of UD13 satellite RNA monomers. Numbers indicate the number of independent isolates/the number of total clones sequenced. Numbers in the ‘Plus’ and ‘Minus’ columns indicate the number of sequenced clones of PCR products derived from free plus-sense ss satellite RNA multimers (Plus) or minus-strands of dsRNA multimers (Minus), respectively. Only the 220 bp PCR product of the minus-strands of dsRNA multimers (Fig. 4, lane 5) was cloned and sequenced.

Fig. 5. Schematic representation and sequence analysis of the junction between monomers of UD13 satellite RNA. The top sequence represents the complete 3′-terminal region joined to the exact 5′ end of UD13 satellite RNA monomers. Numbers indicate the number of independent isolates/the number of total clones sequenced. Numbers in the ‘Plus’ and ‘Minus’ columns indicate the number of sequenced clones of PCR products derived from free plus-sense ss satellite RNA multimers (Plus) or minus-strands of dsRNA multimers (Minus), respectively. Only the 220 bp PCR product of the minus-strands of dsRNA multimers (Fig. 4, lane 5) was cloned and sequenced.

**Effect of time after inoculation on the formation of deletions in the junction region**

Samples were taken at different times after CMV inoculation and amplified using RT–PCR to detect multimeric forms of satellite RNA. Total RNA samples were extracted from inoculated leaves at 3 days and 5 days post-inoculation and from systemically infected leaves 7 days post-inoculation. Only the 220 bp DNA band was detected 3 days after inoculation but the 180 bp DNA was detected, in addition to the 220 bp DNA, 5 or 7 days after inoculation (Fig. 6).

Inoculation of infectious cDNA clones of artificial satellite RNA dimer

To investigate the biological activity of the satellite RNA multimers, we constructed infectious cDNA clones of unit-length satellite RNA (pD13–35S) and tandemly joined unit-length satellite RNA (pD13d–35S). Five µg of pD13–35S or pD13d–35S was co-inoculated with purified CMV-UD13 (without satellite RNA as a helper) onto *N. glutinosa*. Two weeks post-inoculation, dsRNA was extracted and electrophoresed. Unit-length ds satellite RNA and ds multimers were detected and the band pattern of multimeric forms was the same as that of dsRNA purified from tissues infected with CMV-UD13 containing natural UD13 satellite RNA.

The *in vivo* transcribed satellite RNA dimer had biological activity, indicating that satellite RNA multimers could act as templates for the generation of monomeric units.

**Discussion**

Concatemeric forms of plant virus satellite RNAs have been observed in a number of systems (Roossinck *et al*., 1992). The ‘D-type’ satellite of nepovirus and the satellite of sobemovirus, like viroids, produce multimeric forms during replication by a rolling circle mechanism (Kiefer *et al*., 1982; Branch & Robertson, 1984; Forster & Symons, 1987; Mayo *et al*., 1995), suggesting that multimers are intermediates. Multimers of linear satellite RNAs have also been found in plants infected with cucumoviruses (CMV, PSV), TCV and CyRSV (Burgyan & Russo, 1988; Carpenter *et al*., 1991; Linthorst & Kaper, 1984, 1985), but no circular form of satellite RNA has been detected (Linthorst & Kaper, 1984; Burgyan & Russo, 1988; Carpenter *et al*., 1991). DsRNAs of multimeric forms of CMV satellite RNA in infected plants have been reported (Roossinck *et al*., 1992; Wang *et al*., 1990), and are thought to be related to self-cleavage and self-ligation of the dsRNA dimeric and monomeric forms (Roossinck *et al*., 1992). In the case of CyRSV satellite RNA, the putative junction region of oligomers was not amplified by RT–PCR, indicating that ds satellite RNA oligomers were aggregates (Dalmay & Rubino, 1994). In our research, the junction region of multimeric ssRNA of plus-sense CMV satellite RNA was detected by RT–PCR, but
terminal sequence of the UD13 satellite RNA joined to the

In sequencing the 220 bp DNA PCR product a complete 3’

dsRNA samples were used for cDNA synthesis (Fig. 4. lane 5). On the other

hand, the PCR products of the minus-strand junction of the

satellite RNA multimer were detected only when denatured

in plants plus- and minus-sense in vitro transcripts of strict

monomer satellite RNA flanked by extensive 5’ and 3’

sequence when coinoculated with satellite-free CMV RNA. In

our results in vitro transcripts of dimer satellite RNA were also

biologically active to generate monomer satellite RNA. However, the role of multimers of CMV satellite RNA has not

been clear in CMV replication. Furthermore, the mechanism of

CMV satellite RNA replication and the role of multimer

formation must be characterized.

The authors gratefully acknowledge Dr M. A. Mayo for critically

reading this manuscript.

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Fig. 6. RT–PCR analysis of the junction region of the plus-strand multimer of UD13 satellite RNA. Total RNA samples used for cDNA synthesis with

primer 2 were extracted from inoculated leaves at 3 days (lane 3) and at 5 days (lane 2) post-inoculation and from systemic leaves at 7 days (lane 1) post-inoculation, and amplified with primers 2 and 3. Pointers on the

left-hand side indicate the position of junction PCR products. Lane M, 1 kb

ladder (BRL) with band sizes indicated on the right-hand side.

minus-sense was not (Fig. 4), indicating that the multimers

were not formed by aggregation.

From sequencing the clones of PCR products derived from

free plus-sense ss satellite RNA multimers, one-third of the

clones were found to have a perfect 3’ end joined to the exact

5’ terminus, and the deletions of nucleotides were found at the

5’ end in two-thirds of the clones (Fig. 5). The ratio of the

perfect end:deletion junction sequence was thought to depend

on the time after inoculation (Fig. 6). Our results suggest that

multimers can be formed during synthesis of plus-strand

molecules, as described for the formation of TCV satellite RNA

(Carpenter et al., 1991). This model proposed that multimers

are formed by the reinitiation of replication by replicase before

release of the nascent strand (Carpenter et al., 1991). In the

model, plus-strand synthesis is thought to be terminated before full-length synthesis due to an unknown factor, because

almost all sequenced PCR products of the junction region had

deletions of 3’-terminal nucleotides. In our results, few clones

(5/52) had a deletion in the 3’-terminal region (Fig. 5). Recently, junctions of the defective interfering (DI) RNA of

CyRSV and cucumber necrosis tombusvirus (CNV) were shown to have nucleotide deletions in the 5’-rather than in the

3’-terminal region (Dalmay et al., 1995; Finnen & Rochon, 1995), suggesting that the mechanism of formation of CMV

satellite RNA oligomers may be similar to that of the DI s of

CyRSV and CNV rather than TCV satellites. On the other

hand, the PCR products of the minus-strand junction of the

satellite RNA multimer were detected only when denatured dsRNA samples were used for cDNA synthesis (Fig. 4. lane 5).

In sequencing the 220 bp DNA PCR product a complete 3’-

terminal sequence of the UD13 satellite RNA joined to the

exact 5’-terminal sequence was found (Fig. 5). Collmer & Kaper

(1985) showed that an additional unpaired guanosine is present

at the 3’ end of the minus-strand of the ds form of CMV

satellite RNAs. Our results suggest that the ds satellite RNA multimer is not generated as aggregates of the unit-length ds

satellite RNA.

It is known that monomer satellite RNA molecules can be

generated from plants transformed with cDNA of dimer satellite RNA because inoculation of plants with satellite-free

CMV led to the appearance of both dimer and monomer molecules (Baulcombe et al., 1986). In addition, Tousch et al.

(1994) have shown that CMV satellite RNA can be replicated

in plants from plus- and minus-sense in vitro transcripts of strict

monomer satellite RNA because inoculation of plants with satellite-free CMV RNA. In

our results in vitro transcripts of dimer satellite RNA were also

biologically active to generate monomer satellite RNA. However, the role of multimers of CMV satellite RNA has not

been clear in CMV replication. Furthermore, the mechanism of

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The authors gratefully acknowledge Dr M. A. Mayo for critically

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Received 6 June 1996; Accepted 26 November 1996