Studies on the Replication of a Satellite RNA Associated with Cymbidium Ringspot Virus

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SUMMARY

The replication of cymbidium ringspot tomato virus satellite RNA was investigated in Nicotiana clevelandii plants. The temperature at which host plants were grown strongly influenced the synthesis of the satellite RNA; high temperatures were detrimental to, or even inhibited, its multiplication. The most favourable temperature range was 16 to 25 °C. The termini of the satellite RNA were studied and a free hydroxyl group was found at the 3' but not at the 5' end, which may explain why circular forms were not detected in infected tissue. On the other hand, multimers of double-stranded RNA were present, which may have arisen independently of the formation of circular molecules.

Recent studies on cymbidium ringspot virus (CyRSV), a member of the Tombusvirus group (Matthews, 1982), have partially elucidated its molecular biology with special reference to the strategy of expression (Burgyan et al., 1986; Russo et al., 1988). CyRSV particles encapsidate a satellite-like 0.7 kb RNA, which depends on the virus genome for replication and decreases the severity of symptoms induced in infected plants by the helper virus (Gallitelli & Hull, 1985). The modulation of symptom expression may not be due to a specific protein encoded by the satellite RNA since no messenger activity could be detected in vivo or in vitro (Burgyan et al., 1986).

The aim of the present work was primarily to establish the presence in infected tissue of structures of satellite RNA related to its replication. Preliminary experiments were also carried out to define conditions under which satellite RNA multiplied consistently because whereas isolates of CyRSV without satellite RNA remained satellite-free after repeated passages in Nicotiana clevelandii, occasionally there was no production of satellite RNA using an inoculum that in other trials had induced the formation of considerable amounts in infected plants.

Isolates of CyRSV containing or lacking satellite RNA were propagated in N. clevelandii. Virus purification and extraction of RNA from virus particles and infected tissue were done as described (Gallitelli et al., 1985; Russo et al., 1988). Plants were inoculated with purified virus preparations (25 µg/ml in 50 mM-sodium acetate buffer pH 5.5) that contained (Fig. 1 a, lane 1) or did not contain (lane 2) satellite RNA. Ten days after inoculation, total RNA from 200 mg of systemically infected leaf tissue was suspended in 10 µl TE buffer (10 mM-Tris-HCl pH 7.5, 1 mM-EDTA) and 5 µl was loaded onto a 1.2% agarose gel. After electrophoresis in 90 mM-Tris, 90 mM-boric acid, 10 mM-EDTA, satellite RNA was readily detectable in RNA extracts from infected plants (Fig. 1 a, lanes 3 and 4) and these types of extracts were used throughout to assess replication of satellite RNA.

Test plants were grown in a greenhouse or growth chamber. In the greenhouse, from August to October temperatures measured during 10 day periods ranged from 28 to 38 °C, 26 to 36 °C, 18 to 35 °C and 16 to 25 °C. The temperature of the growth chamber was constant at 24 to 26 °C with a 16 h light period (4000 lux).
Fig. 1. Agarose gel electrophoresis and ethidium bromide staining of virus inocula and RNA extracts. (a) RNA composition of the two virus inocula (lane 1, satellite-containing virus; lane 2, satellite-free virus) used in the experiment shown in (b). Lanes 3 and 4 are total RNA extracts from plants inoculated with virus shown in lanes 1 and 2, respectively. G, genomic RNA; sg1 and sg2, subgenomic RNAs; S, satellite RNA. (b) RNA extracted from plants inoculated with satellite-free (lanes 1, 3, 7, 9 and 10) or satellite-containing (lanes 2, 4, 5, 6, 8 and 11) CyRSV and grown under different temperature conditions. Minimum and maximum registered conditions were as follows: lanes 1 and 2, 28 to 38 °C; lanes 3 and 4, 26 to 36 °C; lane 5, as lane 4, kept for additional 10 days at 18 to 35 °C; lanes 6 and 7, 24 to 26 °C; lanes 8 and 9, 18 to 35 °C; lanes 10 and 11, 16 to 25 °C. G and S indicate respectively the position of genomic and satellite RNAs. X is a virus-associated RNA which will be described elsewhere.

As shown in Fig. 1 (b), high temperature conditions with a minimum not below 26 °C did not allow detectable replication of satellite RNA (lanes 2 and 4). However, when the minimum temperature decreased to 18 °C, satellite RNA became detectable (lane 8), notwithstanding the high temperature of the day (35 °C). More satellite RNA was formed when the minimum temperature during the day decreased to 25 °C and the minimum was 16 °C (lane 11). Plants grown at 26 to 36 °C, which apparently did not yield any satellite RNA, produced it in high quantities when grown for an additional 10-day period at 18 to 35 °C (lane 5). It seems that the high temperature conditions under which no satellite RNA could be recovered from infected hosts, rather than eliminating satellite RNA from the inoculum, inhibited its multiplication. Conditions in the growth chamber were not as suitable for multiplication of satellite RNA as those in the greenhouse with a temperature shift from 16 to 25 °C (lane 6). The latter result indicates that satellite RNA multiplication is favoured both by low temperature conditions and marked fluctuations.

The 4 M-LiCl-insoluble fraction (Diaz-Ruiz & Kaper, 1978) of material extracted from plants infected with satellite-containing CyRSV contained double-stranded RNA species other than those related to genomic and subgenomic RNAs (Fig. 2a, lanes 2 and 3). The major species had estimated sizes of 1.4 and 2.8 kbp and are likely to be related to satellite RNA having the size of monomeric and dimeric forms of this RNA. Below the band representing monomeric satellite, there were other bands, the nature of which, i.e. whether they represented degradation products or a true heterogeneity of satellite RNA, is unknown.

After denaturation with formaldehyde and formamide and electrophoresis in 1:2% agarose gels cast in MOPS buffer (20 mM-MOPS, 5 mM-sodium acetate, 1 mM-EDTA) containing 2:2 m-formaldehyde (Maniatis et al., 1982) the putative dsRNA was blotted to nylon membranes (Amersham), and hybridized with two different radioactive probes. One was cDNA of encapsidated satellite RNA, prepared by random priming with sonicated salmon sperm DNA (Taylor et al., 1976), and the other, of the same polarity as encapsidated RNA, was prepared by partial cleavage of RNA in alkaline buffer (50 mM-Tris–HCl pH 9.2) for 20 min at 90 °C
Fig. 2. (a) PAGE (8% polyacrylamide) and ethidium bromide staining of 4 M-LiCl-insoluble material from plants infected with CyRSV minus satellite (lane 3), plus satellite (lane 2), and cucumber mosaic cucumovirus (lane 1). G, genomic RNA; sg1 and sg2, subgenomic RNAs; SM, satellite monomer; SD, satellite dimer RNA. (b) Northern blot analysis of 4 M-LiCl-insoluble material from plant tissue infected with CyRSV plus satellite, and hybridized to cDNA from satellite RNA (lane 1) or to 32P-labelled satellite RNA (lane 2). Molecular sizes (kb) are shown alongside.

and labelling at the 5' ends using T4 polynucleotide kinase (Amersham) and [γ-32P]ATP (3000 Ci/mmol; Amersham). RNAs of the size of monomer and dimer (and occasionally trimer) satellite RNA hybridized with both probes (Fig. 2b), showing that the double-stranded satellite RNA was composed of positive- and negative-sense species.

When total RNA extracts or 4 M-LiCl-insoluble material from plants inoculated with satellite-containing CyRSV were examined for the presence of circular forms by using the two-dimensional electrophoresis method of Schumacher et al. (1983), no such molecules were detected (not shown). Since gels were only stained with silver nitrate, and not blotted, circular RNA forms may have escaped detection. Therefore, the structure of the termini of encapsidated satellite RNA molecules were examined in order to see whether they were compatible with circularization. Labelling at the 3' end was performed by the addition of [5'-32P]pCp (Amersham; 3000 Ci/mmol) in the presence of T4 RNA ligase (Bethesda Research Laboratories, BRL) essentially as described by England & Uhlenbeck (1978). About 30% of the molecules were labelled, as determined by the amount of incorporated radioactivity. These molecules migrated at the position of intact satellite RNA molecules in denaturing sequencing gel electrophoresis (not shown; the position of satellite RNA was identified by staining with ethidium bromide).

The presence of free 3' hydroxyl groups was also tested for polyadenylation. This was done as described by Carrington & Morris (1984) with poly(A) polymerase (BRL) and [3H]ATP (38 Ci/mmol; Amersham). Incorporation increased from 3% after 5 min to 25% after 20 min. Although it was not possible to determine the number of RNA molecules that were polyadenylated the experiment does indicate the absence of a block at the 3' end. Conversely, no phosphorylation was detected when satellite RNA, with or without prior treatment with alkaline phosphatase (Boehringer; used according to the supplier), was treated with T4 polynucleotide kinase and [γ-32P]ATP, as described by Chaconas & Van De Sande (1980). These
results were interpreted as an indication that there is no phosphate or free hydroxyl group at the 5′ end, but that this terminus is protected.

The termini of CyRSV satellite RNA thus lack the structure required for circularization (i.e. 2',3'-cyclic phosphate at the 3′ end) (Konarska et al., 1981), which is present in plant virus satellites or viroids that have a circular form in their replication cycle (Kikuchi et al., 1982; Kiberstis et al., 1985; Buzayan et al., 1986). The inability to detect circular forms of CyRSV satellite RNA may therefore reflect the true situation. If this is the case the replication of CyRSV satellite RNA would not proceed through a rolling circle mode (Branch & Robertson, 1984), but rather according to a linear virus-like mechanism as for satellite RNAs associated with cucumoviruses (Linthorst & Kaper, 1984; Collmer & Kaper, 1985). The question remains how multimeric RNAs are formed and what role they play in the replication process in the absence of a rolling circle mode. Linthorst & Kaper (1984) have suggested that the occurrence of multimers of cucumovirus satellites is an incidental event during replication, but they do not exclude the possibility that oligomerization may occur at each replication cycle. The present results do not clarify this problem; however, they again suggest that oligomers of replicative structures of a satellite RNA may be present without having been derived from a circular molecule.

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REFERENCES


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