Satellite-like Properties of Small Circular RNA Molecules in Particles of Lucerne Transient Streak Virus

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SUMMARY

The isometric particles of lucerne transient streak virus (LTSV) contain linear ssRNA of mol. wt. approx. 1.4 x 10^6 (RNA-1) and ssRNA of mol. wt. approx. 1.2 x 10^5 in both a linear and circular form (RNA-2). Unfractionated LTSV RNA induced necrotic local lesions in leaves of Chenopodium amaranticolor whereas RNA-1, partially separated from RNA-2 by gel electrophoresis, induced many chlorotic local lesions but few necrotic ones. Cultures obtained from either lesion type continued to induce only chlorotic (C isolate) or necrotic lesions (N isolate) on subsequent passage. Apart from the lesion type in Chenopodium species, the isolates were indistinguishable except that particles of isolate N contained both RNA-1 and RNA-2 whereas those of isolate C contained RNA-1 but no RNA-2; RNA-2 was also not detected in leaves inoculated with isolate C. RNA-2 alone did not infect C. amaranticolor but when it was added to inocula of RNA from isolate C, a proportion of the lesions induced were necrotic and this proportion increased with increasing concentration of RNA-2. The infectivity of RNA-1 was destroyed by treatment with snake venom phosphodiesterase or proteinase K but the ability of RNA-2 to alter the lesion type induced by isolate C was unaffected by these treatments, suggesting that the molecules of RNA-2 are biologically functional and do not need a genome-linked protein for this activity. These results suggest that RNA-2 found in particles of LTSV is not part of the virus genome but may be a satellite RNA that affects symptom expression.

INTRODUCTION

Particles of four Australian plant viruses, velvet tobacco mottle (VTMoV) (Randles et al., 1981), Solanum nodiflorum mottle (SNMV) (Gould & Hatta, 1981), subterranean clover mottle and lucerne transient streak (LTSV) (Tien-Po et al., 1981) have been found to contain linear single-stranded RNA of about 1.4 x 10^6 mol. wt. (RNA-1), together with RNA molecules similar to viroids in size, shape and some physicochemical properties. Evidence was obtained that the small circular RNA (RNA-2) of VTMoV and of SNMV is essential for infectivity (Gould et al., 1981; Tien-Po et al., 1981).

In this paper, we confirm that small circular RNA molecules occur in LTSV particles but show that the RNA-1 of LTSV can infect plants on its own and induce the synthesis of virus particles. Our evidence therefore suggests that, unlike the RNA-2 of VTMoV and of SNMV, LTSV RNA-2 may be a satellite RNA.

METHODS

Propagation and purification of LTSV. The New Zealand isolate of lucerne transient streak virus (LTSV-NZ; Forster & Jones, 1979) was propagated in Nicotiana clevelandii under licence from the Department of Agriculture and Fisheries for Scotland. Virus particles were purified from systemically infected leaf tissue essentially as described by Forster & Jones (1979).

Extraction of RNA from virus particles. Purified virus particles were suspended in 0.01 M-Tris-HCl pH 7, 0.05 M-NaCl and mixed vigorously with 0.1 vol. 10% SDS and 1 vol. phenol + m-cresol (9:1, v/v) containing 0.1% 8-hydroxyquinoline. Following a further treatment of the aqueous phase with the phenol mixture, RNA was precipitated with 2.5 vol. ethanol.
Extraction of RNA from leaves. Two methods were used. In the first, leaves were ground in buffer containing SDS and the RNA then extracted by phenol treatment as described above for extracting RNA from virus particles. In the second method, which was used when only low molecular weight RNA was required, leaves were extracted by a modification of the method of Pfannenstiel et al. (1980). Leaves (1 to 3 g) were ground with 2 ml 0.2 M-glycine, 0.1 M-Na2HPO4, 0.6 M-NaCl, 1 % SDS, pH 9.5 containing 1 or 2 drops of thioglycerol. The mixture was extracted with 2 ml phenol mixture (as above) plus 2 ml chloroform : n-pentanol (25:1, v/v), and after a second such extraction, the aqueous phase was mixed with 0.25 vol. 10 M-LiCl. After about 18 h at 4 °C, the insoluble material was removed by centrifugation and the supernatant fluid mixed with 2.5 vol. ethanol to precipitate the RNA.

Gel electrophoresis of RNA. Two methods were used. In the first, samples of about 25 μg RNA were dissolved in 5 % sucrose in 36 mM-Tris, 30 mM-Na2HPO4, 10 mM-EDTA, pH 7.8, containing 0.2 % SDS and analysed by electrophoresis in 2.8 % polyacrylamide gel using the sample buffer without sucrose. In the second, leaf RNA that was soluble in 2 M-LiCl was analysed in 5 % polyacrylamide gel as described by Pfannenstiel et al. (1980) using 40 mM-Tris-acetate, 20 mM-sodium acetate, 1 mM-EDTA, pH 7.2. All samples were suspended in the electrophoresis buffer containing 5 % sucrose and bromophenol blue and xylene cyanol as dye markers, and were centrifuged to remove undissolved solids before use.

Extraction of RNA from acrylamide gels. Bands of RNA were located in cylindrical gels by u.v.-densitometry, excised and extracted with 10 mM-sodium acetate, 1 mM-magnesium acetate, pH 5.5, as described by Harrison et al. (1972).

Treatment of RNA with snake venom phosphodiesterase (SVPDE). Samples of 50 μg RNA were dissolved in 83 μl 10 mM-glycine, 3 mM-MgCl2, pH 9 and mixed either with 17 μl SVPDE (1 mg/ml in 50 % glycerol at 1.5 U/mg; Boehringer) or with 17 μl water. After 60 min at 25 °C, the reaction was stopped by adding 0.9 ml glycine buffer, 0.1 ml 10 % SDS and 1 ml phenol mixture. After shaking and centrifuging the mixture, RNA was precipitated from the aqueous phase by adding 2.5 vol. ethanol.

Treatment of RNA with proteinase K. Samples of 50 μg RNA were mixed with 1 ml 0.5 % SDS, 0.1 M-NaCl, 0.015 M-sodium citrate containing 0.2 mg/ml proteinase K (Boehringer) as described by Harrison & Barker (1978). After overnight incubation at 37 °C, RNA was recovered by phenol treatment and precipitation from the aqueous phase with ethanol.

Infectivity assays. All test plants were grown in an aphid-proof glasshouse at 18 to 25 °C. Assays of virus nucleoprotein and RNA preparations were made in Chenopodium amaranticolor and C. quinoa leaves. RNA inocula, suspended in 0.01 M-Tris–HCl + 0.015 M-NaCl, pH 7.6, containing 0.6 mg washed bentonite per ml, were inoculated with muslin pads. Each inoculum was rubbed on three or four leaves of test plants previously dusted with corundum. Inoculated leaves were then washed with running tap water. Unless otherwise stated, plants were kept after inoculation in controlled environment cabinets at 25 °C with a 16 h photoperiod and light intensity of 5000 lux.

Serological tests. Double-diffusion precipitin tests were done in 0.75 % agarose gels containing 0.85 % NaCl and 0.02 % sodium azide. LTSV antiserum (homologous titre 1/512) prepared in New Zealand (Forster & Jones, 1979; Tien-Po et al., 1981). RNA was dissolved in about 100 μl water and mixed with 0.4 vol. 0.1 M-sodium phosphate pH 7, 2 vol. dimethyl sulphoxide and 0.6 vol. deionized formamide. Following treatment at 50 °C for 1 h, samples were diluted in 50 % formamide in 0.2 M-Tris–HCl, 0.02 M-EDTA, pH 8.5. After adding cytochrome c (20 μg/ml), samples were spread on a hypophase of 20 % formamide, mounted on pyroxylin-filmed grids and stained for 15 s in 0.05 M-uranyl acetate, 0.05 M-HCl in 90 % ethanol. The grids were then rotary-shadowed at an angle of 8° with Pt, stabilized with carbon, and examined in a Philips EM301G electron microscope.

RESULTS

LTSV RNA species

RNA obtained by phenol–SDS extraction of purified LTSV particles was very infective to C. amaranticolor and C. quinoa. When non-denatured RNA was analysed by electrophoresis in polyacrylamide gel or sedimentation in sucrose density gradients, it was shown to contain two major components (RNA-1, RNA-2) corresponding to those reported previously (Forster & Jones, 1979; Tien-Po et al., 1981). Similarly, when denatured RNA was electrophoresed as described by Gould (1981) and Tien-Po et al. (1981), it was separated into three main bands. The two fastest migrating bands presumably corresponded to the linear and circular forms of RNA-2 (Tien-Po et al., 1981). In the electron microscope such denatured RNA preparations were found to contain three principal types of molecule: linear molecules of mol. wt. approx. 1.4 × 106.
Satellite-like circular RNA of LTSV

Fig. 1. Electron micrograph of LTSV-N RNA denatured in glyoxal. Arrows indicate circular molecules. Bar marker represents 0.1 μm.

(presumably RNA-1) and molecules with a mean length corresponding to a mol. wt. of about 0.12 × 10⁶, some linear and some circular (presumably RNA-2) (Fig. 1). In polyacrylamide gels suitable for detecting potato spindle tuber viroid, RNA-2 from LTSV migrated at a rate very similar to xylene cyanol, a marker dye that co-migrates with potato spindle tuber viroid (Pfannenstiel et al., 1980).

Infectivity of LTSV RNA-1 and RNA-2

Each of the two RNA species of LTSV was extracted from polyacrylamide gels after electrophoresis and inoculated to leaves of C. amaranticolor. Only the extracts containing RNA-1 induced lesions. Some of these lesions were small discrete necrotic spots like those induced by the stock LTSV culture, but many were larger, chlorotic, and without a well-defined margin. Diluting the RNA-1 inoculum increased the proportion of chlorotic lesions produced.

When sap from single chlorotic lesions was inoculated to further C. amaranticolor plants, the leaves developed chlorotic lesions only, whereas sap from single necrotic lesions again induced necrotic lesions. A single-lesion culture of each type, referred to as C (chlorotic) and N (necrotic), was selected for further study.

Properties and RNA composition of LTSV cultures C and N

Sap from C. quinoa and N. clevelandii leaves infected either with culture C or with culture N contained LTSV particles which reacted in double-diffusion serological tests with antiserum to the stock culture of LTSV. The precipitin lines produced by the C and N cultures fused without producing a spur. The cultures were indistinguishable in host range and symptomatology from the stock culture except that, in C. amaranticolor and C. quinoa, culture C induced only faint chlorotic local lesions or no symptoms, depending on the environmental conditions, whereas the stock culture and culture N induced discrete necrotic local lesions in both these species under most conditions tested (Fig. 2). However, in the controlled environment cabinets used for infectivity assays, culture C always produced chlorotic lesions and culture N induced necrotic ones.

When nucleic acid extracts of C. amaranticolor leaves infected with each culture were analysed by electrophoresis in 5% polyacrylamide gel, RNA of mol. wt. similar to RNA-2 was detected in samples from leaves inoculated with the N culture but not in those from leaves inoculated with the C culture. Similarly, nucleic acid extracted from purified LTSV particles of culture N contained RNA-2 whereas that from particles of culture C did not (Fig. 3). These results show that RNA-2 is not necessary for the replication of RNA-1 or the production of virus particles. They also suggest that RNA-2 affects the type of lesion produced, and this was studied in more detail.
Fig. 2. Lesions induced in inoculated leaves of *Chenopodium amaranticolor* by cultures of LTSV containing RNA-2 (LTSV-N; left) or lacking RNA-2 (LTSV-C; right).

**Effect of LTSV RNA-2 on the type of lesion induced by RNA-1**

RNA-2 from purified LTSV particles of culture N was freed from RNA-1 either by sedimentation in sucrose density gradients or by precipitation of RNA-1 by two successive treatments with 2 M-LiCl. When such RNA-2 preparations were inoculated to *C. amaranticolor*...
Table 1. Effect of adding RNA-2 to inocula containing RNA-1 (LTSV-C) on the type of lesion induced in Chenopodium amaranticolor leaves

<table>
<thead>
<tr>
<th>RNA in inoculum*</th>
<th>Total no. lesions in four <em>C. amaranticolor</em> leaves</th>
<th>Chlorotic</th>
<th>Necrotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTSV-C + buffer</td>
<td>855</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>RNA-2 (2 μg/ml)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>LTSV-C + RNA-2 (2 μg/ml)</td>
<td>16</td>
<td>983</td>
<td></td>
</tr>
<tr>
<td>LTSV-C + RNA-2 (0.2 μg/ml)</td>
<td>119</td>
<td>439</td>
<td></td>
</tr>
<tr>
<td>LTSV-C + RNA-2 (0.02 μg/ml)</td>
<td>542</td>
<td>147</td>
<td></td>
</tr>
<tr>
<td>LTSV-C + RNA-2 (0.002 μg/ml)</td>
<td>787</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>LTSV-C + RNA-2 (0.0002 μg/ml)</td>
<td>894</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

* LTSV-C: RNA extracted from infected leaves of *Chenopodium amaranticolor*. RNA-2: RNA purified from a preparation of LTSV-N RNA by two treatments with 2 M-LiCl to remove insoluble high mol. wt. RNA (RNA-1).

Table 2. Effect of snake venom phosphodiesterase (SVPDE) and proteinase K (PK) on the biological activity of RNA from LTSV-N

<table>
<thead>
<tr>
<th>RNA in inoculum*</th>
<th>Total no. lesions in three <em>C. amaranticolor</em> leaves</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTSV-C + buffer</td>
<td>1739</td>
<td>0</td>
<td>1133</td>
</tr>
<tr>
<td>LTSV-N + buffer</td>
<td>0</td>
<td>1953</td>
<td>0</td>
</tr>
<tr>
<td>LTSV-C + LTSV-N</td>
<td>0</td>
<td>2996</td>
<td>0</td>
</tr>
<tr>
<td>LTSV-N (SVPDE) + buffer</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LTSV-C + LTSV-N (SVPDE)</td>
<td>845</td>
<td>693</td>
<td>565</td>
</tr>
<tr>
<td>LTSV-N (PK) + buffer</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>LTSV-C + LTSV-N (PK)</td>
<td>0</td>
<td>1669</td>
<td>NT</td>
</tr>
</tbody>
</table>

* LTSV-N: as Table 2. LTSV-N (5 μg/ml): RNA extracted from a purified preparation of LTSV-N. † NT, Not tested.

and *C. quinoa* at about 2 to 4 μg/ml, no symptoms developed. However, whereas nucleic acid extracts of *C. amaranticolor* leaves containing culture C induced only chlorotic lesions in *C. amaranticolor* leaves, similar extracts to which RNA-2 was added induced mostly necrotic lesions; the proportion of necrotic lesions increased with increasing concentration of RNA-2 in the inoculum (Table 1).

**Effect of SVPDE and of proteinase K on the biological activity of RNA-2**

The infectivity of potato spindle tuber viroid is unaffected by treatment with SVPDE because the circular molecules are infective (Diener, 1971). Treatment of RNA from LTSV-N particles with SVPDE abolished its ability to infect plants but the treated RNA retained the ability to alter the chlorotic lesions of LTSV-C to necrotic ones (Table 2). The infectivity of LTSV RNA is also greatly decreased by treatment with proteinase K (Table 2) as is LTSV-C RNA, probably because RNA-1 is linked to a protein that facilitates infection (Mayo & Jones, 1981). However, LTSV-N RNA rendered non-infective by proteinase K treatment was able to convert the lesion type induced by LTSV-C from chlorotic to necrotic (Table 2). These results suggest that the molecules of RNA-2 from LTSV-N particles remained biologically functional after SVPDE treatment and that protein, if present at all on RNA-2, is not needed for altering the type of lesion induced by RNA-1.

Tests to detect replication of RNA-2 independently of RNA-1

Purified RNA-2 was inoculated at approx. 2 to 8 μg/ml (depending on the experiment) to plants of *C. amaranticolor, C. quinoa, Cucumis sativus* cv. National Pickling, *Nicotiana clevelandii,*
**DISCUSSION**

Our results show that LTSV RNA-2 is not necessary for the replication of LTSV RNA-1 or the production of virus particles, implying that RNA-2 is not an essential part of the virus genome. Furthermore, we obtained no evidence that RNA-2 replicated in plants independently of RNA-1, suggesting that RNA-2 is not an independent viroid associated with LTSV RNA-1. However, when RNA-2 was added to cultures containing only RNA-1, the severity of symptoms in *C. amaranticolor* and *C. quinoa* was increased. Thus, RNA-2 appears to be a satellite RNA, dependent on RNA-1 for replication and able to be packaged in LTSV coat protein. Proof that RNA-2 is a satellite RNA awaits the demonstration that its nucleotide sequence is unique and not part of that of RNA-1. In velvet tobacco mottle virus (VTMoV), another virus with small circular RNA molecules (Randles et al., 1981), the sequence of the RNA-2 is distinct from the RNA-1 (Gould, 1981). However, neither the RNA-1 nor the RNA-2 of VTMoV seems able to replicate independently in plants, and it was concluded that RNA-2 is neither a satellite RNA nor an independent viroid (Gould et al., 1981).

Our results with LTSV therefore contrast with those obtained with VTMoV in Australia and indicate that small circular RNA molecules seem to act in one of three alternative ways: as (i) independently self-replicating pathogenic viroids, (ii) satellite RNA molecules dependent on a virus for replication and assembly into virus-like particles, or (iii) a part of the virus genome that is needed for infectivity. Whether these different activities reflect degrees of association of viroids, or viroid-like molecules, with plant viruses is not known. However, a deeper understanding of such associations may cast light on whether viroids are derived from plants or from plant viruses.

Finally, it has been suggested that the viruses with these viroid-like RNA molecules should be assigned to a new virus group (Tien-Po et al., 1981). We consider this premature until more information is available. Indeed, our data on LTSV are not in conflict with the earlier suggestion that this virus should be included in the sobemovirus group (Forster & Jones, 1979, 1980).

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**REFERENCES**


Satellite-like circular RNA of LTSV


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