A Satellite RNA in Grapevine Fanleaf Virus Strain F13

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SUMMARY

The F13 isolate of grapevine fanleaf virus (GFLV) differs from other isolates in that
it induces severe symptoms on Chenopodium quinoa. We show here that its particles
contain three RNA species with sizes, estimated by electrophoresis in agarose gels
containing formaldehyde, of 6800 nucleotides (RNA1), 3900 nucleotides (RNA2) and
1150 nucleotides (RNA3). The three RNA species are polyadenylated and probably
have a genome-linked protein at their 5' end. RNA1 and RNA2 are known to be
 genomic RNAs. Evidence for the satellite nature of RNA3 came from Northern blot
analysis with DNA probes. A probe originating from the 3' end region of RNA3 and
corresponding to one-third of the molecule did not hybridize with either RNA1 or
RNA2. Conversely 3'-terminal cDNA probes of RNA1 and RNA2 did not hybridize
significantly to RNA3. Further proof of the satellite nature of RNA3 is that it depends
on RNA1 and RNA2 for its multiplication in C. quinoa. RNA3 acts as mRNA in
wheatgerm extract and directs the synthesis of a Mr 39000 protein.

One of the most widespread and damaging virus diseases affecting grapevines is induced
mainly by two nepoviruses, grapevine fanleaf virus (GFLV) and arabis mosaic virus (ArMV).
Nepoviruses have a bipartite genome of positive-sense, single-stranded RNA molecules which
are separately encapsidated. The RNAs are polyadenylated at their 3' end and are covalently
attached, probably at their 5' end, to a small protein (VPg). Gel electrophoresis determinations
made in denaturing conditions (Murant et al., 1981) revealed differences between nepoviruses in
the Mr of RNA1 (2.7 x 10^6 to 2.8 x 10^6) and especially of RNA2 (1.3 x 10^6 to 2.4 x 10^6).
In addition, some nepoviruses encapsidate smaller RNAs which have been shown to be satellites or
satellite-like and have M_r between 0.08 x 10^6 for ArMV and 0.5 x 10^6 for grapevine Bulgarian
latent virus (Francki, 1985). The small satellite-like RNA in a strain of ArMV isolated from
hops is about 230 nucleotides in length and represents up to 80% of the viral nucleic acid in
plants showing nettlehead symptoms (Davies & Clark, 1983). These authors compared the
symptoms induced by various ArMV isolates on Chenopodium quinoa and suggested that this
small RNA is in part responsible for the severity of symptoms.

GFLV is transmitted by nematodes (Hewitt et al., 1958) and several isolates of GFLV, which
vary in the severity of the symptoms they induce, have been described or obtained in the
laboratory (Quacquarelli et al., 1976; B. Walter, unpublished results). We have examined the
F13 isolate of GFLV (Vuittenez et al., 1964) which differs from other GFLV isolates in the
severity of symptoms induced in C. quinoa; whereas milder strains induce a weak mosaic which
disappears after 2 to 3 weeks, F13 induces a strong persistent mosaic which leads to leaf
deformation and stunting of the host plant. Analysis of the RNA content of the F13 isolate
revealed the presence of an extra RNA species (RNA3) which we have found to have properties
suggesting that it is a satellite RNA.

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The F13 isolate of GFLV was propagated in *C. quinoa* and virus was extracted from systemically infected leaves harvested 15 days after infection. Frozen leaves were triturated in 0.1 M-sodium phosphate buffer pH 7.2, 0.1 M-ascorbic acid, 0.01 M-EDTA, in the presence of 8.5% (w/v) butanol. After clarification of the extracts at 10,000 g for 20 min, the virus was precipitated by the addition of polyethylene glycol (PEG 20,000) to 100 g/l and NaCl to 0.1 M, collected by low speed centrifugation and dissolved in 0.2 M-sodium citrate pH 6.0. After centrifugation at 145,000 g for 120 min the virus pellet was resuspended in 0.02 M-sodium citrate pH 6.0 containing 0.02% sodium azide. Insoluble material was removed by low speed centrifugation. The RNA extracted with phenol-SDS from GFLV, either purified by this procedure or by conventional methods (Quacquarelli *et al.*, 1976), always contained polydisperse low *M* material which in some cases accounted for 80% of the u.v. absorbance of the sample (Fig. 1a, lane 2). This material was partly eliminated by sedimentation of the virus through a 20 ml cushion of 20% sucrose in 0.02 M-sodium citrate pH 6.0, at 35,000 r.p.m. for 5 h in a Beckman rotor 42 before the RNA was extracted. However, the most efficient method was to treat the virus preparations with 1 mM-EDTA and 1% SDS at pH 7.5 to 8 (adjusted with NaOH) for 15 min at 55 °C in the presence of 1 mg/ml proteinase K before extraction twice with phenol. RNA was precipitated from 70% ethanol, washed twice with 3 M-sodium acetate, once with 75% ethanol and dissolved in sterile water.

The sizes of GFLV F13 RNAs were determined under denaturing conditions. Samples of RNA (2 to 10 µg) were heated for 5 min at 65 °C in 10 µl of 20 mM-HEPES, 1 mM-EDTA pH 7.8, containing 50% deionized formamide and 6% formaldehyde and electrophoresed in a 1% agarose slab gel, made up in the same buffer lacking formamide (Gustafson *et al.*, 1982). After electrophoresis the gels were stained for 2 min in 0.05% toluidine blue O, destained in 1% acetic acid for 1 h and dried on a sheet of Whatman 3MM paper. Quantitative analysis was done by reflection densitometry of dried gels at 535 nm, using a Shimadzu CS-930 scanner. Fig. 1(a) shows the electrophoretic pattern in denaturing conditions of RNA obtained by phenol-SDS
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extraction from virus purified by conventional methods without purification through a sucrose cushion (lane 2) or from proteinase K-treated virus (lane 6). It appeared that treatment with proteinase K yielded RNA preparations nearly free of the low Mr contaminant shown in lane 2 and therefore allowed accurate determination of viral RNA concentration. Comparison of lanes 2 and 6 with lane 5 (C. quinoa RNA) shows that the level of viral RNA1 and RNA2 in total RNA from infected plants was too low to be detectable by staining among the host plant RNAs. The weight proportion of each GFLV RNA species in the mixture (lane 6) was evaluated from the densitometer tracing of the stained gel. The mean values from six samples were 26% ± 3% for RNA1, 42% ± 6% for RNA2 and 32% ± 8% for RNA3.

The number of nucleotides in each GFLV RNA was calculated from a calibration curve established with values from the literature for the RNAs used as markers: alfalfa mosaic virus (AlMV) strain S (Ravelonandro et al., 1984), beet necrotic yellow vein virus (BNYVV) strain F13 (Bouzoubaa et al., 1986) and tomato black ring virus (TBRV) (Meyer et al., 1986). For each F13 RNA, two well defined marker RNAs, closely related in size, were used: BNYVV RNA1 (6746 nucleotides) and TBRV RNA1 (7356 nucleotides) for RNA1 (C. Greif, personal communication), TBRV RNA2 (4662 nucleotides) and AlMV RNA1 (3644 nucleotides) for RNA2 and TBRV RNA3 (1375 nucleotides) and AlMV RNA4 (881 nucleotides) for RNA3. The values for BNYVV and TBRV RNAs do not take into account their poly(A) tail. A linear relationship between the mobilities of the marker RNAs and the logarithm of their nucleotide number under the migration conditions enabled us to determine the sizes and the Mr of GFLV RNAs. Assuming an average nucleotide molecular weight of 343 (sodium salt) the RNA species had 6800 nucleotides (Mr 2.29 x 10^6; RNA1), 3900 nucleotides (Mr 1.31 x 10^6; RNA2) and 1150 nucleotides (Mr 0.38 x 10^6; RNA3). Thus the molar proportions of these RNAs in the virus were 8% for RNA1, 24% for RNA2 and 68% for RNA3. Size comparison with other nepovirus RNAs showed that GFLV F13 RNAs were among the smallest genomic RNAs known, together with chicory yellow mottle virus RNA1 and ArMV RNA2 (Murant & Mayo, 1982). For RNA1 the near comigration of BNYVV RNA1 (6746 nucleotides) and GFLV RNA1 made size estimation very accurate. The size of RNA3 is close to that of strawberry latent ringspot virus satellite RNA (Mayo et al., 1982).

Identification of the terminal structures of F13 RNAs was done for the 3' end after ligation of [32P]pCp with T4 RNA ligase as described by Devos et al. (1976). Analysis of the labelled material by the wandering spot method (Lockard et al., 1978) and by RNA sequencing (Donis-Keller, 1979) revealed a typical 3'-terminal poly(A) sequence for each RNA (not shown). Attempts to 5' end-label GFLV F13 RNAs with [γ-32P]ATP using polynucleotide kinase on untreated RNA or RNA treated with alkaline phosphatase or tobacco acid pyrophosphatase according to the labelling procedures of Efstradiatis et al. (1977) were unsuccessful. This indicated that the 5' ends of these RNAs were blocked, but were neither phosphorylated nor capped. Indeed, the 5' end of nepovirus RNA is thought to have a VPg. Indication of the presence of this structure on each GFLV F13 RNA came from analysis of RNA labelled with 125I as described by Koenig & Fritsch (1982). Autoradiographic analysis of the labelled RNAs after gel electrophoresis distinctly showed three bands corresponding to each of the three GFLV RNAs (Fig. 1 b). The nearly equal labelling of these bands, on a molar basis, for the three RNAs reinforces the suggestion of a VPg on each F13 RNA.

GFLV RNA3 is either a possible satellite RNA with no significant homology with RNA1 and RNA2 or a subgenomic RNA with a high degree of homology with one of the viral RNAs. To investigate this possibility, cDNA copies of each GFLV RNA were used. Viral RNAs (5 µg) were annealed to linearized oligo(dT)-tailed pUC9 plasmid DNA (2 µg). Clones corresponding to the GFLV F13 RNAs were obtained by the procedure of Heidecker & Messing (1983) and amplified in Escherichia coli JM103. Several cDNA copies were analysed by classical sequencing methods (Maxam & Gilbert, 1980) and the restriction maps established. The clones were screened both for the length of their inserts and for the specificity of the cloned DNA. After digestion of the recombinant plasmid DNA with HindIII and BamHI, the length of the inserted DNA was determined by electrophoresis in 1-2% agarose gels. The clones containing long inserts were nick-translated (Rigby et al., 1977) and the GFLV F13 RNAs were fractionated in
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RNA1  poly(A)  Probe 1

RNA2  poly(A)  Probe 2

RNA3  poly(A)  Probe 3

Fig. 2. Location of the cDNA probes on GFLV F13 RNAs. The heavy lines correspond to the position of the cDNA probe on each RNA. The restriction enzymes used to excise these DNA fragments are indicated. The scale is indicated at the lower left.

Fig. 3. Autoradiographic detection of viral RNAs with $^{32}$P-labelled cDNA probes corresponding to RNAs 1, 2 and 3. $^{32}$P-labelled cDNA probes corresponding to RNA1 (a), RNA2 (b) or RNA3 (c) were hybridized to triplicate Northern blots of GFLV F13 RNA (2 µg, lanes 1), total RNA from healthy C. quinoa (5 µg, lanes 2) or from GFLV F13-infected plants harvested 12 days after infection (5 µg, lanes 3). These RNAs were extracted from leaves which had been ground to a fine powder in liquid nitrogen or dry ice, mixed with 2 vol. of the extraction buffer described by Jackson & Larkins (1976) and extracted with phenol–chloroform–isoamyl alcohol. Lanes V and H are stained gels corresponding respectively to lanes 1 and 2.

In order to improve hybridization of these cDNA probes with viral RNAs extracted from infected plants and to decrease non-specific background, their poly(T) tails were removed using the appropriate restriction sites as shown in Fig. 2. Three probes were selected to correspond to each GFLV F13 RNA. They were labelled with $[^{32}$P]dCTP (3000 Ci/mmol) by nick translation and their specificity was tested by hybridization to triplicate Northern blots (Fig. 3a to c) of viral RNAs (lanes 1) or RNA extracted from healthy (lanes 2) or GFLV-infected C. quinoa (lanes 3). The toluidine blue O-stained viral (lane V) or plant (lane H) RNAs were used as markers. Under the hybridization conditions used, the probes corresponding to RNAs 1, 2 and 3 hybridized to RNA from purified virus or from infected C. quinoa (lanes 1 and 3) but not to RNA from healthy plants (lanes 2). The very low degree of cross-reactivity between the three purified GFLV RNAs shown in lanes 1 was not detectable in the RNA of infected plants (lanes 3), due to
the small amount of viral RNA. Each probe reacted faintly with the two non-homologous RNAs except the one for RNA2 (b) which did not appear to hybridize to RNA3. This cross-hybridization cannot be due to the poly(A) or the adjacent non-coding sequences at the 3' end of GFLV RNAs [which could contain short homologies as reported for TBRV satellite RNA and RNA2 (Meyer et al., 1986)] since none of the probes was complementary to these sequences (Fig. 2). The lack of hybridization between RNA1 or RNA2 and the DNA probe corresponding to one-third of RNA3, mainly in the coding part of the molecule, proves that RNA3 is not subgenomic and therefore could be a satellite RNA.

Additional evidence strongly suggesting that RNA3 is a satellite RNA came from RNA3 multiplication assays. Purified RNA3 was inoculated alone or together with RNAs of GFLV TU (a GFLV isolate which contained no RNA3) on one leaf of C. quinoa. Eight days after inoculation, the RNAs were extracted from the inoculated and uninoculated leaves. Using the 32P-labelled RNA3-specific cDNA probe, no RNA3 could be detected in uninoculated leaves after infection with RNA3 alone. RNA3 multiplication occurred only when it had been coinoculated with GFLV TU RNAs. These RNAs acted as helpers for RNA3 multiplication as expected for a helper virus for an authentic satellite RNA.

The messenger properties of GFLV F13 RNAs were examined in a wheatgerm extract, containing [35S]methionine (Godefroy-Colburn et al., 1985). The viral RNA species used were separated by electrophoresis in 1% agarose slab gels, 180 × 150 × 1.5 mm, for 4 h at 120 V in 100 mM-Tris–borate, 2 mM-EDTA pH 8.3, without preliminary denaturing treatment of the RNA.

![Fig. 4. Translation products of GFLV F13 RNAs in wheatgerm extract. The 35S-labelled translation products of fractionated or unfractionated GFLV F13 RNAs were analysed by SDS–PAGE in 12.5% (lanes 1 and 2) or 6% gels (lanes 3 to 6). The added mRNAs were: lane 1, purified RNA3 (0.1 µg); lane 2, unfractionated GFLV F13 RNAs (0.7 µg); lane 3, RNA2 (0.3 µg); lane 4, RNA1 (0.2 µg); lane 5, unfractionated RNA from GFLV F13 (0.75 µg); lane 6, none. The positions and sizes (Mr × 10^-3) of the markers are indicated at each side. P1, P2 and P3 indicate the positions in the two gels of the major translation products of RNA1, RNA2 and RNA3 respectively.](image-url)
samples. RNAs were located by staining small gel strips on the edges of the gel with toluidine blue O. The RNA-containing bands were excised and RNA was electroeluted in Tris–borate buffer, extracted repeatedly with phenol–chloroform and precipitated from 70% ethanol. The Mg²⁺ concentration was 1.8 mM for optimum incorporation of [³⁵S]methionine. The translation products were analysed by SDS–PAGE in 12.5% or 6% gels in the buffer system of Laemmli (1970) and detected by autoradiography as shown in Fig. 4. The largest and most abundant product directed by RNA3, P3, has an Mₐ of 39000 (39K) which corresponds to nearly the full coding capacity of this RNA (lane 1). This protein is similar in size to the 38K translation product of strawberry latent ringspot virus satellite RNA (Mayo et al., 1982). The major translation product of RNA2 was a protein of 127K, P2 (lane 3), and that of RNA1, P1, was the largest, with an Mₐ of about 225K (lane 4). All these products were also obtained by translation of total GFLV RNAs (lanes 2 and 5). The Mₐ of P1 and P2 are similar to those of the two proteins directed by GFLV RNA1 and RNA2 in reticulocyte lysates (Morris-Krsinich et al., 1983).

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