Isolation of Three Viroids and a Circular RNA from Grapevines

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SUMMARY

Analysis of nucleic acids from grapevine tissues by two-dimensional gel electrophoresis demonstrated the presence of two bands of circular RNA. The smaller RNA contained about 300 nucleotide residues and was identified as hop stunt viroid by nucleotide sequencing. The larger RNA band was a mixture of species and contained similar amounts of two components, referred to as RNA 1a and RNA 1b, and in addition a trace amount of citrus exocortis viroid (CEV) which became detectable only after inoculation of the mixture to tomato. The identity of CEV was determined by probe hybridization and nucleotide sequencing. Both RNAs 1a and 1b are distinct from CEV and have estimated sizes larger than those of CEV and other viroids reported so far. RNA 1a preparations were infectious in cucumber and in tomato and the recovered viroid had unique properties. We have provisionally named this viroid Australian grapevine viroid. Evidence for the autonomous replication of RNA 1b was not obtained.

INTRODUCTION

A number of graft-transmissible diseases whose causal agents have not been identified infect grapevine worldwide. These include leafroll, stem pitting and corky bark which cause significant damage (Bovey et al., 1980). We are studying the possible involvement of viroids in these grapevine diseases.

Two viroids have been isolated from grapevines by other workers. Sano et al. (1985a) have reported the presence of an isolate of hop stunt viroid (HSV) and Flores et al. (1985) have isolated a viroid which has been shown to have 89% to 92% sequence homology with citrus exocortis viroid (CEV; Garcia-Arenal et al., 1987). In this paper we report the isolation of a mixture of four distinct species of circular RNAs from grapevine tissues which were identified as HSV, CEV, a new viroid provisionally called Australian grapevine viroid (AGV) and a circular RNA similar in size to viroids, referred to as RNA 1b, for which evidence of replication was not obtained.

METHODS

Plants. Grapevine (Vitis vinifera L.) cultivars were grown either in the field or in a growth cabinet at 30 °C ± 1 °C with a 16 h light period. Healthy and inoculated tomato (Lycopersicon esculentum Mill cv. Rutgers) and cucumber (Cucumis sativus L. cv. Supermarket and cv. Suyo) plants were also maintained in cabinets as above. In infectivity studies, tomato and cucumber plants were inoculated mechanically at the cotyledon stage. When purified circular RNA preparations were used as inocula, the RNA concentration was approximately 5 µg/ml. CEV-A (Visvader & Symons, 1983) was propagated in tomato in a separate growth cabinet.

Extraction and purification of nucleic acids. Plant tissues were extracted by the method of Palukaitis & Symons (1980) up to and including ethanol precipitation of crude extracts, with minor modifications. MgCl₂ was omitted from the medium and 5 mM-EDTA was added to minimize cleavage of circular RNA to the linear form (Sänger et al., 1979). When extracting RNA from grapevine tissues, 4 vol. of the extraction medium were used and no NaCl was added. The crude pellets obtained by ethanol precipitation were stirred for 2 h in half-strength solution A containing 1% 2-mercaptoethanol (Palukaitis & Symons, 1980) and 0.5 vol. buffer-saturated phenol. The aqueous phase was recovered by centrifugation, adjusted to 35% with ethanol and mixed with cellulose (CF11) for 30 min.
to bind the nucleic acids (Franklin, 1966). The cellulose was recovered by centrifugation and washed three times by resuspension in an excess volume of ethanol: STE (50 mM-Tris·HCl pH 7, 100 mM-NaCl, 1 mM-EDTA), 0.25 M by subsequent centrifugation. The cellulose was transferred to a column and washed further with the ethanol:STE solution until no further u.v.-absorbing material was eluted. Nucleic acids were then eluted with STE, precipitated with ethanol and resuspended in a small volume of TE (10 mM-Tris·HCl pH 7-5, 0.1 mM-EDTA).

**Purification of circular RNA.** Purified total nucleic acids obtained by cellulose chromatography were fractionated with 1-5 M-NaCl (Rezaian & Francki, 1973) which enriched the amount of viroid by 14-fold. RNA from the salt-soluble fraction was precipitated by adding 2 vol. of ethanol and circular RNA was isolated from it by two-dimensional (2-D) electrophoresis in polyacrylamide gels (170 × 170 × 3 mm; Schumacher et al., 1983). Following electrophoresis, the nucleic acids were stained with ethidium bromide and regions of the gel containing circular RNA bands were excised. Nucleic acids were eluted by incubating the gel slices at 37 °C overnight in the elution buffer containing 0.5 M-ammonium acetate, 0.1% SDS as used by Maxam & Gilbert (1980) except that magnesium acetate was omitted to avoid nicking the circular RNA (Sänger et al., 1979). The circular RNAs were recovered by precipitation with ethanol. When spectrophotometric measurement of the circular RNA was required, it was often necessary to purify the eluted RNA further by spermine precipitation (Hoopes & McClure, 1981). Spermine (5 mM) was added to the RNA preparations in TE and they were left on ice for 15 min. The RNA precipitates were collected by low-speed centrifugation, washed with 75% ethanol containing 0.3 M-sodium acetate and 0.01 M-magnesium acetate followed by 75% ethanol, dried and resuspended.

For analytical purposes, 2-D electrophoresis was carried out on a 1 mm thick polyacrylamide gel (Schumacher et al., 1983) and nucleic acids were detected by silver staining (Merril et al., 1981).

**Prolonged electrophoresis of RNA.** Samples of circular RNAs were applied to 1 mm thick 6% polyacrylamide gels in 89 mM-Tris, 89 mM-boric acid, 2 mM-EDTA, 4 M-urea (Maniatis et al., 1982) and electrophoresed at approximately 16 W for three times the time taken for the bromophenol blue tracking dye to reach the bottom of the gel. The surface temperature of the gel plates was maintained at 52 °C.

**Dot-blot hybridization.** Samples of nucleic acids in TE were mixed with 3 vol. of 10 × SSC (1.5 M-NaCl, 0.15 M-trisodium citrate) containing 20% formaldehyde (White & Bancroft, 1982). The mixture was heated at 65 °C for 15 min, then at 90 °C for 2 min and applied to a nitrocellulose filter (Schleicher and Schüll, 0.45 μm). The filter was baked in vacuo at 80 °C and the RNA was detected by hybridization with 32P probes in a buffer containing 50% formamide (Maniatis et al., 1982). 32P-labelled cDNA to grapevine RNA 1 was prepared by oligomer-primed synthesis using the synthetic primer (5' TAGCGGGGGTTCGGGG 3') in the presence of 25 μM-[32P]dATP (20 μCi; Rezaian & Jackson, 1981). The CEV probe was made by transcribing a full-length plus-sense CEV clone in the bacteriophage M13, using the Klenow fragment of DNA polymerase I and the universal 17-mer M13 primer (Maniatis et al., 1982).

**RNA sequencing.** RNA sequencing by the dideoxy chain termination method using reverse transcriptase and a synthetic primer was carried out as outlined before (Rezaian et al., 1984). Direct RNA sequencing by the partial enzymic cleavage method (Haseloff & Symons, 1981) was carried out on fragments of viroids generated by digestion with RNase T1 or U2 which were end-labelled with [7-32p]ATP using polynucleotide kinase (Gould & Symons, 1982). Sequence data obtained were analysed using MBIS and MTX computer packages (Bucholtz & Reisner, 1986; Reisner & Bucholtz, 1986). Stretches of 12 nucleotides with a minimum of 65% match were the parameters used when searching for sequence homologies.

**RESULTS**

**Detection of circular RNAs in grapevine**

Analysis of purified nucleic acids from a number of grapevine sources by 2-D gel electrophoresis and silver staining revealed the presence of two circular nucleic acid bands migrating more slowly than the plant linear RNAs which form a diagonal line in this gel system (Fig. 1a). These nucleic acids were purified by preparative 2-D gel electrophoresis and were found to be sensitive to RNase and resistant to DNase (results not shown). They were therefore RNA species and will be initially referred to as RNA 1 (later found to be a mixture) and RNA 2 (later found to be HSV) in the order of increasing electrophoretic mobility as shown in Fig. 1.

The occurrence of circular RNAs in field material seemed to be common as most varieties used for RNA analysis contained one or both of the RNAs (Table 1). Marked variations were observed between the circular RNA contents of field samples harvested at different times of the year, and the highest yields were obtained in the two hottest months of summer (January and February). Tissue collected from the field was unsatisfactory for viroid purification due to the low yields. Grapevines maintained at 30 °C in a growth cabinet produced a higher yield of the
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Fig. 1. Analysis of purified grapevine nucleic acid extracts by 2-D gel electrophoresis. The sources of grapevines were Cabernet franc (a) and clones regenerated by fragmented shoot apex culture (b). Purified nucleic acids (75 μg) were analysed and stained with silver. The circular nucleic acid bands detected are indicated as grapevine RNA 1 (1) and RNA 2 (2). Direction of non-denaturing electrophoresis in the first dimension was from right to left and in the second denaturing dimension was from top to bottom.

Fig. 2. Prolonged electrophoresis of grapevine RNA 1 and RNA 2 in polyacrylamide gel. Grapevine RNA 1 (lanes 1 and 2) and RNA 2 (lanes 3 and 4) were isolated from 2-D gels as shown in Fig. 1 and were subsequently electrophoresed as outlined in Methods. In lanes 2 and 4 the RNA was incubated with 5 mM-MgCl₂ at 80 °C for 12 min prior to electrophoresis. RNAs 1a and 1b are the components of grapevine RNA 1. The small quantities of linear RNAs shown were generated from the circular forms. Size markers used, from the top of the gel downwards, are CEV (371 residues; Visvader & Symons, 1983), one of the RNA forms of CCCV (296 residues; Haseloff et al., 1982) and avocado sunblotch viroid (247 residues; Symons, 1981).

Table 1. Grapevine cultivars assayed for the presence of circular RNAs

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>RNA detected*</th>
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<tbody>
<tr>
<td>Cabernet franc</td>
<td>1 and 2</td>
</tr>
<tr>
<td>Cabernet franc regenerated by fragmented shoot apex culture</td>
<td>1</td>
</tr>
<tr>
<td>Knight's centennial</td>
<td>1 and 2</td>
</tr>
<tr>
<td>Waltham cross</td>
<td>1 and 2</td>
</tr>
<tr>
<td>Mission seedling No. 1</td>
<td>2</td>
</tr>
<tr>
<td>Mataro</td>
<td>None</td>
</tr>
<tr>
<td>Sultana clone H₄</td>
<td>1</td>
</tr>
<tr>
<td>Sultana clone DI H</td>
<td>1 and 2</td>
</tr>
</tbody>
</table>

* RNAs 1 and 2 were detected by 2-D gel electrophoresis and silver staining as described in Methods.

two RNAs and it was possible to recover about 2 μg of each circular RNA from 1 kg of tissue. One of the grapevine sources, a Cabernet franc clone generated from fragmented shoots (Barlass et al., 1982), did not contain RNA 2 in quantities detectable by silver staining (Fig. 1b) but infectivity tests in cucumber showed that this grapevine source did contain low levels of RNA 2.

The sizes of RNA 1 and RNA 2 were estimated, by electrophoresis in 6% polyacrylamide under denaturing conditions, to be approximately 370 and 300 nucleotides respectively as they
migrated close to either CEV (371 nucleotides; Visvader & Symons, 1983) or coconut cadang cadang viroid (CCCV, 296 nucleotides; Haseloff et al., 1982). On a few occasions, RNA 1 could be resolved as two closely migrating components in denaturing gels. After further investigation we found that prolonged electrophoresis resolved RNA 1 into two components, RNA 1a and RNA 1b (Fig. 2, lane 1), both with an electrophoretic mobility slightly less than CEV. RNA 2 remained a single species under the same electrophoretic conditions (Fig. 2, lane 3). Separation of RNAs 1a and 1b was not possible by the 2-D gel system because RNA bands move vertically in the second dimension and the duration of electrophoresis is relatively short in the first dimension. However, the 2-D system has the advantage that circular RNA can be separated from plant nucleic acids unambiguously. The term RNA 1 used in the following sections refers to the circular RNA species of about 370 nucleotides isolated from 2-D gels as shown in Fig. 1 and does not identify a particular species. Thus RNA 1 isolated from grapevine, tomato and cucumber are not the same and each contains a mixture as is outlined in the following sections.

Most purified preparations of the circular RNAs contained small amounts of RNA species which migrated much faster than the circular RNAs in denaturing gels. Presumably these were linear forms produced by random cleavage of the circular RNAs (Fig. 2, lane 1). Generation of these species was increased by incubation of RNAs 1 and 2 in buffer containing magnesium (Fig. 2), a treatment known to convert circular RNA to the linear form (Sanger et al., 1979).

Data given above demonstrated the presence of at least three circular RNAs of viroid size in grapevine. The nature of these RNAs was studied by a combination of electrophoretic analysis, infectivity tests, probe hybridization and nucleotide sequencing.

The circular RNA 2 is an isolate of HSV

Purified preparations of RNA 2 from grapevine were infectious on cucumber and induced severe stunting, rugosity and vein clearing 17 to 20 days after infection. Nucleic acids from infected plants contained an RNA species corresponding to RNA 2 in the 2-D gel system (Fig. 3a). This RNA band was absent in healthy plants (Fig. 3a). The electrophoretic mobilities of RNA 2 from grapevine and from infected cucumber were indistinguishable. These results demonstrated that the circular RNA 2 from grapevine is capable of autonomous replication and hence can be classified as a viroid. RNA 2 was propagated in cucumber and further characterized.

RNA 2 was partially digested with RNase T1 and U2 and the resulting fragments were sequenced by the enzymic digestion procedure. A comparison of the partial sequence data obtained with the known sequences of other viroids demonstrated complete sequence homology with the grapevine isolate of HSV, HSVg (Fig. 3b) reported by Sano et al. (1985b) including the single residue change that is specific to HSVg. Fig. 3(b) shows the sequences of 203 nucleotides of RNA 2 which were determined in this study and it identifies the positions of these sequences in relation to the published sequence of HSV from grapevine (Sano et al., 1985b). The estimated size of RNA 2 shown in Fig. 2 is also consistent with the size of HSVg determined to be 297 nucleotide residues. It is concluded that RNA 2, the smaller RNA shown in Fig. 1(a), is HSV, a viroid not previously reported in Australia.

Infectivity of the purified circular RNA 1 from grapevine

Grapevine RNA 1 (containing RNAs 1a and 1b) isolated by preparative 2-D gel electrophoresis was mechanically inoculated to tomato and cucumber cotyledons and the plants were maintained at about 30 °C for 4 weeks. All inoculated plants showed stunting, cucumber leaves showed clearing around the main radial veins, and tomatoes developed severe tip necrosis. Nucleic acids were extracted from the infected plants and analysed by 2-D gel electrophoresis. Both inoculated tomatoes and cucumbers contained two circular RNAs corresponding to RNA 1 and to HSV (Fig. 4). The latter RNA was indistinguishable from the HSV extracted directly from grapevine when compared by prolonged electrophoresis. The presence of HSV in plants inoculated with grapevine RNA 1 was unexpected. Since HSV is unrelated to RNA 1 (see below), trace quantities of this viroid must be present in RNA 1 preparations either due to contamination during electrophoresis or because of the possible
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Fig. 3. (a) 2-D gel electrophoresis of healthy (H) and infected (I) cucumbers inoculated with grapevine RNA 2 (2). Electrophoresis was carried out as in Fig. 1. (b) Nucleotide sequences of fragments of grapevine RNA 2. The sequences determined in this study are aligned on the published sequence of HSVg (Sano et al., 1985b). The boxed regions show the sequences which were not determined. The arrow indicates the single residue specific to HSVg.

Fig. 4. 2-D gel electrophoresis of purified nucleic acids from cucumber and tomato. Plants used for extraction were either healthy tomato (a), tomato inoculated with grapevine RNA 1 (b), cucumber inoculated with grapevine RNA 1 (c), tomato inoculated with grapevine RNA unfractionated by 2-D gel electrophoresis (d) and cucumber inoculated with unfractionated grapevine RNA (e). Electrophoresis was carried out as in Fig. 1. Positions of the circular RNAs 1 and 2 of tomato and cucumber are indicated as 1 and 2, respectively.
existence of more than unit length molecules of HSV. Other circular RNAs have been shown to exist in oligomeric forms (Hutchins et al., 1985). We have observed that when unfractionated grapevine RNA containing both HSV and RNA 1 was used as inoculum, production of RNA 1 in cucumber and in tomato was inhibited to undetectable levels and only HSV was produced (Fig. 4d, e). It appears that HSV replicates much more efficiently than the RNA 1 in cucumber and in tomato and, therefore, the presence of small proportions of HSV in the RNA 1 inoculum results in the production of a significant amount of this viroid.

Fig. 5. A comparison of RNA 1 obtained from different plants, by electrophoresis. RNA 1 from grapevine (lane 3) which includes RNA 1a and 1b was used as the inoculum and the RNA 1 bands were isolated from inoculated cucumber (lane 1) and tomato (lane 2) and then analysed electrophoretically as described in Fig. 2. A sample of CEV was also electrophoresed as a marker (lane 4). The linear forms were probably generated by random cleavage as all the circular RNAs were prepared by preparative 2-D gel electrophoresis. The major band in lane 2 was shown to be CEV and is distinct from RNA 1b in lane 3.

Fig. 6. (a) Nucleotide sequence of a region of RNA 1 from inoculated tomato. The sequence of 66 residues obtained is identical to the CEV sequence from residues 18 to 83 (Visvader & Symons, 1983). (b) Dot-blot hybridization of nucleic acids of healthy and infected plants with 32P-labelled DNA probes. The probes were an M13 transcript of CEV and a cDNA made by priming grapevine RNA 1 (containing components 1a and 1b) with an oligodeoxynucleotide complementary to one of the sequenced RNA 1 fragments. The amount of RNA applied per spot was 50 ng of the salt-soluble fraction of healthy tomato RNA, approximately 3 ng of purified RNA 1 from tomato and 1 ng each of the remaining RNAs.

Analysis of the RNA 1 band from infected cucumbers: detection of a new viroid

Prolonged electrophoresis of RNA 1 preparations from cucumber infected with grapevine RNA 1 showed the presence of one major component with the same mobility as RNA 1a from grapevine (Fig. 5, lane 1). This observation indicated that RNA 1a obtained from cucumber is a viroid since it is capable of autonomous replication. From its electrophoretic mobility in denaturing gels RNA 1a appeared to be larger than the 371 residue CEV-A (Fig. 5). RNA 1a is
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thus one of the largest viroids reported since the largest viroid sequenced to date is CEV-G with 375 nucleotide residues (Visvader & Symons, 1985).

Characterization of grapevine RNAs 1a and 1b has proved difficult because we have not been able to obtain sufficient quantities of pure material as it is difficult to separate large quantities of RNAs 1a and 1b electrophoretically. Inoculation of cucumber with an inoculum containing equal proportions of RNAs 1a and 1b resulted in a predominance of RNA 1a but a minute quantity of a band corresponding to RNA 1b was just visible in some stained gels (not evident in Fig. 5, lane 1).

We also performed direct RNA sequencing on the grapevine RNA 1 which contains both RNAs 1a and 1b, before we recognized that RNA 1 contained two species. A total of 207 residues was determined in nine non-overlapping fragments. Comparisons of the nucleotide sequences of these fragments with all reported sequences of viroids (Keese & Symons, 1985) and circular RNA satellites (Haseloff & Symons, 1982; Keese et al., 1983) and the satellite of tobacco ringspot virus (Buzayan et al., 1986) which occurs in circular form in plant cells (Linthorst & Kaper, 1984) showed no extended regions of homology. The complete primary structures of RNAs 1a and 1b are currently being determined by cloning techniques.

A synthetic oligodeoxynucleotide primer (17-mer), complementary to one of the grapevine RNA 1 fragments sequenced, was obtained and used to make cDNA. Since the RNA is known to be a mixture of at least two components it was not known to which of the two this particular cDNA corresponded. This cDNA hybridized to dot-blots of grapevine RNA 1, containing both RNAs 1a and 1b but failed to hybridize with the RNA 1a component purified from infected cucumbers (Fig. 6b). This lack of hybridization with cucumber RNA 1a suggests that the $^{[32P]}$cDNA probe synthesized is complementary to a component of the unfractionated grapevine RNA 1 other than that which replicates in cucumber. The same cDNA also failed to hybridize with HSV and CEV (Fig. 6). Both RNAs 1a and 1b were confirmed to be unrelated to CEV as they failed to hybridize with the CEV probe DNA (Fig. 6). The observation that RNA 1a but not 1b is readily detectable in cucumber extracts (Fig. 5) indicates that these RNAs are not two electrophoretic forms of the same species.

We conclude that RNA 1a which is capable of replication in cucumber and in tomato (see below) is a viroid with a size comparable to CEV, the largest viroid sequenced (Keese & Symons, 1985; Riesner & Gross, 1985), but is unrelated to CEV (Fig. 6). Because of these unique properties the self-replicating RNA 1a will be referred to, for the present, as AGV.

A trace amount of CEV is present in grapevine RNA 1 preparations

RNA 1 produced in tomatoes inoculated with grapevine RNA 1 was compared with samples of the grapevine RNA used as the initial inoculum, by electrophoresis in denaturing gels (Fig. 5). The main component of tomato RNA 1 had a faster mobility than either AGV or RNA 1b of grapevine (Fig. 5, lanes 2 and 3). However, a minor component was also present which had a mobility corresponding to AGV (Fig. 5, lane 2). This experiment indicated that another viroid which replicated in tomato was also present in the grapevine RNA 1 preparation, in addition to AGV. Since the mobility of the major RNA 1 component replicating in tomato coincided with the mobility of CEV (Fig. 5, compare lanes 2 and 4), further experiments were carried out to examine whether CEV was present in tomato and grapevine RNA 1 preparations.

Primer extension using reverse transcriptase and the oligodeoxynucleotide 5'GTTTCCCCGGGGATCC 3' which is complementary to the central conserved region of most viroids (Keese & Symons, 1985) failed to synthesize any significant amount of cDNA on the grapevine RNA 1 template but produced cDNA on both CEV-A and RNA 1 from inoculated tomato. Using the above primer in chain termination sequencing reactions, the nucleotide sequence of 66 residues of tomato RNA 1 was determined (Fig. 6a) and these corresponded to the sequence of CEV between residues 18 and 83 (Visvader & Symons, 1983). Moreover, dot blot hybridization using an M13 transcript of a plus CEV insert as a probe confirmed that the major component in tomato RNA 1 was CEV (Fig. 6b).

A trace of CEV was also detected in RNA 1 from inoculated cucumber both by prolonged electrophoresis (Fig. 5, lane 1) and by the weak hybridization signal observed when a CEV
probe was used in dot blot analysis (Fig. 6). It is noteworthy that CEV was not detected in dot-blots of grapevine RNA 1 preparations. Thus CEV must be present in extremely small quantities in the initial grapevine RNA 1 inoculum.

DISCUSSION

We have detected a mixture of viroids in grapevine and have described a complex pattern of infectivity produced in herbaceous plants by identifying the viroids involved. Nucleotide sequencing combined with probe hybridization and electrophoresis enabled unambiguous identification of HSV and CEV as well as two other RNAs with unique properties. One of the RNAs has been provisionally named AGV and is a new viroid capable of replication in cucumber and in tomato, and the other, RNA 1b, resembles a viroid in size and circular structure but clear evidence for its autonomous replication is lacking.

Infectivity tests of these viroids were complicated because their complete isolation from each other was not possible by electrophoretic means. This was due in part to the similarity in the sizes of CEV, AGV and RNA 1b but even HSV, which is about 70 nucleotide residues shorter than these three RNAs, could not be totally removed from the cultures. It remains to be determined whether RNA 1b is capable of replication in the absence of the contaminating viroid species.

HSV has been found in grapevines in Japan (Sano et al., 1985b) but this is the first time that it has been reported in a crop in Australia. A viroid with 92% sequence homology with CEV-A has also been isolated from grapevine (Garcia-Arenal et al., 1987), but a self-replicating RNA with the characteristics of AGV has not been reported before.

Flores et al. (1985) have also characterized a viroid-like RNA, designated GV-f, present in grapevine extracts. The electrophoretic mobility of GV-f under denaturing conditions is indistinguishable from CEV but its linear form moves at a rate much faster than the linear CEV (Flores et al., 1985). RNA 1b and AGV reported here have a slightly slower electrophoretic mobility than CEV and their linear forms have about the same mobility as the linear form of CEV. Although the electrophoretic mobilities indicate that GV-f may differ from RNA 1b and AGV, direct evidence is needed to establish the relationship of these RNAs. More recently, Semancik et al. (1987) have detected viroid-like RNAs termed GV-1, GV-2 and GV-3 in grapevines. Semancik et al. (1987) have suggested that GV-1 may be related to GV-f reported by Flores et al. (1985) and have noted that GV-3 may be related to HSV. As yet infectivity data for these viroid-like RNAs have not been provided.

Evidence given here shows co-replication of different viroids in the same host and indicates competition in viroid replication, depending on the host and viroids involved. HSV for example replicates successfully in grapevine (Fig. 1b, Table 1), in cucumber (Fig. 3 and 4) and in tomato (Fig. 4) in the presence of the other viroids. The presence of trace quantities of HSV in gel-purified grapevine RNA 1 preparations at levels undetectable by silver staining leads to a significant build-up of HSV in cucumber (Fig. 4b) and in tomato (Fig. 4c) with co-replication of AGV and CEV, respectively, in these tissues. In fact, our observations have shown that the presence of comparable concentrations of HSV and RNA 1 in unfractionated grapevine RNA inocula lead to the replication of only HSV in cucumber and in tomato. These data indicate that the replication of AGV in cucumber and CEV in tomato is inhibited by the presence of HSV. It was not possible to test this possibility directly by mixing inocula as purified preparations completely free of the contaminating species could not be obtained.

Co-infection of plants with different viroids and with strains of the same viroid has been studied previously and has been found to result in cross-protection with some viroids (Fernow, 1967; Niblett et al., 1978). The competition of HSV with CEV and with AGV observed here resembles a cross-protection phenomenon and may be due to competition for a common biological process as suggested by Niblett et al. (1978).

In grapevine, AGV and RNA 1b are present together, in concentrations comparable to HSV but CEV is undetectable by probe hybridization. However, CEV multiplies to high levels when an RNA containing supposedly only AGV and RNA 1b is inoculated on tomato. Taken together
these observations show that a combination of biological testing and other detection methods such as probe hybridization is needed for accurate identification of viroids present in a particular RNA extract.

The relationship of the viroids and the circular RNA 1b reported here to grapevine diseases is unclear. The grapevine cultivars used in this study are of known disease status and analysis of viroids present in these vines may produce evidence for their possible involvement in the diseases.

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