Two Related Viroids Cause Grapevine Yellow Speckle Disease Independently

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SUMMARY

We have confirmed that two closely related circular RNA molecules previously named grapevine yellow speckle viroid (GYSV) and grapevine viroid 1B (GV1B) are indeed viroids. Electron microscopy after spreading under non-denaturing conditions revealed that GYSV has a rod-like structure typical of viroids. Purified GYSV and GV1B replicated independently in inoculated grapevine seedlings and some of the infected plants developed yellow speckle symptoms indicating that both viroids can cause grapevine yellow speckle disease. Plus-sense RNA transcripts derived from a dimeric GYSV cDNA clone induced yellow speckle symptoms in a grapevine seedling confirming the role of GYSV in the yellow speckle disease. Two oligonucleotide probes were synthesized for the detection of the two related viroids. The probes which could detect each viroid individually were used to assess correlations between the occurrence of these viroids and the incidence of the disease.

INTRODUCTION

The sequences of two new viroid RNAs from grapevine tissue which we have tentatively named grapevine yellow speckle viroid (GYSV; Koltunow & Rezaian, 1988) and grapevine viroid 1B (GV1B; Koltunow & Rezaian, 1989a) have recently been determined. GYSV and GV1B are composed of 367 and 363 nucleotide residues, respectively, and have 73% sequence homology (Koltunow & Rezaian, 1989a). GYSV and GV1B both possess a central sequence which is also found in apple scar skin viroid (ASSV; Hashimoto & Koganezawa, 1987) and which differs from that found in other viroids. On this basis it was proposed that GV1B, GYSV and ASSV form a new viroid group represented by ASSV (Koltunow & Rezaian, 1988, 1989b).

It has been suggested from observations of their molecular properties (Koltunow & Rezaian, 1988, 1989a) that GYSV and GV1B are viroids. In this paper we demonstrate that GYSV and GV1B are viroids by infectivity studies in viroid-free grapevine seedlings and by electron microscopy. Since these two viroids are so closely related that they cannot be differentiated unambiguously by probes produced from M13 clones, we have used synthetic oligonucleotides for their identification. These probes were used to determine the distribution of GYSV and GV1B in grapevine cultivars of known disease status. The results of the survey and the infectivity tests show that both GYSV and GV1B can cause the grapevine yellow speckle disease independently.

METHODS

Nucleic acid extraction and viroid purification. Young terminal leaves were sampled during the hotter months of summer from grapevines grown in the field or in a glasshouse. These tissues were snap-frozen in liquid nitrogen and stored at −70 °C. Procedures for the extraction of total nucleic acids and subsequent viroid purification were as described by Rezaian et al. (1988). Purified preparations of GYSV, GV1B, citrus exocortis viroid (CEV), hop stunt viroid (HSV) and Australian grapevine viroid (AGV) were obtained from sources described by Koltunow & Rezaian (1988) and Rezaian et al. (1988).

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Northern blot and dot blot analysis. Northern blot analysis was carried out as described previously (Koltunow & Rezaian, 1988) except that synthetic oligonucleotides specific for the detection of GYSV (5' GGACGCGAACGTGAATAGG 3') and GV1B (5' ACCGGCTTCGGAGATAGAAG 3') were 32P-labelled at their 5' ends using T4 polynucleotide kinase (Maniatis et al., 1982) and used as hybridization probes. The hybridization buffer (Thomas, 1980) included 10 ng/ml of labelled oligonucleotide and the filter was incubated at 34 °C for 12 h. The filter was washed twice in 6 × SSC, 0.1% SDS (Thomas, 1980) at room temperature for 10 min followed by two washes in 6 × SSC, 0.1% SDS for 20 min at 30 °C. Dot blot analysis was used for purified viroid samples as described (Rezaian et al., 1988). Conditions for the use of labelled synthetic oligonucleotides were the same as those described for Northern blot analysis.

Electron microscopy. Non-denatured RNA samples were spread and rotary-shadowed, as described by Randles & Hatta (1979). Electron micrographs were taken in a Philips EM 400 at a magnification of 10,500 × and the negatives were enlarged 10-fold.

Dimeric GYSV transcripts. A full-length GYSV cDNA clone (referred to as J1), and a partial length GYSV cDNA clone (referred to as 27) constructed previously (Koltunow & Rezaian, 1988) were used to make a dimeric GYSV cDNA clone in M13mp19, as illustrated in Fig. 1. The dimeric GYSV cDNA was re-cut and inserted into the Smal site of pGem2 (Fig. 1), generating pGem2-2 which was used as a template for the synthesis of plus and minus sense dimeric GYSV transcripts. Unlabelled plus-sense dimeric GYSV transcripts were obtained by digesting pGem2-2 with BamHI and using SP6 RNA polymerase to synthesize transcripts from the SP6 promoter. Minus-sense dimeric GYSV transcripts were obtained by digesting pGem2-2 with EcoRI and synthesizing transcripts from the T7 promoter using T7 polymerase. Reactions were carried out as described by Melton et al. (1984). Transcripts were re-suspended in 1 mM-Tris–HCl pH 7.5, 0.01 mM-EDTA and transcript integrity was assessed by PAGE under denaturing conditions and subsequent silver staining (Rezaian et al., 1988).

Viroid inoculations. Viroid-free seedlings of Emperor grapevines were inoculated by injection. A micro-syringe was used to deliver 1 μl of viroid inoculum (Table 1) into the cortex of green stem tissue. Inoculum purity was assayed prior to injection by probe hybridization using the specific oligonucleotide probes. All inocula were re-suspended in 1 mM-Tris–HCl pH 7.5, 0.01 mM-EDTA; two injections were given to each plant. The punctures were wrapped with grafting tape and all foliage and visible lateral buds below the point of inoculation were removed. Inoculated plants were grown at about 30 °C either in a glasshouse or a growth room under daily illumination of 16 h (200 to 300 μE/m²·s). Tissue was sampled 3-5 months post-inoculation. All plants were subsequently placed outside under natural summer conditions to induce symptom expression. The average minimum temperature over the 2 months of observation was 9.5 °C and the average maximum temperature was 20 °C.
**RESULTS**

**Electron microscopy of GYSV**

GYSV, GV1B and ASSV belong to a new viroid group on the basis of their common conserved core sequence which is distinct from that found in other viroids (Koltunow & Rezaian, 1988, 1989b). Rod-like secondary structures have been postulated but not demonstrated for the members of this viroid group. However, both the upper and lower strands of the conserved core sequence of these viroids have the potential to form hairpin loop structures; therefore these viroids could also exist as cruciform structures (Fig. 2a). We had sufficient quantities of GYSV for spreading and compared its structure with that of CEV (Visvader & Symons, 1985). CEV is only four residues larger than GYSV and has been demonstrated to exist as a rod-like structure when spread under non-denaturing conditions (Sänger et al., 1976). Fig. 2(c) shows that GYSV molecules have a rod-like structure when spread under non-denaturing conditions and that the size of GYSV is similar to that observed for CEV as expected (Fig. 2b). No GYSV molecules in a cruciform conformation could be detected. Therefore it is probable that GV1B and ASSV, which both possess the same central core sequence as GYSV, exist also as rod-like structures under these conditions.

**Construction of specific probes for GYSV and GV1B**

GYSV was named as such tentatively because its presence correlated well, but not perfectly, with the grapevine yellow speckle disease when grapevine cultivars of known disease status were screened with a full-length GYSV probe (Koltunow & Rezaian, 1988). However we found that this GYSV probe also hybridized with purified GV1B RNA (Koltunow & Rezaian, 1988), indicating that probes specific to particular sequences of GYSV and GV1B were required to distinguish between these viroids and to assess their role in grapevine disease syndromes.

The degree of similarity between the sequences of GYSV and GV1B is lowest in their right terminal (T2) regions (Koltunow & Rezaian, 1989a). Hence unique sequences from the T2 region of each viroid were chosen for the synthesis of the oligonucleotide probes. These oligonucleotides were labelled at their 5' ends with 32P and their specificity was tested by dot blot hybridization.

Samples of GYSV and GV1B RNA purified from grapevine plants were spotted onto duplicate nitrocellulose filters together with samples of CEV, AGV and the grapevine isolate of HSV, which were used as controls. The filters were then probed with labelled oligonucleotides specific to either GYSV or GV1B. The GYSV oligonucleotide probe hybridized specifically to GYSV and the GV1B probe hybridized specifically with GV1B (Fig. 3). No cross-hybridization was observed with any of the other viroids.

**Infectivity of GYSV and GV1B in grapevine**

**Viroid detection**

To confirm that GYSV and GV1B are true viroids, it was necessary to demonstrate that purified GYSV and GV1B RNA could replicate autonomously when inoculated into viroid-free plants. We had observed previously that GYSV and GV1B could not be transmitted to either cucumber or tomato plants (Rezaian et al., 1988; Koltunow & Rezaian, 1988). Therefore viroid-free grapevine seedlings were inoculated with various inocula containing GYSV and GV1B (Table 1), examined for viroid replication and observed for symptom expression.

The presence of viroid RNA in the inoculated plants was determined by oligonucleotide probe hybridization. Both the GYSV and the GV1B probes were used to determine the viroid content of each plant. This test also acted as a check on the purity of the initial inocula. Both GYSV and GV1B could be transmitted independently to viroid-free grapevines as detected by probe hybridization (Table 1). None of the nine control seedlings gave a positive signal for GYSV or GV1B when tested with the two viroid probes (Table 1, no. 7). Of the 25 plants injected with inoculum containing plus-sense GYSV RNA, 13 contained GYSV (Table 1, no. 1, 2 and 5) and of the nine plants injected with GV1B inoculum, there were seven that contained this viroid (Table 1, no. 3). When a mixed inoculum containing both viroids was injected into
Fig. 2. Analysis of the structure of viroids in the ASSV group. (a) Diagrams of the cruciform structures that all viroids in the ASSV group can potentially form. In all cases the upper and lower strands of the conserved core sequence cap the top and the bottom vertical rod of each cruciform, respectively. (b, c and d) Electron microscopic analysis of viroids and DNA after spreading under non-denaturing conditions. (b) CEV. (c) GYSV. (d) DNBA. The bar represents 100 nm.
Viroids causing grapevine yellow speckle

Table 1. Infectivity of GYSV and GV1B in viroid-free grapevine seedlings

<table>
<thead>
<tr>
<th>No.</th>
<th>Inoculum</th>
<th>Number of plants</th>
<th>Viroid detected</th>
<th>Number of plants with yellow speckle symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GYSV (6 ng/plant)</td>
<td>16</td>
<td>8</td>
<td>-*</td>
</tr>
<tr>
<td>2</td>
<td>Total nucleic acid containing GYSV (34 μg/plant)</td>
<td>5</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>GV1B (6 ng/plant)</td>
<td>9</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>GYSV + GV1B (6 ng/plant)</td>
<td>5</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>GYSV plus transcript (200 ng/plant)</td>
<td>4</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>GYSV minus transcript (200 ng/plant)</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>None</td>
<td>9</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* - , Not detected.

grapevines (Table 1, no. 4) both viroids replicated in three out of the five inoculated plants, whereas in only one of these plants was GV1B found alone (Table 1, no. 4). These results show that GV1B and GYSV can replicate autonomously in inoculated grapevines and that both viroids can also replicate together within the same plant.

Symptom expression

One of the four plants which had been injected with plus sense dimeric GYSV transcripts contained monomeric GYSV, as observed by Northern blot analysis (Table 1, no. 5). A yellowish-green speckling indicative of severe yellow speckle disease (Taylor & Woodham, 1972) was observed along the veins on some of the leaves of this plant (Fig. 4a). This provided direct evidence that the molecule we have sequenced and named GYSV is involved in yellow speckle disease. Plants injected with minus sense dimeric GYSV transcript did not contain GYSV and were symptomless (Table 1, no. 6).

All of the inoculated and uninoculated plants were maintained under natural summer conditions and observed for the expression of yellow speckle disease symptoms. In total, yellow speckle symptoms were observed on nine inoculated plants, four of which had been inoculated with GV1B only (Table 1). Thus only plants in which GV1B or GYSV had replicated expressed yellow speckle disease symptoms; no symptoms were observed on any of the nine control plants.
Fig. 4. Yellow speckle symptoms observed on the leaves of inoculated grapevines. (a) Leaf from a plant inoculated with plus-sense dimeric GYSV transcript showing severe yellow speckle symptoms. (b) Leaf from a plant inoculated with GV1B showing symptoms of yellow speckle disease with relatively few scattered speckles.

(Table 1, no. 7) nor on those inoculated plants which contained neither of the two viroids. The frequency of symptom expression was low compared with the number of inoculated plants in which the viroids had been detected. The degree of symptom expression on the leaves of the inoculated plants ranged from a few chrome yellow spots to yellowish-green vein banding (Fig. 4). These symptoms are similar to the field symptoms of yellow speckle which are variable within individual plants of the same grapevine cultivar and between seasons (Krake & Woodham, 1983). Despite these problems, our results show that both GYSV and GV1B can independently induce symptoms resembling the grapevine yellow speckle disease.

Field occurrence of GYSV and GV1B in grapevine cultivars

The GYSV- and GV1B-specific probes were used to detect GYSV and GV1B in grapevine cultivars of known disease status as determined by graft indexing. Table 2 shows that GYSV is widespread in grapevine cultivars grown in Australia and that it also occurred in plants which had been indexed as negative for yellow speckle disease. However GV1B correlated more closely with the results obtained by graft indexing as GV1B was observed in nearly all plants that had been indexed positive for yellow speckle disease (Table 2). The only exception were plants which had been regenerated from fragmented shoot apex culture at a culture temperature of 35°C (Barlass et al., 1982). These grapevines expressed yellow speckle disease and contained GYSV but not GV1B (Table 2) as observed in our experimental transmission studies. In the surveyed plants GV1B was always found together with GYSV. However the replication of GV1B is nevertheless independent from that of GYSV as they can replicate autonomously in grapevines (Table 1).

DISCUSSION

We have confirmed that GYSV exists as a rod-like molecule when spread under non-denaturing conditions and visualized by electron microscopy. We have confirmed that GYSV and GV1B are viroids by demonstrating their infectivity in experimental transmission tests using grapevine seedlings inoculated with purified viroid samples. These infectivity studies showed that each viroid is capable of replicating independently in grapevine and inducing symptoms of the yellow speckle disease. Plus-sense RNA transcripts of a dimeric GYSV cDNA clone produced monomeric viroid progeny and yellow speckle symptoms in one inoculated plant
indicating that the GYSV molecule we have sequenced is directly involved in the yellow speckle disease. The use of an in vitro synthesized RNA transcript for inoculation rules out the possibility of a contaminating viroid being present in the inoculum.

Not all of the plants inoculated with GYSV and GV1B became infected. It is not clear whether this reflects either a variation between the test plants, which are open pollinated, genetically heterogeneous seedlings, or the inefficiency of the mechanical inoculation procedure.

The screening of grapevines of known disease status with probes specific for the detection of GYSV and GV1B showed a close relationship with the yellow speckle disease, although GYSV was also detected in cultivars which had been indexed as negative for this disease. There are at least three reasons for the inconsistencies observed between graft indexing and probe hybridization. One reason may be the existence of mild or symptomless variants of GYSV in the surveyed plants. We have observed that sequence variants of GYSV exist both within a particular GYSV source and between sources of GYSV (Koltunow & Rezaian, 1988; A. M. Koltunow & M. A. Rezaian, unpublished results). The existence of sequence variants may explain the differences in the severity of symptoms which have been observed on clonal
indicators (Taylor & Woodham, 1972; Shanmuganathan & Fletcher, 1980). The oligonucleotide probe for GYSV would detect GYSV sequence variants provided that significant residue changes did not occur in the region corresponding to probe hybridization.

A second reason for the inconsistencies observed is that indexing for yellow speckle is difficult as the expression of this symptom is erratic (Taylor & Woodham, 1972; Shanmuganathan & Fletcher, 1980). Usually only a few leaves express symptoms on an infected plant and symptom expression in a plant may be non-reproducible from one year to another (Taylor & Woodham, 1972; Shanmuganathan & Fletcher, 1980). For these reasons indexing for yellow speckle disease is carried out for at least two growing seasons. Thirdly, the observed inconsistencies between the indexing and the probe hybridization survey may relate to the horizontal spreading of yellow speckle disease after the indexing had been conducted. This is possible, as a low incidence of natural transmission of this disease has been observed in the field (Woodham & Krake, 1982).

Three viroids, named GV1, GV2 and GV3 have been isolated from grapevine cultivars in California (Semancik et al., 1987; Szchowskii et al., 1988). The size and electrophoretic behaviour of GV1, GV2 and GV3 suggest that they are similar to GYSV (Koltunow & Rezaian, 1988), GV1B (Koltunow & Rezaian, 1989a) and HSV (Sano et al., 1985; Rezaian et al., 1988) respectively. If GV1 and GV2 are related to GYSV and GV1B respectively, they may be involved in yellow speckle disease. The latter occurs in California but is mostly latent in the field. Mink & Parsons (1975) demonstrated the disease in Californian grapevine cultivars by indexing in controlled environment chambers. Taylor & Woodham (1972) reported that clones of grape cultivars which were symptomless in California expressed yellow speckle symptoms when grown in Merbein, Australia. The characterization of GV1 and GV2 by sequencing will establish the degree of relatedness between these two viroids and GYSV and GV1B.

REFERENCES


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