Relationships among the viroids derived from grapevines

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There have been numerous reports of grapevine viroids, describing physical and biological properties suggestive of similar or identical molecular forms. With consideration of these properties and the application of random-primed and specific cDNA probes, four major groups of grapevine viroids have been defined. Designations which can be used to describe distinct viroids within the four groups include (i) CEVd-g, a grapevine isolate of citrus exocortis viroid, (ii) GVd-c, a grapevine viroid recovered from cucumber, and AGVd, Australian grapevine viroid, (iii) GYSVd-1 and GYSVd-2, two viroids inducing yellow speckle disease and (iv) HSVd-g, a grapevine isolate of hop stunt viroid.

Introduction

Different descriptions of grapevine viroids reported previously have created the illusion of a larger number of viroids than probably exists. Degrees of relatedness among individual grapevine viroids have been suggested but not rationalized. Nucleotide sequence similarity of a grapevine viroid (GVd) (Sano et al., 1986) with hop stunt viroid (HSVd) has led to HSVd-grapevine (HSVd-g) being accepted as an isolate of HSVd. Other variants of HSVd-g have also been reported by Duran-Vila et al. (1987) and Puchała et al. (1988). Similarly, grapevine viroid-slow (GVd-s) (Flores et al., 1985) is a variant of citrus exocortis viroid (CEVd) designated as CEVd-grapevine (CEVd-g) by Garcia-Arenal et al. (1987). The original designation of grapevine viroid-fast (GVd-f) (Flores et al., 1985) was recognized as GVd-1 after detection of a fourth grapevine viroid, GVd-2 (Semancik et al., 1987).

With the active world-wide exchange of vegetatively propagated grapevine materials, it is reasonable to suspect that many of the viroids inferred to be distinct agents probably represent identical biological entities. This view is supported by a recent study (Szychowski et al., 1991) indicating a high degree of similarity among viroids isolated from sources in California and Europe.

The relationships suggested by common physical and biological properties among several of the grapevine viroids have yet to be tested. The characteristic electrophoretic properties of GVd-1 are shared by grapevine yellow speckle viroid (GYSVd) (Koltunow et al., 1989). Similarly, the physical properties and pattern of occurrence in grapevine cultivars of GVd-2 and GVd-1B (Koltunow & Rezaian, 1989) suggest a close relationship between these two viroids. The host range and partial relatedness to CEVd-g and GYSVd described for Australian grapevine viroid (AGVd) (Rezaian, 1990) are similar to the properties of GVd-c, reported here.

Utilizing the parameters of biological screening by differential hosts, electrophoretic properties and molecular hybridization with cDNA probes, relationships among the grapevine viroids emerge. In the study reported here, properties of grapevine viroids have been analysed and correlated to provide the basis for a grouping system. Portions of this proposal have already been introduced (Semancik & Szychowski, 1991).

Methods

Viroid culture and purification. Plant materials were produced and processed as previously reported (Szychowski et al., 1988). Sequential PAGE was performed according to the procedure of Rivera-Bustamante et al. (1986). The final purification of the isolated viroids included electrophoresis of the viroid from denaturing gels containing 8 M-urea.

Molecular hybridization. Radioactive \(^{32}P\)CTP-labelled cDNA probes were synthesized from either purified viroid templates using the random priming reverse transcriptase procedure (Maniatis et al., 1982), or oligonucleotides specific to known viroids (Table 1) and \(^{32}P\)ATP end-labelled by T4 polynucleotide kinase. Hybridization was performed against either formaldehyde-denatured purified viroids slot-blotted onto nitrocellulose and Nytran membranes or partially purified viroid preparations after electrotransfer of the viroid-containing region directly from denaturing gels onto Nytran membranes (Semancik et al., 1988).

Hybridization assays were performed under conditions (i) routinely employed with random probes (Garger et al., 1983) and (ii) as recommended with end-labelled oligonucleotides (Koltunow et al., 1989). Condition (i) included hybridization in 50% formamide and 5 x saline-sodium phosphate-EDTA at 42 °C for 24 h with two
Table 1. Relative identity among grapevine viroids based on hybridization analyses with cDNA probes

<table>
<thead>
<tr>
<th>Grapevine viroid</th>
<th>GVd-1</th>
<th>GVd-2</th>
<th>GVd-3</th>
<th>CEVd-g</th>
<th>GVd-c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random probes*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+t</td>
<td>-</td>
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<tr>
<td>GVd-1</td>
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<tr>
<td>GVd-2</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>GVd-3</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>CEVd-g</td>
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<td>+</td>
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<tr>
<td>GVd-c</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Specific probes†</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CEVd</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>HSVd</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>GVd-1B</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GYSVd</td>
<td>+</td>
<td>-</td>
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</tbody>
</table>

* Complementary DNA was produced by reverse transcriptase synthesis from a purified viroid template randomly primed with herring sperm DNA fragments.
† Relative hybridization was determined by evaluation of intensity on autoradiographs following slot-blot and electrotransfer hybridization.
‡ Specific oligonucleotide probes were synthesized to the indicated viroids: CEVd probe, a 35-mer (cDNA to 214 to 248), a gift of M. Bar-Joseph; HSVd-1 probe, a 17-mer (cDNA to 72 to 88), Sano et al. (1988); GVd-1B probe, a 20-mer, Koltunow et al. (1989); GYSVd probe, a 19-mer, Koltunow et al. (1989).

washes each in 2 x SSC, 0-1% SDS at room temperature for 10 min and 0-1 x SSC, 0-1% SDS at 55 °C for 20 min. Condition (ii) included hybridization in 50% formamide and 5 x SSC at 34 °C for 24 h with two washes each in 6 x SSC, 0-1% SDS at room temperature for 10 min and at 30 °C for 20 min.

Results

A new viroid from grapevines, GVd-c, detected following biological amplification in cucumber

When an inoculum preparation containing a partially purified mixture of only GVd-1 and GVd-2 from the cultivar Cardinal was inoculated to cucumber, a viroid identified here as grapevine viroid-cucumber (GVd-c) was shown to be distinct from GVd-1 and GVd-2.

Under non-denaturing PAGE conditions, migration of GVd-c was similar to that of GVd-1 but distinctly separable from CEVd-g (Fig. 1). Thus, GVd-c could also be confused with a GVd-f form (Flores et al., 1985). These properties also differentiate GVd-c from AGVd, another viroid recovered from cucumber, but reported to be indistinguishable from CEVd in size and electrophoretic properties (Koltunow & Rezaian, 1988).

When compared with the reaction of GVd-c with its homologous probe, weak but positive signals were observed with random-primed probes to GVd-1 and CEVd-g (Fig. 2). The GVd-c random-primed probe, however, gave only an extremely weak signal with GVd-l and none with CEVd. These reactions, summarized in Table 1, suggest minor similarity of GVd-c to GVd-1 and CEVd-g.
Relationships among grapevine viroids by molecular probes and electrophoretic properties

(i) Nucleotide sequence identity from slot-blot hybridization with purified viroid preparations

The five grapevine viroids contained in the California collection, GVd-1, GVd-2, GVd-3, CEVd-g and GVd-c, were utilized to construct a series of cDNA probes by the random priming procedure. Oligonucleotide probes reported to be specific for HSVd, CEVd, GYSVd and GVd-1B (Table 1) were also tested against the viroid collection. In all tests, isotope levels of the end-labelled oligonucleotides were in 10- to 20-fold excess over the random primed probes.

A comparison was made of hybridization reactions with slot blots of pure GVd-1 against either random-primed cDNA synthesized to a GVd-1 template or end-labelled oligonucleotide specific to GYSVd (Fig. 3). When tested on nitrocellulose membranes as previously described (Rezaian et al., 1988), the GYSVd-specific probe displayed only faint and inconsistent signals with very high background. This background was not observed with either the GVd-1B- or HSVd-specific oligonucleotide probes synthesized by the same protocol.

Although the reason for the reactivity of the GYSVd-specific oligonucleotide with nitrocellulose was not determined, the high background could be obviated by employing a nylon-based membrane (Nytran). A low but positive signal was confirmed with GVd-1 from several sources. Oligonucleotide probes reported to be specific to GVd-1B and HSVd were also tested against GVd-2 and GVd-3, respectively. A positive reaction was registered between the GVd-1B-specific probe and GVd-2 under condition (ii) (see Methods), whereas the GVd-2 probe synthesized by the random-priming procedure registered a strong positive signal under both conditions.

Both the HSVd-specific probe (Sano et al., 1988) as well as the random-primed GVd-3 probe reacted positively against GVd-3 under both conditions. These reactions identify GVd-3 as a member of the HSVd group. The signals produced by the GYSVd and GVd-1B probes were consistently weaker than those of the random-primed GVd-1 and GVd-2 probes. When high levels (1 x 10^6 c.p.m.) of the latter two probes were used, weak heterologous reactions could also be detected suggesting partial sequence identity.

(ii) Correlation of sequence identity with electrophoretic properties

Added confidence in the interpretation of signals from the slot-blotted preparations was derived from hybridization of viroids electrotransferred directly from denaturing gels. In this way, evidence for both the relative molecular size and sequence identity could be evaluated.

This can best be illustrated utilizing the GVd-2 probe.

Fig. 3. (a) Autoradiograph of slot-blot containing pure GVd-1 from Cabernet franc in two reaction sets of 1 x, 1/2 x and 1/4 x after hybridization on nitrocellulose with either GVd-1 random-primed probe at 4 x 10^3 c.p.m./ml of hybridization buffer or GYSVd-specific oligonucleotide at 5 x 10^6 c.p.m./ml under conditions (i) and (ii) (upper panel) (see Methods). Autoradiograph of slot blot containing pure GVd-1 from the two cultivars Cabernet franc and Mission in two reaction sets of 1 x and 1/2 x after hybridization on a Nytran membrane with either GVd-1 random primed probe at 1 x 10^5 c.p.m./ml or GYSVd-specific oligonucleotide at 1 x 10^6 c.p.m./ml under condition (ii) (lower panel). Note marked reduction in background on the Nytran membrane with GYSVd probe. (b) Autoradiograph of slot-blot containing pure GVd-2 from the rootstock 039-16 in two reaction sets of 1 x, 1/2 x and 1/4 x after hybridization on nitrocellulose with either random-primed GVd-2 at 1 x 10^6 c.p.m./ml or GVd-lB-specific oligonucleotide at 1 x 10^6 c.p.m./ml under conditions (i) and (ii). (c) Autoradiograph of slot-blot containing pure GVd-3 from Cabernet Sauvignon in two reaction sets of 1 x, 1/2 x and 1/4 x after hybridization on nitrocellulose with either random-primed GVd-3 at 2.5 x 10^5 c.p.m./ml or HSVd-specific oligonucleotide at 1 x 10^6 c.p.m./ml probes under conditions (i) and (ii).
against extracts containing different viroid profiles as shown in Fig. 4. Both the Gvd-2 and Gvd-1B probes reacted positively with Gvd-2 providing strong evidence for the identity of these two viroids. The random-primed Gvd-2 probe also reacted with extracts containing Gvd-1 (Fig. 4, lanes 1 and 4). Thus, partial sequence identity between Gvd-1 and Gvd-2 is implied.

When Gvd-1 probes with high specific activity were employed against preparations with high concentrations of Gvd-2, weak signals were recorded, again indicating partial sequence similarity between Gvd-1 and Gvd-2. The GYSVd-specific oligonucleotide probe was consistently negative in tests with electrotransferred samples of grapevine viroids. The synthesized oligonucleotide probes specific for HSVd and CEVd reacted strongly with Gvd-3 and CEVd-g, respectively. A summary of the hybridization reactions for the California grapevine viroid collection-following both slot-blot and electrotransfer procedures is presented in Table 1.

Discussion

A general plan has been offered (Semancik & Szychowski, 1991) for the clustering of viroids based upon their recovery from specific hosts. Viroids isolated directly from infected grapevines, or apparent viroids, include Gvd-1, GYSVd, Gvd-2, Gvd-1B and HSVd-g. Enhanced viroids include CEVd-g, AGVd, and Gvd-c which are recovered from alternative hosts following inoculation of a grapevine extract containing trace amounts of viroid. This broad clustering can now be expanded into more specific groups defined by properties describing molecular size, sequence identity and disease relationships.

A proposal for the organization of grapevine viroids into four groups is presented in Table 2. Implicit in this scheme is the prospect that each group will inevitably include a series of closely related variants derived either from grapevines or by selection in different hosts, as appears common for the HSVd group. Specific designations can be assigned to individual viroids within each group as properties dictate.

Group 1 and group 4 define two grapevine variants, CEVd-g and HSVd-g, of well characterized viroids. With the deviation of nucleotide sequence exhibited by these variants and lack of evidence of biological equivalence, it seems prudent to retain a reference to grapevine. The suffix -g is used to denote grapevine as the source.

Group 2 contains two enhanced viroids, AGVd and Gvd-c, both of which are amplified in cucumber. AGVd cannot be differentiated from CEVd-g either electrophoretically (Koltunow & Rezaian, 1988) or in nucleotide number (Garcia-Arenal et al., 1987; Rezaian, 1990), whereas Gvd-c has been shown to be electrophoretically distinct from CEVd-g. However, these enhanced viroids can become apparent in grapevines after introduction into viroid-free vines.

Group 3 is based on symptom expression in grapevines. GYSVd and Gvd-1B have been reported to induce yellow speckle disease symptoms (Koltunow et al., 1989) and the distribution of Gvd-1 in grapevines also suggests an association with the disease (Flores et al., 1985).

The relationship between Gvd-1 and GYSVd is sustained by the reaction of the GYSVd-specific probe with Gvd-1 and the unusually rapid migration under non-denaturing conditions of PAGE. This property shared by GYSVd and Gvd-1 mark the molecules as possessing a most atypical conformation (Semancik, 1986) and strongly supports a commonality for these viroids. Other data supporting this conclusion are the widespread occurrence of Gvd-1 (Semancik et al., 1987; Minafra et al., 1990; Szychowski et al., 1991) and...
GYSVd (Koltunow et al., 1989) across a wide range of grapevine cultivars. The designations GYSVd-1 and GYSVd-2 have been offered by Semancik & Szychowski (1991) for GYSVd and GVd-1B, respectively.

The similar biological activity of the two viroids GYSVd-1 and GYSVd-2 in the presence of only partial sequence similarity may offer an explanation for the extreme variability in symptom expression reported for the yellow speckle disease (Shanmuganathan & Fletcher, 1980).

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References


