Detection of Viroid and Viroid-like RNAs from Grapevine

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SUMMARY

Analysis by polyacrylamide gel electrophoresis of nucleic acid preparations, obtained from several varieties of grapevine by a procedure designed to isolate and purify viroids, revealed the presence of RNA species with some of the characteristic physical properties of viroids. Under non-denaturing conditions, a band with a mobility faster than that of citrus exocortis viroid (CEV) was detected, and under fully denaturing conditions two bands were observed, one co-migrating with the circular forms of CEV and a second migrating faster than the linear forms of this viroid. This RNA species did not hybridize with a cDNA probe to CEV. Some of the grapevine preparations were infective for Gynura aurantiaca, inducing symptoms similar to those caused by CEV, and the appearance of an RNA which had the same mobility as CEV in denaturing and non-denaturing electrophoretic systems and hybridized with cDNA to CEV. These results suggest that viroid-like and viroid RNAs can be recovered from grapevine, the former (with no detectable sequence homology to CEV) at a concentration sufficient to be observed as a physical entity in gels, and the latter (with close sequence homology to CEV) whose presence could only be revealed by bioassay. The possible involvement of these RNAs in some grapevine diseases of unknown aetiology is discussed.

INTRODUCTION

Grapevine, an important crop in many areas of the world, is affected by a number of diseases whose aetiological agents have not yet been isolated and identified (Bovey et al., 1980). This group of diseases, including leafroll, corky bark, stem pitting (legno riccio) and yellow speckle, are distributed world-wide and cause significant economic losses. They are considered to be virus-like diseases on the basis of their transmissibility and because the symptoms caused are like those induced by other virus maladies. Nevertheless, it is possible that a viroid, rather than a virus, could be the causal agent of some of these diseases. Viroids are a group of small pathogenic RNAs of plants endowed with autonomous replication (Diener, 1979; Semancik, 1979), which have been proved to cause an increasing number of diseases of higher plants (Sänger, 1982). In this article we report data indicating the presence of viroid and viroid-like RNA molecules in grapevine, and we discuss the possible relationships between these RNAs and some grapevine diseases.

METHODS

Extraction of nucleic acids from grapevine tissue. Young apical leaves and stems were taken from field plants in the summer of 1983 and the spring and summer of 1984. Spanish samples were, unless otherwise stated, of the local red variety Bobal and collected in two different areas of Valencia (Spain). American samples were kindly provided by Dr A. C. Goheen from the collection of the University of California at Davis (U.S.A.). Nucleic acids were extracted as described by Marton et al. (1982). Fresh or frozen tissue (150 g) was mixed with 500 ml water-saturated phenol (adjusted to pH 7 with NH₄OH), 10 ml 2-mercaptoethanol and 200 ml extraction medium (EM).
The EM was prepared by mixing 1 vol. 5% SDS, 1 vol. 0.1 mM-EDTA pH 7, 0.4 vol. 2 mM-Tris—HCl pH 8.9, 1.5 vol. 20% polyvinylpyrrolidone (PVP-10, Sigma), 0.6 vol. 10 mM-LiCl and 1.5 vol. distilled water. The mixture was homogenized at room temperature with an Osterizer liquefier-blender. After centrifugation at 8000 g for 15 min, the upper aqueous phase was re-extracted with 0.5 vol. water-saturated phenol. The nucleic acids in the final aqueous phase were precipitated with 2.5 vol. ethanol at -20 °C for several hours. The pellet was collected by centrifugation at 8000 g for 15 min, dried, resuspended in 40 ml TKM buffer (10 mM-Tris—HCl pH 7.4, 10 mM-KCl, 0.1 mM-MgCl₂) and dialysed overnight against the same buffer containing 10 mM-2-mercaptoethanol. The non-resuspended material was removed by centrifuging at 8000 g for 15 min.

Cellulose chromatography. The procedure of Franklin (1966) was followed with some minor modifications. The cleared supernatant from the resuspended ethanol precipitate was adjusted to STE (50 mM-Tris-HCl pH 7.2, 100 mM-NaCl, 1 mM-EDTA) and 35% ethanol, and applied to a column of 5 to 7 g cellulose (CF-11, Whatman) prewashed with 35% ethanol in STE, and then washed with 250 ml of the same solution, 250 ml 25% ethanol in STE and finally with 30 ml STE. The nucleic acids eluted in the last wash were precipitated by adding 0.1 vol. 3 M-sodium acetate pH 5.5 and 2.5 vol. ethanol. The pellet was recovered by centrifugation, dried and resuspended in 0.5 ml TKM. In order to complete rRNA precipitation, 1 vol. 4 M-LiCl was added and the resulting solution was allowed to stand at 4 °C overnight. The pellet obtained by centrifugation (8000 g for 15 min) was discarded and soluble nucleic acids were recovered by precipitation with 3 vol. ethanol. The pellets were collected, dried and resuspended as before. In some cases a second cellulose chromatography was performed on a smaller column (2 to 3 g) in order to enrich for viroid-like and dsRNAs.

Polyacrylamide gel electrophoresis. The method of Schumacher et al. (1983), as modified by Semancik & Harper (1984), was followed. The solution of nucleic acids obtained after the cellulose chromatography was subjected to one cycle of 5% polyacrylamide gel electrophoresis under non-denaturing conditions (Morris & Wright, 1975). A preparation of citrus exocortis viroid (CEV), used as a marker, was electrophoresed in the two outside lanes. After staining with ethidium bromide (0.5 μg/ml in water for 10 min), a 1 cm segment of the gel containing CEV and about 0.8 cm below it was cut and applied directly on top of a 5% fully denaturing polyacrylamide gel (Sänger et al., 1979). Following electrophoresis it was stained with ethidium bromide. The same gel was stained subsequently with silver according to the procedure of Sammons et al. (1981) with the modifications of Igloi (1983). For preparative purposes, the visible bands after ethidium bromide staining in the zones corresponding to the circular and linear forms were cut and both types of molecules isolated by electroelution in small dialysis bags.

Preparation of a complementary DNA probe to CEV. Complementary DNA (cDNA) was synthesized by the method of Taylor et al. (1976) as modified by Maniatis et al. (1982). Reaction mixtures of a final volume of 50 μl contained 100 mM-Tris—HCl pH 8.3, 10 mM-MgCl₂, 100 mM-KCl, 10 mM-dithiothreitol, 25 units of human placental ribonuclease inhibitor (Amersham), 1:2 mM each of dATP, dGTP and dTTP, 50 μCi [α-32P]dCTP (Amersham, sp. act. 3000 Ci/mmol), 1 μg CEV RNA, 35 μg calf thymus deoxynucleotide primers purified by passage through DEAE-cellulose, and 32 units avian myeloblastosis virus reverse transcriptase (Boehringer). CEV RNA and DNA primers were mixed, heated for 1 min at 100 °C, and quenched in an ice water-bath before addition to the synthesis reaction (Owens, 1978). After adding the RNA-dependent DNA polymerase, the reaction mixture was incubated at 37 °C for 3 h and then stopped by the addition of 2 μl 0.5 mM-EDTA pH 8 and 25 μl 150 mM-NaOH. During a subsequent incubation for 1 h at 65 °C the RNA template was hydrolysed. The resulting solution was neutralized with 25 μl 1 M-Tris—HCl pH 8 and 25 μl 1 M-HCl and then chromatographed on a small column of Sephadex G-50 (fine) to separate the cDNA from low molecular weight material. The cDNA was concentrated by ethanol precipitation, using tRNA from yeast as a carrier, and resuspended in a small volume of distilled water. An aliquot of this solution was applied to a glass fibre filter, which was then washed to determine the radioactive material insoluble in cold 5% TCA.

RNA analysis by dot-blot hybridization. Purified preparations of viroid and viroid-like RNAs were denatured with formaldehyde according to White & Bancroft (1982) and 2.5 μl spots with several dilutions were applied to nitrocellulose membranes (2 × 5 cm) which had been pretreated with distilled water, equilibrated with 20 × SSC (3 M-NaCl, 0.3 mM-trisodium citrate, pH 7.0), and dried with blotting paper and then under a lamp (Thomas, 1983). The spots were allowed to dry at room temperature and after baking the membranes for 2 h at 80 °C, they were prehybridized for 2 to 3 h at 42 or 55 °C with 1 ml of a solution consisting of 50% formamide, 5 × SSPE (1 × SSPE = 0.12 M-NaCl, 0.015 M-trisodium citrate, 0.013 M-Na₂HPO₄ pH 6.5, 2 mM-EDTA), 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.1% SDS and 100 μg/ml of sonicated and denatured calf thymus DNA (Garger et al., 1983). The membranes were then hybridized at 42 or 55 °C with 0.5 ml of a solution containing the radioactive probe (1 × 10⁶ to 1.5 × 10⁶ c.p.m.), plus the same ingredients as in the previous solution except that the calf thymus DNA was not included. After hybridization the membranes were washed three times at room temperature for 15 min in 2 × SSC, 0.1% SDS, twice at 55 °C for 15 min in 0.1 × SSC, 0.1% SDS, and exposed to X-ray film at 4 °C overnight using Dupont Cronex Lightning Plus intensifying screens.

Biocassay. Preparations of nucleic acids were assayed for infectivity by inoculation to Gymnura aurantiaca DC plants (Semancik & Weathers, 1972). Plants were observed for 30 days after inoculation, topped to promote growing of new shoots, and observed for an additional period of 30 days.
RESULTS
Detection of a viroid-like RNA in grapevine

One band with an electrophoretic mobility slightly greater than that of CEV was observed in some grapevine preparations of nucleic acids partially purified by cellulose chromatography, and run in non-denaturing gels stained with ethidium bromide (Fig. 1 a). In order to confirm the viroid-like nature of this band, a segment of the gel containing the CEV position was cut and applied to a fully denaturing gel. Staining with ethidium bromide showed the presence in the grapevine extracts of one band migrating in the zone of circular CEV (Fig. 1 b), strongly suggesting a viroid-like nature, since this zone is very characteristic of circular viroid RNA molecules. Other bands that were not studied further could also be seen in the upper part of non-denaturing gels of grapevine extracts; they correspond probably to RNAs with high degrees of secondary structure. The extraction of nucleic acids was found to be a difficult process with the grapevine tissue, because it contains high levels of interfering substances which affected the recovery of nucleic acids in general. With some samples it was impossible to observe the viroid-like RNA following the procedure stated above. In all cases, a further staining with silver revealed more intense bands with mobilities similar to circular and linear CEV (CEV_c and CEV_L) (Fig. 1 c). Both bands were RNAs, since they did not withstand the action of RNase A (data not shown).

![Fig. 1. (a) Electrophoretic patterns in a non-denaturing polyacrylamide gel stained with ethidium bromide of two preparations of nucleic acids from grapevine (lanes 1 and 2), and one preparation of nucleic acids from G. aurantiaca plants infected with CEV (lane 3). (b) The segment of the previous gel containing CEV and about 0.8 cm below it, as indicated within dashed lines, was cut and applied on top of a denaturing gel which was subjected to electrophoresis and then stained with ethidium bromide. (c) The same gel presented in (b) after silver staining.](image)

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* Samples of the Bobal variety were processed as stated in Methods. The nucleic acids eluting with STE buffer without ethanol from the cellulose chromatography were used for inoculation.
† Number of plants with symptoms/total (five) inoculated.
Fig. 2. Polyacrylamide gel electrophoresis under non-denaturing conditions of: lane 1, viroid obtained from *G. aurantiaca* plants after their infection with nucleic acid preparations from grapevine; lane 2, viroid-like RNA isolated directly from grapevine; lane 3, mixture of lane 2 and lane 4 samples; lane 4, CEV. Gel was stained with silver.

Fig. 3. Polyacrylamide gel electrophoresis under denaturing conditions of linear (a) and circular (b) forms of: lane 1, viroid obtained from *G. aurantiaca* plants after their infection with nucleic acid preparations from grapevine; lane 2, viroid-like RNA isolated directly from grapevine; lane 3, mixture of lane 2 and lane 4 samples; lane 4, CEV. Gel was stained with silver.

**Infectivity of grapevine extracts and evidence of viroid RNA**

In order to investigate the possibility that the viroid-like RNA isolated from grapevine could be infectious, *G. aurantiaca* plants were inoculated by slashing the stems with nucleic acid preparations collected after the cellulose chromatography. Low levels of transmission to this host were observed but only in some preparations (Table 1). The symptoms induced in *G. aurantiaca* were indistinguishable from those caused by CEV, and an infectious RNA with the same electrophoretic mobility as the CEV could be re-isolated (Fig. 2 and 3). These figures present the results of a detailed analysis of the mobilities in different electrophoretic systems of the viroid-like and viroid RNAs obtained from grapevine compared with CEV. Under non-denaturing conditions it was confirmed that the viroid-like RNA from grapevine had a faster mobility than CEV (Fig. 2, lanes 2, 3 and 4). Moreover, the viroid purified from *G. aurantiaca* plants showing symptoms after being inoculated with grapevine preparations of nucleic acids had the same mobility as CEV (Fig. 2, lanes 1 and 2) and, therefore, it was physically distinct from the viroid-like RNA isolated directly from grapevine. The same conclusions could be reached when the purified linear forms of RNA described above were analysed in polyacrylamide gels under denaturing conditions (Fig. 3a), but no differences were observed in the case of the circular forms (Fig. 3b). The viroid-like RNA observed in polyacrylamide gels of grapevine extracts was termed grapevine viroid fast (GV-f), and the viroid isolated from *G. aurantiaca* plants was termed grapevine viroid slow (GV-s). GV-f has been found in Spanish samples of three other red grapevine varieties (Tintorera, Monastrell and Cabernet-Sauvignon) as well as in the Bobal variety, whereas GV-s has only been detected in Bobal samples. In order to discount the possibility that chance contamination of the grapevine preparations with CEV could explain the finding of GV-s, the experiments were carried out independently by two collaborating groups working in different laboratories and using different greenhouses.
Comparative analysis of GV-f, GV-s and CEV by dot-blot hybridization

The results of an analysis of this kind are presented in Fig. 4. GV-s and CEV showed no differences in their ability to hybridize with a probe of cDNA to CEV at 42 °C, which is a standard temperature for hybridization experiments carried out in the presence of 50% formamide (Fig. 4a). In order to study the behaviour of these two RNAs under more stringent conditions, the temperature of hybridization was increased to 55 °C, since Macquaire et al. (1984) have observed that a cDNA to potato spindle tuber viroid (PSTV) is able to hybridize with both PSTV and chrysanthemum stunt viroid (CSV) RNAs at 42 °C, but only with PSTV at 55 °C. Nevertheless, GV-s and CEV hybridized to the same extent with cDNA to CEV at both temperatures (Fig. 4a, b). Taking into account the PSTV and CSV sequence homology of more than 65% (Haseloff & Symons, 1981; Sanger, 1982), and the experimental conditions of hybridization and washing used, which were essentially similar to those reported by Macquaire et al. (1984), we can conclude that GV-s and CEV have a sequence homology of more than 65%.

Conversely, no hybridization could be detected between GV-f and a probe of cDNA to CEV (Fig. 4c). Negative results were also obtained when the stringency of the washing conditions was decreased (results not shown), and, therefore, it can be assumed that GV-f is clearly different from GV-s and CEV in terms of base sequence.

Possible relationships between GV-f, GV-s and grapevine diseases

Grapevine tissue taken from the virus collection at the University of California at Davis (UCD) included the varieties Cabernet-Sauvignon, Cabernet Franc, Emperor, Melon and Zinfandel. These samples contained the agents of the leafroll disease as well as the latent yellow speckle disease, but had been indexed as free of all other known virus-like diseases. Nucleic acid preparations from all varieties were demonstrated to contain an RNA species behaving as the GV-f component, when analysed on denaturing and non-denaturing gels (Fig. 5). Similar results were observed in extracts of healthy Cabernet Franc tissue collected from the UCD Foundation vineyard and known to be free of the leafroll disease. None of the nucleic acid preparations was infectious for G. aurantiaca, thus suggesting that the detection of GV-f and GV-s may represent a complex peculiar to the Spanish variety Bobal.
Analysis of the nucleic acids from several varieties of grapevine demonstrated the presence of apparent circular and linear molecules of small RNAs which are characteristic of viroids. Inoculation of *G. aurantiaca* plants with some of the nucleic acid preparations from grapevine resulted in the onset of the typical symptoms induced in this host by CEV and some other known viroids and, concomitantly, in the appearance in the nucleic acid profile of an infectious RNA with the electrophoretic properties of CEV. A comparative study of the electrophoretic mobilities revealed two patterns. First, the viroid-like molecules detected in grapevine extracts (GV-f) moved faster than those of CEV under native conditions. Under denaturing conditions a behaviour similar to that exhibited in native gels was presented by the linear forms of both RNAs, whereas no differences were observed in the case of the circular forms. Second, the viroid obtained from *G. aurantiaca* plants, as a consequence of their inoculation with nucleic acid preparations from grapevine (GV-s), co-migrated with CEV in both of the electrophoresis systems used. The differences in electrophoretic mobilities between GV-f and GV-s could be explained by assuming that GV-s has a higher molecular weight than GV-f, although these different sizes were not detected in the case of the circular forms under denaturing conditions, maybe due to the low mobility of viroid circular forms under these conditions. Alternatively, differences in conformation rather than in size could also provide a basis for the dual electrophoretic behaviour of GV-f and GV-s.

The results obtained from hybridization studies revealed a close sequence homology between GV-s and CEV, consistent with their indistinguishable size and the type of syndrome that both induced in *G. aurantiaca*. On the other hand, the lack of detectable homology between GV-f and CEV was also consistent with the absence of transmissibility of GV-f to the herbaceous diagnostic host of CEV, *G. aurantiaca*.

Are GV-f and/or GV-s the aetiological agents of some grapevine diseases? Leafroll, corky bark, stem pitting (legno riccio), yellow speckle, fleck, vein mosaic and vein necrosis are examples of maladies of grapevine whose pathogens have not yet been identified, and are assumed to be viruses on the grounds of graft transmission and similarity of symptoms with those of other virus diseases (Bovey *et al.*, 1980). GV-f has been found in leafroll-free samples of the variety Cabernet Franc and therefore it does not seem to be related to this malady, but may be related to the yellow speckle disease (as suggested by Dr A. C. Goheen). Moreover, GV-f is also present in symptomless samples from Spanish field plants of several red varieties (Bobal,
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Tintorera, Monastrell) as well as in ‘healthy’ Cabernet-Sauvignon plants, an indicator of leafroll. These observations suggest that GV-f could be the causal agent of one of the grapevine diseases mentioned above, that are latent in some cultivars and widely dispersed among cultivars and rootstocks (Hewitt & Bovey, 1979; Martelli, 1979). It is well known that viroids are able to replicate at higher temperatures than most viruses (Sanger, 1982), and in this respect it is interesting to point out that yellow speckle is heat-resistant and that yellow speckle-free plants can be produced in vitro at a culture temperature of 27/20°C but not at 35°C, which has been considered as circumstantial evidence in favour of a viroid as the causal agent of this disease (Barlass et al., 1982). The same aforementioned suggestions concerning an hypothetical relationship between some grapevine diseases and GV-f can be extended to GV-s.

In summary, we report here evidence for the presence in grapevine of a viroid-like RNA (GV-f) physically different from CEV and not transmissible to G. aurantiaca as well as an infectious viroid RNA (GV-s) resembling CEV in some physical and biological properties. From a consideration of the detection of GV-f in extracts from various grapevine samples of known diseases, a direct correlation with yellow speckle disease or a hitherto unknown malady can be suggested.

When this manuscript was being prepared for submission, Shikata et al. (1984) and Sano et al. (1985) reported an infectious low molecular weight RNA in grapevines with close sequence homology to hop stunt viroid (HSV) cDNA. The infectivity was located in native polyacrylamide gels in a position where no visible specific band was seen, although this position coincided with that of HSV. The authors suggest that the infectious low molecular weight RNA is a viroid-like RNA resembling HSV. From our results it appears that neither GV-s nor GV-f is the same size as HSV, since this viroid co-migrates in 5% native polyacrylamide gels with 7S RNA (Sano et al., 1982), whereas in this type of gel both GV-s and GV-f showed mobilities slower than 7S RNA (see Fig. 1a and Fig. 5a). Moreover, as stated above, GV-s appears to be closely related to CEV, whereas GV-f does not appear to be infective for cucumber (data not shown). In any case, it can be concluded that both GV-s and GV-f are entities different from the viroid-like RNA detected previously with a cDNA probe to HSV in grapevines.

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