

Citrus B Viroid Identified as a Strain of Hop Stunt Viroid

T. O. DIENER, Research Plant Pathologist, D. R. SMITH, Microbiologist, and ROSEMARIE H. HAMMOND, Research Associate, Microbiology and Plant Pathology Laboratory, USDA-ARS, Beltsville, MD 20705; GIULIANA ALBANESE, Research Associate, ROSA LA ROSA, Research Associate, and M. DAVINO, Professor, Istituto di Patologia Vegetale, Università, Catania, Italy

ABSTRACT

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Citrus B viroid (CBV), differing from the citrus exocortis viroid in host range, symptom expression, and reaction with viroid-specific molecular probes, has been identified in many commercial citrus trees in Sicily. Citrus B viroid replicates in cucumber (*Cucumis sativus* L. 'Suyo') plants and produces symptoms similar to those induced in cucumber by the hop stunt viroid (HSV). Molecular hybridization with HSV-specific RNA probes has demonstrated that the nucleotide sequence of CBV is closely homologous to that of HSV, indicating that the viroid is a strain of HSV. Citrus B viroid, alone or in conjunction with other viroids, may be responsible for inducing the citrus cachexia (xyloporosis) disease.

A number of viroidlike RNAs distinct from citrus exocortis viroid (CEV) have been identified recently in commercial citrus trees (1,3,5,15,16). One of these, citrus B viroid (CBV), occurs either associated with CEV or alone in a number of citrus species and cultivars in Sicily (1). Under denaturing conditions, its electrophoretic mobility is between those of CEV and the fast form of coconut cadang-cadang viroid RNA 1(1). Citrus B viroid replicates in zucchini squash (*Cucurbita pepo* L. 'Italica'), but not in 10 other hosts of CEV (1). In molecular hybridization (dot blot) tests, CBV did not react with full-length CEV or potato spindle tuber viroid (PSTV) cDNA probes, nor with CEV, PSTV, tomato apical stunt viroid, or tomato planta macho viroid RNA probes (1). These and other properties of CBV suggested to us that the viroid might be a strain of hop stunt viroid (HSV) (8,14). We report here that CBV indeed replicates in cucumber (*Cucumis sativus* L.) plants that display symptoms typical of HSV, and that in molecular hybridization CBV strongly reacts with HSV-specific cDNA or RNA probes.

MATERIALS AND METHODS

Source plants and biological tests. Samples were obtained from the same trees used earlier in the investigation of CBV (1), and propagation, isolation, and

inoculation procedures were as described before.

In brief, tissue samples were taken from citrus trees located in different citrus areas in Sicily that had previously been indexed for psorosis, cachexia-xyloporosis, and exocortis diseases by routine tests and were propagated on volkameriana lemon rootstock or grafted-inoculated onto Arizona 861-S-1 citron and grown in the greenhouse at 30-37 C. A severe CEV isolate (PV-194) from the American Type Culture Collection was inoculated into Arizona 861-S-1 citron and into volkameriana lemon as a control.

Young bark of citrus shoots collected in the field (during spring or autumn) or in the greenhouse was used to extract nucleic acids by phenol-chloroform extraction and 2 M LiCl-fractionation (2). Nucleic acids from herbaceous plants were extracted by the method of Macquaire et al (6). Nucleic acids extracted from citrus trees were dissolved in 0.04 M K_2HPO_4 (pH 8.0) buffer and inoculated by stem-slashing into herbaceous hosts.

Molecular hybridization. To prepare ^{32}P -labeled DNA or RNA probes, double-stranded cDNAs of the CEV variant "d" (18) and of the cucumber isolate of HSV (13) were separately inserted into appropriate restriction endonuclease sites (specific for each viroid) of vector pSP65 and amplified in *Escherichia coli* strain OM 83. High specific activity radioactive probes were prepared either by nick-translation using a commercial kit or by in vitro synthesis of an RNA probe using SP6 RNA polymerase and a linearized DNA template.

Samples were prehybridized in 2 ml of buffer containing 40% (v/v) formamide, 0.18M NaCl, 10 mM sodium cacodylate, 1 mM EDTA, 0.1% sodium dodecyl

sulfate (SDS), and calf thymus DNA (300 μ g/ml) at pH 7.0 per 35 cm² of membrane area (nitrocellulose, Schleicher and Schuell, Inc., Keene, NH) at 55 C for 30 min to 1 hr. Dextran sulfate was then added to 10% (w/v) and the membranes were further incubated for 30 min. Hybridization was performed for 18 hr at 42 or 55 C in the presence of 10% dextran sulfate (19) and ^{32}P -labeled probes (1-2 $\times 10^6$ cpm/ml for DNA probes and 0.3-0.5 $\times 10^6$ cpm/ml for RNA probes). The membranes were then washed at 55 C with three changes (10 min/change) of buffer (0.36 M NaCl, 0.02 M Tris, 0.1% SDS, pH 7.0) and then with three changes of a tenfold dilution of the above buffer. Membranes hybridized with RNA probes were given an additional stringent wash at 65 C with three changes (20 min/change) of 0.1 \times SSC (SSC = 0.15 M NaCl, 0.015 M Na citrate, pH 7.0) with 0.1% SDS.

Membranes hybridized with RNA probes were treated with RNase A (1 μ g/ml) in 2 \times SSC at room temperature for 15 min and then rinsed at 50 C for 30 min in 0.1 \times SSC with 0.1% SDS. Autoradiography was carried out overnight at -70 C with Kodak XAR-5 film and an intensifying screen.

RESULTS

Biological tests. Nucleic acids extracted from CEV- and CBV-infected citrus trees were inoculated into cucumber (*C. sativus* 'Suyo') seedlings. Symptoms typical of those of infection with HSV (14) appeared on all plants that had been inoculated with nucleic acid from trees known to be infected with CBV or with CBV and CEV (1), whereas plants inoculated with samples from trees known to contain CEV, but not CBV, remained healthy. Tomato (*Lycopersicon esculentum* Mill. 'Rutgers') seedlings inoculated with samples from trees known to be infected with CEV or with CEV and CBV (1) developed mild symptoms typical of CEV infection (17), whereas plants inoculated with samples from trees known to be infected with CBV, but not CEV, remained symptomless. Only CEV, not CBV, could be recovered from tomato plants inoculated with both CEV and CBV. As in earlier experiments (1), no viroids could be recovered from tomato plants inoculated with CBV alone.

Molecular hybridization. Northern

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blots with samples from trees known to be infected with either CEV or CBV alone, or with both viroids showed that, as in earlier experiments, only RNA bands in samples from trees containing CEV or both viroids reacted with a CEV-specific cDNA probe; no reaction was

obtained with samples from trees infected with CBV alone (Fig. 1, left).

In contrast, when the same membrane was hybridized to an HSV-specific RNA probe, only RNA bands in samples from trees containing CBV or both viroids reacted with the probe; no reaction was

obtained with purified PSTV or with samples from trees infected with CEV alone (Fig. 1, right).

These results were confirmed in dot blot assays in which CEV- and HSV-specific RNA probes were used (Fig. 2). Again, only samples from trees known to contain CBV reacted with the HSV-specific probe. No hybridization was obtained with samples from tomato plants inoculated with both CEV and CBV that were reacted with the HSV-specific probe (Fig. 2), confirming the results of the biological tests described above.

To rule out nonspecific hybridization of nucleic acids with the RNA probes, membranes were treated with RNase A as described in Materials and Methods, followed by autoradiography. Although spot densities were somewhat reduced in some cases, this treatment did not reduce spot densities obtained by hybridizing samples containing CBV with the HSV-specific RNA probe (data not shown).

DISCUSSION

Results presented demonstrate that citrus B viroid is a strain of HSV. Comparison of its electrophoretic mobility under denaturing conditions with those of known viroids suggests that CBV consists of approximately 300 nucleotides, a size that is compatible with the size of HSV (297 nucleotides) (8) or with its cucumber isolate (cucumber pale fruit viroid, 303 nucleotides) (13).

An HSV-like viroid has been reported to occur in symptomless Etrog citron plants grown in Japan (9). Whether CBV is identical with this viroid remains to be determined. An HSV-like viroid has also been reported to occur in almost 90% of grapevines from Europe, the United States, and Japan (10,12). Its nucleotide sequence differs from that of HSV at one position only (11). In Spain, a viroid related to severe isolates of CEV has been isolated from grapevine and its sequence has been determined (4).

The presence in grapevines of HSV in Japan and of CEV in Spain has been correlated with the proximity of commercial grapevine and hop plantations in Japan and of grapevine and citrus plantations in Spain (4).

The properties of CBV reported earlier (1), as well as those described here, suggest similarity with a viroid that has been found associated with the cachexia (xyloporosis) disease of citrus (16) and a citrus viroidlike RNA IIa (3). Like CBV, these viroids consist of about 300 nucleotides, display no homology to CEV, and could not be recovered from CEV hosts such as *Gynura aurantiaca* (Blume) DC or tomato. Like CBV, the viroids could be recovered from *C. sativus* 'Suyo' plants. Contrary to our observations, however, the cachexia-associated viroid did not cause symptoms in cucumber (16).

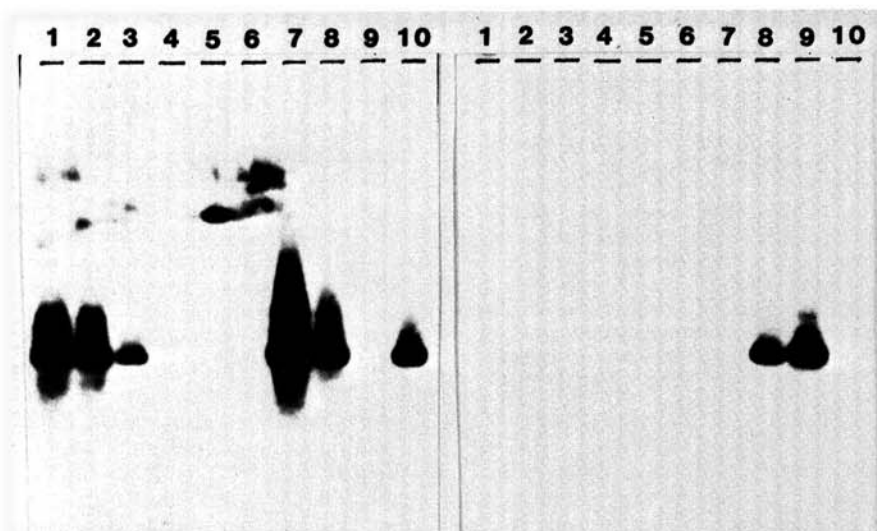


Fig. 1. Autoradiographs of Northern blots with glyoxylated nucleic acid extracts (7) of: 1-5, potato spindle tuber viroid-inoculated tomato at increasing dilutions; 6, healthy tomato; 7, citron inoculated with citrus exocortis viroid (ATCC PV-194); 8, Navelina 8 sweet orange infected with citrus exocortis viroid and citrus B viroid; 9, citron inoculated with Navelina 1 sweet orange infected with citrus B viroid; 10, gel-purified potato spindle tuber viroid. Samples electrophoresed in 1.5% agarose gel. Left: hybridization with citrus exocortis viroid-specific cDNA probe; right, same membrane hybridized with hop stunt viroid-specific RNA probe.

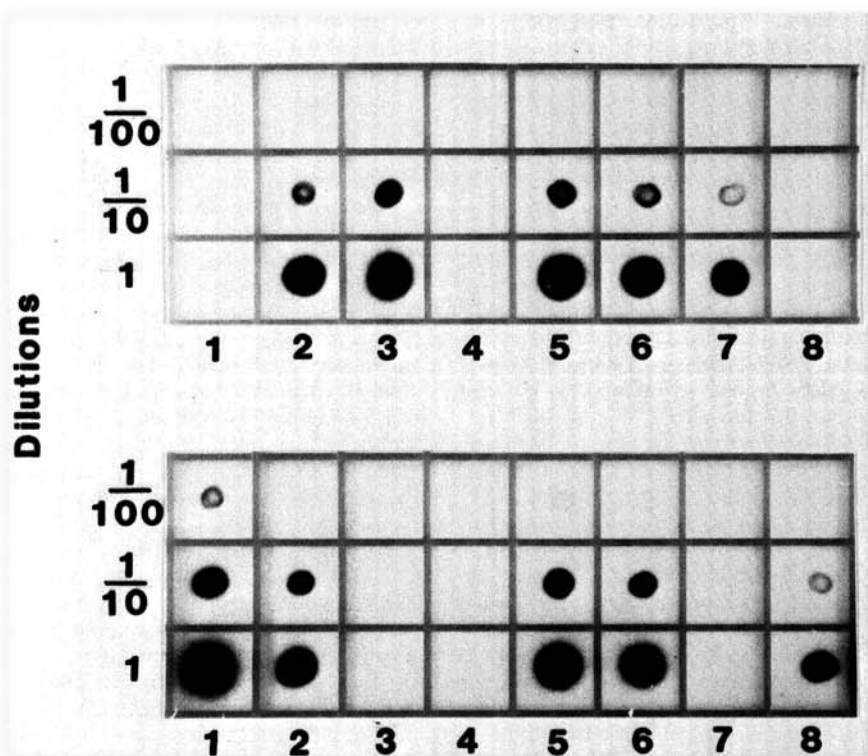


Fig. 2. Autoradiographs of nitrocellulose dot blots with RNA (in 4.6 M formaldehyde and 7.5 X SSC) extracted from: 1, citrus exocortis viroid-infected citrus; 2, 5, 6, citrus exocortis and citrus B viroid-infected citrus; 3, 7, citrus B viroid-infected citrus; 4, healthy citrus; 8, citrus exocortis and citrus B viroid-inoculated tomato. Hybridization with (top) hop stunt viroid-specific RNA probe, (bottom) citrus exocortis viroid-specific RNA probe.

In view of these findings it appears possible that a strain of HSV, either alone or in conjunction with one or more other viroids, causes the cachexia (xyloporosis) disease of citrus. It is evident that only complete nucleotide sequencing of the various viroids detected in citrus trees will elucidate their mutual relationships. Such work is presently in progress.

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