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Hybridization Analysis of Citrus Viroids with Citrus Exocortis Viroid- and Hop Stunt Viroid-Specific Probes

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ABSTRACT. Nucleic acids extracted from viroid-infected commercial citrus trees were analyzed by bidirectional electrophoresis in 7% polyacrylamide gels with the second-direction run performed under denaturing conditions either at homogeneous pH (8.3) or at discontinuous pH (8.3-6.5). Electrotransfer of gel samples to nylon membranes, followed by molecular hybridization tests with citrus exocortis viroid (CEVd)- or hop stunt viroid (HSVd)-specific nucleic acid probes revealed that, of all the viroids detected, only CEVd reacted with the CEVd-specific probes, whereas two viroids reacted with the HSVd-specific probes. Identity of these viroids is discussed in comparison with results of other investigators.

Several new viroids have been detected during recent years in citrus trees and some of their biological, biochemical, and physical properties determined (4, 5, 6, 7, 8, 13, 14). From commercial citrus trees in Sicily, we have isolated a viroid, the citrus B viroid (CBVd), that migrates in bidirectional 5% polyacrylamide gels [with the second direction run made under denaturing conditions] between citrus exocortis viroid (CEVd) and the fast form of coconut cadang-cadang viroid (CCCVd) RNA 1 (1). Dot blot and Northern blot hybridization analysis with CEVd- and HSVd-specific nucleic acid probes showed that the nucleotide sequence of CBVd is similar to that of HSVd but not CEVd (1, 3). In addition to CEVd and CBVd, we have detected several additional viroids that migrate in bidirectional polyacrylamide gels faster than CEVd, but slower than CCCVd RNA 1. We report here on further investigations to characterize these viroids and to compare their properties with those of citrus viroids described by others.

MATERIALS AND METHODS

Samples from viroid-infected field citrus trees were extracted as previously reported (1) and the resulting nucleic acid preparations loaded in triplicate onto 5% polyacrylamide gels and electrophoresed in the first direction as described (7). For the run in

the second direction, the viroid-containing region of each gel was excised and placed below the bottom of 7% polyacrylamide gels; and the gels were electrophoresed in either of two buffer systems, as follows: 1) gels prepared in TBE buffer (90mM Tris, 90mM boric acid, 3mM EDTA, pH 8.3) were electrophoresed for 6 hr at 37mA; 2) gels prepared in TAE buffer (12mM Tris, 6mM Na-acetate, 1mM EDTA, pH 6.5) were electrophoresed as described by Rivera-Bustamante *et al.* (11).

With either system, runs were made in TBE buffer. Avocado sunblotch viroid (ASBVd) or CCCVd RNA 1 (fast or slow) were coelectrophoresed as markers. In each experiment, the nucleic acids in one of the gels run in triplicate were silver stained, whereas those in the other two were electroblotted to nylon membranes (Hybond-N, Amersham) with a Bio-Rad transblot apparatus.

DNA probes were prepared from cDNA of the cucumber isolate of HSVd (kindly supplied by Dr. E. Shikata, Hokkaido University, Sapporo, Japan) and the Australian isolate of CEVd, variant "d" (kindly supplied by Dr. R. H. Symons, University of Adelaide, South Australia), cloned in the pSP65 vector and amplified in *Escherichia coli*, strain OM 83 (3).

To prepare DNA probes, vectors carrying viroid cDNAs were digested by appropriate restriction endonu-

cleases; inserts were eluted from agarose gels by DEAE membranes (NA45—Schleicher and Schuell) and nick-translated using a commercial kit (BRL) and $\alpha^{32}\text{P}$ -CTP. Probe-specific activity was 1×10^6 cpm/ μg .

RNA probes were obtained by *in vitro* synthesis using linearized chimeric pSP65, SP6 RNA polymerase, and $\alpha^{32}\text{P}$ -UTP. Prehybridizations and hybridizations were performed at 42 C in 50% formamide, as per instructions (Gen Screen, New England Nuclear, Du Pont, method I). Autoradiography was carried out at 70 C with Amersham Hyperfilm— βmax .

RESULTS

As shown in Fig. 1 and 2, a number of putative viroids migrating between the CEVd and ASBVd markers were identified. Some of these RNAs were barely separated when electrophoresed at homogeneous pH, but electrophoresis at discontinuous pH enhanced their resolution. Determination of their electrophoretic migration rates relative to those of CEVd, ASBVd, or CCCVd showed that these RNAs migrated at rates identical with those of citrus viroids (CVd) I, II, III, and IV, as described by Duran-Vila, *et al.* (5). Consequently, the terminology proposed by these investigators was accepted.

Molecular hybridization analysis showed that, in samples containing CEVd, both circular and linear forms could be detected with CEVd-specific DNA or RNA probes (Fig. 1 and 3). None of the other RNAs reacted with the CEVd probes; however, some bands at positions below that of linear CEVd (thus presumably smaller than CEVd) reacted with the CEVd probes (Fig. 1). Although the identity of these small RNAs is unknown, they could represent degradation products of CEVd or fragments of citrus genomic DNA homologous to CEVd. Only two RNAs reacted with the HSVd probes (Fig. 1); on the basis of their relative electrophoretic mobilities, both of these are considered to be members

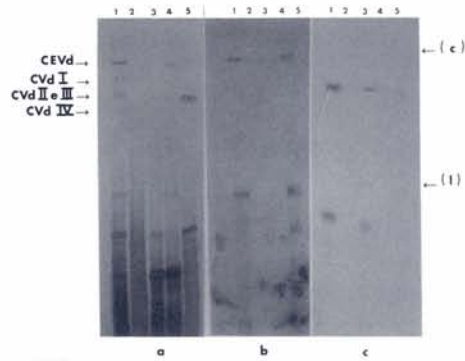


Fig. 1. a) Silver-stained 7% polyacrylamide gel after bidirectional electrophoresis with the second direction run performed under denaturing conditions; b) autoradiograph of a transblot from a gel identical to (a) after hybridization with a CEVd-specific cDNA probe; c) same, but hybridization with an HSVd-specific cDNA probe. Samples were: 1) Redblood sweet orange infected with group I to IV citrus viroids (CVd), the concentration of CEVd in the gel is approximately 150 μg ; 2) healthy citrus; 3) Navelina 2 sweet orange infected with CVd II and III; 4) CEVd-infected Navelina 7 sweet orange; 5) CCCVd RNA1 slow marker. Arrows on left indicate the circular viroid forms, on right circular (c) and linear (l) forms of CEVd.

of CVd II, as defined by Duran-Vila *et al.* (5). RNAs with electrophoretic properties coinciding with those of CVd III did not react with the HSVd probes, as is clearly shown in Fig. 2. RNAs presumably belonging to other

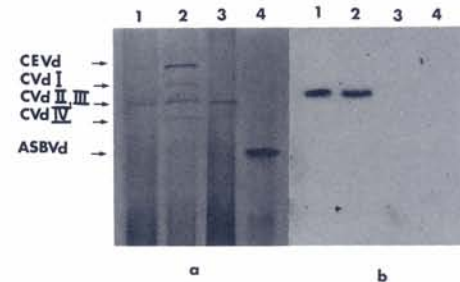


Fig. 2. a) Silver-stained 7% polyacrylamide gel after bidirectional electrophoresis with the second direction run performed under denaturing conditions; b) autoradiograph of a transblot from a gel identical to (a) after hybridization with an HSVd-specific cDNA probe. Samples were: 1-3) nucleic acid extracts from trees infected by group I to IV citrus viroids (CVd); 4) ASBVd marker. Arrows indicate the circular viroid forms.

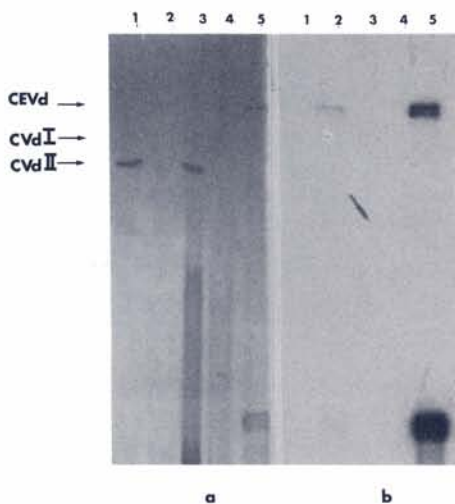


Fig. 3. a) Silver-stained 7% polyacrylamide gel after bidirectional electrophoresis with the second direction run performed under denaturing conditions; b) autoradiograph of a transblot from a gel identical to (a) after hybridization with a CEVd-specific cRNA probe. Samples were: 1) CCCVd RNA1 slow; 2) Redblood sweet orange infected with groups I to IV citrus viroids (CVd), the concentration of CEVd is approximately 20 pg, other citrus viroids are less concentrated and not detectable; 3) Citrus viroid II-infected citrus; 4) Citrus viroid I-infected citrus; 5) CEVd-infected Navelina 7 sweet orange. Arrows indicate the circular citrus viroid forms.

CVd groups also did not react with the HSVd probes.

RNA probes were found to be more suitable than DNA probes to detect small amounts of viroids. Under our hybridization conditions, we could detect as little as 20-40 pg of pure viroid with RNA probes (Fig. 3, sample 2); whereas with DNA probes, 100-200 pg of pure viroid were required (Fig. 1, sample 1).

DISCUSSION

Electrophoresis of our preparations in a discontinuous pH system (11) made it possible to compare our results with those of other investigators (5, 6, 14).

CVd Ia and Ib were detected in some of the source trees we tested. Molecular hybridization with these

viroids showed that they have little, if any, sequence similarities with either CEVd or HSVd.

In a previous paper (1), we described a viroid from citrus trees that differs from CEVd in its symptoms on citron, its molecular size as estimated by its electrophoretic mobility in denaturing gels, and its host range. This viroid, which we named citrus B viroid (CBVd), replicates and gives symptoms in cucumber cv. Suvo (3). On the basis of dot blot and Northern blot assays, in which the viroid disclosed close sequence similarities with HSVd, it was considered as a strain of HSVd (3). Results reported in this paper indicate that CBVd is identical with CVd-IIa. The other member of CVd group II, CVd-IIb, migrates very close to CVd-IIa and also is closely related in its nucleotide sequence to HSVd (2). This viroid has been identified as the causative agent of the citrus cachexia disease (14).

A viroid that appears to be identical with CVd-IIIa was found in some of our samples. Although this viroid migrates very close to viroids of the CVd group II, it is very different from these, in that it does not react with HSVd-specific probes.

Viroids apparently belonging to CVd-group IV also were detected and these did not react with CEVd- or HSVd-specific probes.

Our results are in accord with previous findings. They show that, aside from CEVd, many citrus trees contain a population of viroids, some of which are very similar to HSVd, whereas others show neither nucleotide sequence similarities with CEVd nor with HSVd. Our data are compatible with a report showing positive hybridization between a CVd group II viroid and an HSVd probe (6), as well as with other recent reports of HSVd strains isolated from citrus trees (10, 12). To clarify the role of these various viroids in the etiology of citrus diseases and their potential risk or actual damage to the citrus industry, it will be necessary to study more thoroughly their

biological and biochemical properties and their modes of transmission.

Finally, our results demonstrate that RNA probes are superior to

DNA probes for the detection of very small amounts of viroids, thus confirming previous results (9).

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