Detection and Characterization of Citrus Viroids in Uruguay

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ABSTRACT. Citrus trees in Uruguay are mainly grafted on trifoliate orange after the dissemination of citrus tristeza virus in the 1940s. Trifoliate orange is very susceptible to citrus exocortis viroid (CEVd), but the occurrence of typical rootstock symptoms are difficult to find in citrus orchards in Uruguay. Several samples from orange and grapefruit trees grafted on trifoliate orange, with dwarfing and rootstock bark scaling were chosen, as well as asymptomatic trees to determine the presence of citrus viroids. The detection of viroids was done by biological assays on Etrog citron Arizona 861, herbaceous hosts and sequential polyacryamide gel electrophoresis. Several cDNA probes labeled with ^{32}P or digoxigenin were tested and were hybridized with samples from different isolates collected from citrus orchards. The cDNA probe was produced from a severe Uruguayan CEVd isolate (CEVd-Uy1) which had been previously cloned and sequenced. The following citrus viroids were found: CEVd, CVd-Ia, CVd-Ib, CVd-IIa, CVd-IIIa and CVd-IIIb; no CVd-IV was found in the samples collected. Several viroid combinations were found in the same trees. Characterization of the citrus viroids is an important step for the control and prevention of these agents in Uruguayan citrus industry that is 90% grafted on a susceptible rootstock.

Index words. Citrus viroid complex; exocortis; nucleic acids hybridization; sPAGE; slot blot; imprint.

Citrus in Uruguay is second in agriculture exports. The area dedicated to citrus increased 23% from 1980 to 1996 and the amount of citrus exports increased 326%. Moreover, the value of fresh fruit exported in 1997 was more than US$57 million (2).

Today, ninety percent of citrus orchards in Uruguay are grafted onto *Poncirus trifoliata* rootstock after citrus tristeza virus (CTV) killed most trees on sour orange in the 1940s. Virus and viroid-induced diseases (tristeza, psorosis, exocortis, etc.) are important problems of the citrus industry. The low diversity of roostocks represents a potential risk because trifoliate orange and its hybrids are very susceptible to citrus exocortis viroid (CEVd). This disease produces dwarfing, weakness, rootstock bark scaling, and reduction in yield, fruit quality and the life of producing trees (8).

Control of introduced and propagated plant material is very important to avoid viroid dissemination. The current citrus certification program uses woody and herbaceous hosts for biological indexing of citrus viroids. The most important host for indexing CEVd is Etrog citron Arizona 861 (3) because of its great sensibility and rapid symptom expression (16).

In addition to CEVd, four discrete viroid groups have been proposed: CVd-I, CVd-II, CVd-III, and CVd-IV. These species are based on molecular size, conformation and sequence relatedness, as well as host range and symptom reaction on Etrog citron. Nucleic acid analysis of inoculated citrons using sequential polyacrylamide gel electrophoresis (sPAGE) and silver staining improves diagnosic sensitivity and shortens the time of assay (6, 7).

The purpose of this work was the detection and characterization of the viroid complex found in citrus in Uruguay by means of biological indexing, sPAGE and nucleic acid hybridization techniques.

MATERIALS AND METHODS

Viroid sources and biological indexing. Twenty samples were collected from different mature trees (15- to 20-yr-old) of Marsh seedless

grapefruit, Valencia Late and Washington navel orange. Fifteen trees showed bark scaling and dwarfing symptoms and five were symptomless (Washington Navel oranges) trees. Bark tissue from branches was collected from field samples and grafted onto Etrog citron Arizona 861 for bioassay and nucleic acid extractions. *Gynura aurantica* was grafted with the inoculum source following the procedure of Weathers et al. (22).

Healthy citrons and *Gynura* were used as negative controls. Well-characterized viroid standards were provided by Dr. N. Duran-Vila, IVIA (Valencia) and Dr. J. Semancik, University of California (Riverside) and maintained in the viroid bank of the Facultad de Agronomía (Montevideo). All isolates were maintained in the greenhouse at 18 to 25°C, and 2 wk later were transferred to a growth chamber at 30 to 32°C and 16 h daylight to promote viroid replication and symptom expression.

Nucleic acid extraction and viroid detection. Tissue samples (5 g each) of young leaves were collected from inoculated plants with symptoms and pulverized in liquid nitrogen. Nucleic acids were extracted according to the procedure of Duran-Vila et al. (5) and Semancik et al. (20). After partition in 2 M LiCl, the soluble fraction was concentrated by ethanol precipitation and used in sPAGE (15, 19). Viroid bands were visualized by silver staining (12) and compared with standards mentioned.

Electrotransfer, slot-blot and imprint hybridization. Sections of the denaturing gel (8 M urea TBE) containing the viroid RNAs were electrotransferred to polyvinylidene difluoride membranes (PVDF) (Inmobilon™-N, Millipore) using TBE buffer (40 mM Tris; 40 mM H_3BO_3; 1 mM EDTA, pH 8.3). The membranes were pre-treated with 70% ethanol for 3 sec, water for 2 min and TBE for 15 min. The electrotransfer was performed at 60V for 1 h, and the filters were dried at 80°C for 2 h.

For the slot-blot assay, 10 µl of the nucleic acid preparations were denatured with 40% formaldehyde at 60°C for ca. 15 min. The nylon filters (Boehringer Mannheim) pretreated with 20X SSPE buffer (18), were then loaded under vacuum and the nucleic acid immobilized using an UV crosslinker. The slot blot and electrotransfer filters were hybridized with cDNA probes labeled with digoxigenin (DIG).

Tissue imprints were made on positively charged nylon membranes (Boehringer). Freshly cut sections of young leaves and stems from field samples (with and without bark scaling symptoms), and graft-inoculated citron and *Gynura* were used for imprinting. The nucleic acids were fixed at 80°C for ca. 2 h, and filters were hybridized with the cloned CEVd-Uruguayan (CEVd-Uy) probe labeled with radioactivity.

Labeling and hybridization. DIG-labeled probes were produced from the recombinant plasmid pT7-Blue (Novagen) which contain the complete viroid monomeric DNA. These probes were synthesized by PCR amplification in a total reaction volume of 50 µl containing 0.5 mM of each specific viroid species primer (13), 1.5 mM $MgCl_2$, 120 mM of each dNTPs (with DIG labeled UTP) and 1 U of *Taq* DNA polymerase. The reaction consisted of an initial 5 min denaturing step at 95°C, 35 cycles (10 sec at 92°C, 10 sec at 50°C and 20 sec at 72°C) and a final extension of 5 min at 72°C.

The prehybridization and hybridization were performed in 50% formamide and 20X SSPE. The filters were prehybridized at 42°C for 2 to 4 h then hybridized at 50°C overnight. Two washing steps were done with 2X SSC, 0.1% SDS for 15 min at room temperature, followed by one wash in 0.1X SSC, 0.1% SDS for 60 min at 60°C. The DIG-labeled nucleic acids were detected with an anti-DIG-alka-

line phosphatase conjugate (Fab fragment) and visualized with either the NBT (4-nitro-blue-tetrazolium-chloride) substrate and X-phosphatase or the Chemioluminiscent CSPD (DIG Nucleic Acid Detection Kit, Boehringer Mannheim) substrate.

A radioactive probe was labeled using the clones of CEVd-Uy1 (14) with ^{32}PdCTP using the Multiprime DNA Labeling System kit from Amersham. The membranes were prehybridized for 1 h at 65°C with 50 ml of Church buffer (0.5 M Na-phosphate buffer, pH 7.2, 1 mM EDTA, and 7% SDS) and were then hybridized at 65°C for 16 h. After hybridization the membranes were washed for 30 min at 60°C twice in 1 mM EDTA, 5% SDS, in 0.5 M Na-phosphate buffer, pH 7.2, followed by another wash step of 15 min at 65°C with the same buffer containing 1% SDS.

RESULTS

Biological indexing. Etrog citron inoculated with field material from the 20 sources showed different symptoms 3 to 4 mo after inoculation. The symptoms observed in the bioassay on citron using orange and grapefruit tissue collected from plants having dwarfing and bark scaling were size reduction, epinasty, vein necrosis, petiole wrinkle and necrotic spots. The five samples of Washington navel orange collected from field samples without bark scaling and dwarfing also caused the same symptoms described above in the biological indexing in Etrog citron.

Viroid Detection. The results from sPAGE analysis show the presence of several viroids from different species when compared to the mobility of well-characterized viroid standards (Fig. 1). The numerous bands observed in each sample demonstrate the presence of mixed infections in the same trees. Using the Northern-blot technique, we were able to determine the proper viroid sizes and the precise identification of the band to the different species using specific probes. Both techniques confirmed the presence of the CEVd and allowed the detection of viroids corresponding to the following groups: CVd-I, CVd-II and CVd-III. The species CVd-IV was not found in any of the 20 analyzed samples. CEVd was detected in different combinations with the three viroid species mentioned above, either in samples from citrons inoculated with symptomatic or symptomless field trees.

Viroids CVd-Ia, CVd-Ib, CVd-IIa, CVd-IIIa and CVd-IIIb were found by comparing the mobility of the samples with well characterized standards. The results are summarized in Table 1.

The hybridization with the CVd-II specific probe in slot blots confirmed the infection with viroids from the CVd-II species (Fig. 2). Strong signals were obtained with several Uruguayan samples. From plants obtained from the shoot-tip grafting and indexed in citron to check the sanitary status, only one sample was detected which was still infected with the viroid. This is an important technique to use in rapid screening of mother plants in the Sanitation Program and is necessary for the production of viroid-free materials.

In addition to this assay, slot-blot hybridizations and Northern-blots were done using probes specific for CEVd, CEV-I, CV-III and CV-IV. The results are summarized in Table 1. Using Northern-blot hybridization, no signal was obtained when the CV-IV specific probe was tested with the 20 Uruguayan samples, and a positive result was detected with the CVd-IV viroid reference provided by N. Duran.

Direct plant tissue imprints. Plant imprint hybridization from isolates grafted on Etrog citron maintained in the greenhouse gave a strong positive signal with the

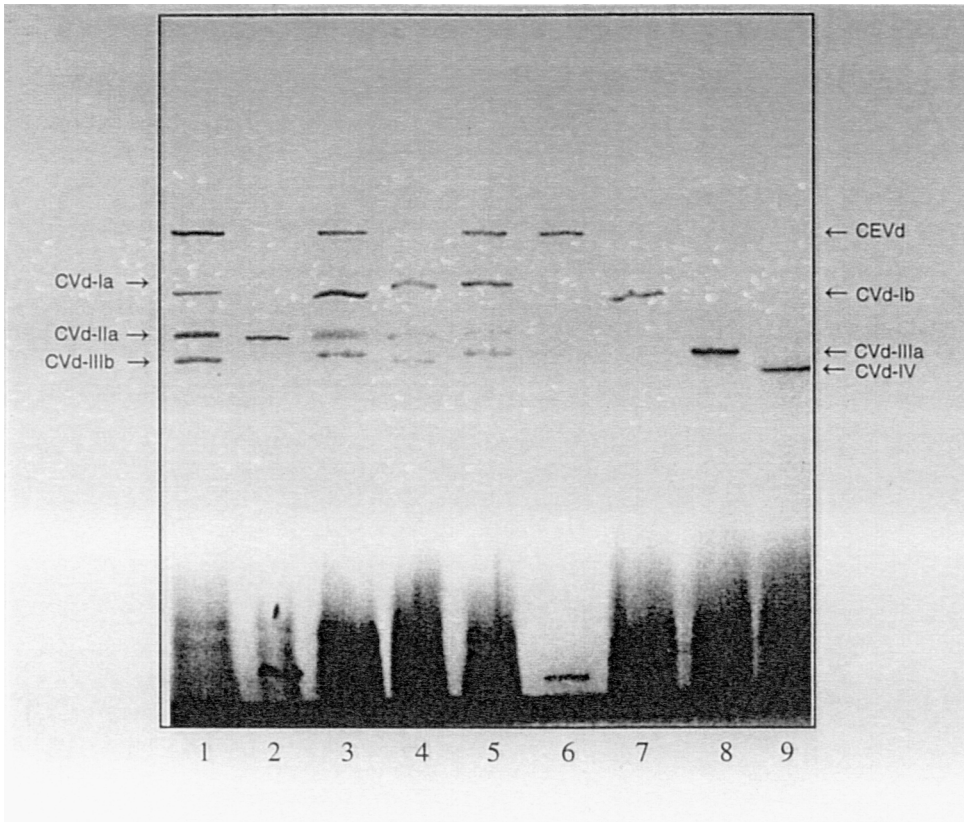

Fig. 1. Section of a silver-stained 8 M denaturing polyacrylamide gel after electophoresis of nucleic acid extracts from viroid-infected citron. The Uruguayan samples correspond to isolates 146, 147 and 030 (lanes 1, 3 and 5); positive controls, Cvd-Ia, Cvd-IIa, CVd-IIIb (lane 4); pure isolates of CEVd; CVd-Ib; CVIIa; CVd-IIIa; and CVd-IV from Spain (lanes 6, 7, 2, 8 and 9, respectively).

sample of *Gynura* inoculated with CEVd from California (2A) and with the CEVd-Uy (2B) and with the two citron samples inoculated with field isolates (3A and 5B). A moderate signal could be observed in the autoradiography on the sample corresponding to CVd-IV (4B) at 42 h of exposure which was imperceptible in Fig. 3 (taken 16 h after exposure). Figure 3 shows the results obtained with direct tissue imprints using the CEV-Uy1 cloned probe.

A positive signal was observed on the citron sample (3A) and *Gynura* infected with CEVd, from California sample (2A) and also Uruguay isolate (2B) maintained in the greenhouse under high temperature conditions. Imprints made using infected tree material from the field gave negative results.

DISCUSSION

The reaction observed in indicator hosts when inoculated with samples from trees showing symptoms was similar to those previously described by Duran-Vila et al. (7), Gillings et al. (11) and Semancik et al. (21). Nevertheless, the same symptoms were obtained from five different field sources that did not show dwarfing nor rootstock bark scaling on trifoliate orange. This result could be attributed to a late mechanical infection of the mature tree or to the

TABLE 1
SUMMARY OF CITRUS VIROIDS FOUND IN URUGUAYAN SAMPLES USING SPAGE AND HYBRIDIZATION

Variety[z] and identification	CEV d	CV-Ia	CV-Ib	CV-IIa	CV-IIIa	CV-IIIb
Marsh Seedless						
030, 031, 043, 044, 052, 054, 055, 056	+	+	—	+	+	—
032	+	—	+	+	+	—
Washington Navel						
147, 151, 201, 205, 207, 208	+	—	+	+	+	—
146	+	—	+	+	—	+
149	+	+	—	+	+	+
150	+	—	+	+	+	+
206	+	+	+	—	+	—
Valencia						
012	+	+	—	+	+	—

[z]Grafted into *Poncirus trifoliata*.

presence of strains of CEVd that do not induce bark scaling symptoms on trifoliate rootstock (N. Duran-Vila, pers. comm.). Mild forms of exocortis characterized by bark scaling without dwarfing or dwarfing without bark scaling, have been reported by Fraser and Levitt (1, 10).

The analysis of the viroids by sPAGE and hybridization of graft-inoculated citron with different field samples from trees with or without typical CEVd bark scaling symptoms, confirmed the presence of the CEVd. This work identifies for the first time in Uruguay of other RNAs of lower molecular weight than CEVd-RNA belonging to three other citrus viroid species, CVd-I, CVd-II and CVd-III (Table 1). The smaller species, CVd-IV, was not found in any of the 20 samples tested. According with the comparison of well characterized standards the following viroids were found: CEVd, CVd-Ia, CVd-Ib, CVd-IIa, CVd-IIIa and CVd-IIIb. The samples presented different combinations of viroids (Table 1). It is necessary to

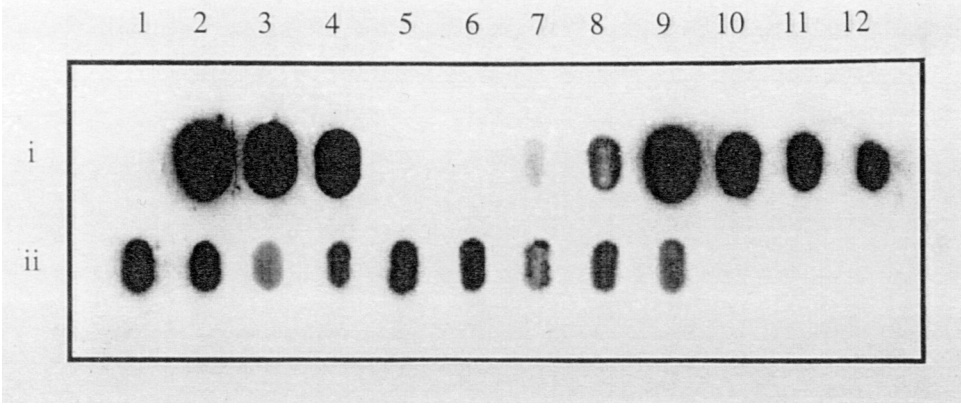

Fig. 2. Autoradiography of the slot blot from samples extracted from citron and *Gynura* after hybridization with cDNA probes from CVd-II viroid labeled with digoxigenin (DIG). Row i: 1) negative control (clonal citron); 2) positive control CVd-II; 3) Viroid standard CVd-Ia, CVd-IIa, CVd-IIb, CVd-IIIb (E821); 4) pure isolate CVd-II; 5) pure isolate CV-I; 6 and 7) micrograft previous to introduction indexing from 8 to 12 citron inoculated with Uruguayan samples; 8) symptomless field sample (201); 9) 030; 10) 031; 11) 043; 12) 044. Row ii: from 1 to 9 citrons inoculated with Uruguayan samples: 1) 146; 2) 147; 3) 149; 4) 151; 5) 052; 6) 012; 7) 054; 8) 055; 9) 056.

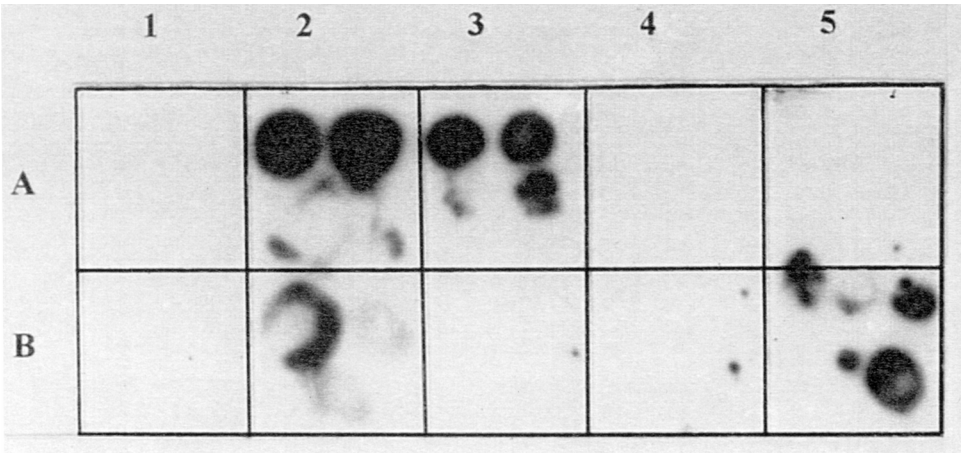

Fig. 3. Hybridization of cross-section imprints of tissue. Row A): 1) Healthy *Gynura*; 2) *Gynura* inoculated with CEVd from California; 3-4) citrons inoculated with CEVd-Uy and 821 samples respectively. 5) Symptomless field Washington navel orange. Row B): 1) Healthy citron; 2) *Gynura* inoculated with CEVd-Uy isolate; 3) Field Washington navel orange with bark scaling; 4) Citron inoculated with CVd-IV isolate; 5) Citron inoculated with field isolate.

use other complementary techniques to the biological assays mentioned above to detect this viroid mixture because the severe epinasty produced by CEVd masks the expression of the milder symptoms produced by the other viroid species. The use of sPAGE and nucleic acid hybridization with specific viroid species probes is a suitable method to know the composition of the viroid species present in one tree.

The labeling techniques using non-radioactive substances (13, 17) can reach the same level of sensitivity as radioactive-labeled probes and could be adapted for the use in massive testing of samples since it is safer.

The positive results obtained with direct impression of tissue without any denaturation steps indicates that inside the plant the viroid molecule presents some region in the form of a single strand that is accessible for hybridization with the viroid cDNA probe. In the native form, without chemical or physical treatment, the viroid molecule does not behave as a rigid rod covalently linked as is described by Diener (4), instead it is probable that the viroid RNA in the plant adopts a secondary structure with single strand loops as suggested by Francis et al. (9).

The results obtained with the direct tissue imprints are significant because they could represent an easier way to prepare large quantity of samples and to reduce the cost and time for the analysis.

This is the first report on the characterization and diagnosis of the different citrus viroid groups in Uruguay. The technology developed for this assay is very convenient to the future application in the Uruguay Citrus Certification Program.

The possibility to complement the biological indexing with other rapid molecular techniques based on the analysis of nucleic acids could be very useful for reducing the time of the bioassay, allowing large numbers of samples to be examined and to identify each citrus viroid species.

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