Detection of Citrus Exocortis Viroid by Polyacrylamide Gel Electrophoresis[1]

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ABSTRACT. A polyacrylamide gel electrophoresis procedure was developed to detect citrus exocortis viroid (CEV) from infected citrus tissue. The procedure included extraction of tissue with organic solvents, partial purification by CF-11 cellulose chromatography, and electrophoresis on polyacrylamide gels. The viroid band location in the gel was confirmed through infectivity tests on citrus, *Gynura aurantiaca*, and tomato. Citrus exocortis viroid was detected consistently from 20 g of citrus leaves 3 weeks after plants were inoculated with CEV-infected buds. The three severe and seven mild CEV isolates tested were all consistently detected in greenhouse-grown citron plants. We detected CEV from 50-g samples of flush tissue collected in summer from CEV-infected field trees of sweet orange, or grapefruit, but did not consistently detect CEV in winter. The relative amounts of CEV recovered from infected citrus tissue were compared by quantifying the UV absorbancy (260 nm) of viroid bands after electrophoresis on polyacrylamide gels. The amount of CEV was greater in young, succulent, expanding citron leaves than in old, fully expanded leaves. The amount of CEV extracted from plants infected with severe isolates was consistently greater than that extracted from plants infected with mild isolates.

Citrus exocortis viroid (CEV) is distributed worldwide (29) and severe isolates are debilitating to susceptible citrus stionic combinations. Since CEV causes no obvious symptoms in tolerant stionic combinations, it can go undetected and may be spread inadvertently by contaminated tools (10, 31) and propagation.

Citrus exocortis viroid can only be detected in tolerant trees by indexing, and there has been a long-standing need for rapid, reliable CEV detection procedures. Biological methods based on symptomatology in indicator plants were first developed using trifoliate orange (1) and Rangpur lime (20) indicators. More rapid biological indexing was obtained using selections of Etrog citron (2, 13), and this has become the standard procedure for detecting CEV. Exocortis symptoms are manifested 1-3 months after inoculation in sensitive citron selections (1). However, mild CEV isolates do not produce conspicuous symptoms in Etrog citron and require more time for symptom expression. There have been attempts to detect CEV by nonbiological methods (4, 7, 9, 22), but these have not been used widely because of unreliability or technical difficulty.

Viroids are small strands of pathogenic RNA which is largely, but not completely double stranded (5). By exploiting the unique molecular properties of viroids, detection procedures employing polyacrylamide gel electrophoresis (PAGE) have been developed to detect potato spindle tuber viroid (PSTV) (16, 17, 18), chrysanthemum stunt viroid (14), coconut cadang-cadang viroid (19) and avocado sunblotch viroid (28). Although PAGE had been used to isolate the infectious entity of the exocortis disease (25), a reliable PAGE procedure to index CEV from citrus tissues has not been reported. We report here the development of a PAGE procedure to detect CEV from citron and from field-grown sweet orange and grapefruit trees. We also compare amounts of CEV extracted from young and old tissues and from plants infected with severe and mild CEV isolates.

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MATERIALS AND METHODS

Plant materials. Arizona 861 and OES 4 Etrog citron selections, Madam Vinous and Valencia sweet orange, and Marsh grapefruit were used as source plants. Citron plants were graft-inoculated with three budchips from infected source plants and were not topped after inoculation unless specifically noted. Citron root and leaf tissues were harvested at various intervals after inoculation. Leaves on citron plants sampled for CEV detection by PAGE were mostly from young flushes, but older leaves were also harvested sometimes to obtain a desired weight. Root samples included the feeder root system and portions of larger roots. All tools were decontaminated with a solution containing 2% formaldehyde (v/v) and 2% sodium hydroxide (w/v) (11).

Rutgers tomatos. *Gynura aurantiaca* DC, and citron were used as indicator plants to identify which bands on gels were infectious.

Plants were grown in Pro-mix BX® (Premier Brands, Inc., New Rochelle NY 10810) and maintained in a glasshouse, or in some experiments in a shade house. Summer temperatures ranged from 25-35°C and winter temperatures ranged from 15-30°C.

Viroid isolates. Ten CEV isolates were used: three (E9, E16B, E22) caused severe leaf epinasty and stunting on citron and severe stunting in plants on trifoliate orange rootstocks, one (E14B) caused moderate leaf epinasty and stunting on citron, and six (E6A, E7A, E10, E11, E30, E32) caused mild leaf epinasty and little stunting on citron.

Detection of CEV using PAGE. Citrus exocortis viroid was detected in citrus tissue by modifying the procedure of Morris and Dodds (15) for isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue. Tissue was homogenized in a Waring Blendor® at a ratio of 20 g of tissue to 180 ml of extraction buffer (0.2 M glycine, 0.1 M Na_2HPO_4, and 0.6 M NaCl, pH 9.6) plus 100 ml of phenol saturated with 0.2 M Tris buffer containing 0.8% hydroxyquinoline (w/v), 100 ml of chloroform/pentanol (25:1 v/v), 5 ml of 2-mercaptoethanol, 5 ml of 10% sodium lauryl sulfate (w/v), and 0.5 g of bentonite.

The homogenate was centrifuged for 15 min. at 10,000 *g* at 4°C. The aqueous phase was removed and an equal volume of STE buffer (0.05 M Tris, 0.001 M Na_2EDTA, 0.1 M NaCl, pH 6.9) containing 40% (v/v) ethanol was added to the aqueous phase. Two grams of CF-11 Whatman® cellulose powder (Reeve Angel, Clifton, NJ 07014) were added while stirring; the suspension was incubated for 10 min. at 4°C with gentle stirring, then centrifuged at 10,000 *g* for 10 min. at 4°C. The cellulose precipitate was washed two times with 50 ml of STE buffer containing 20% (v/v) ethanol, then poured into a chromatographic column (1.5 x 30 cm) and washed with 150 ml of STE buffer containing 20% (v/v) ethanol.

Materials bound to the cellulose were eluted with 10 ml of STE buffer, collected in autoclaved tubes, precipitated with two volumes of cold 95% ethanol and three drops of 3.0 M sodium acetate and stored overnight at –20°C. The precipitate was collected by centrifugation at 11,000 *g* for 10 min. at 4°C. The pellet was dried under N_2 gas and resuspended in 0.4 ml of sterile, distilled water. One-tenth ml of saturated sucrose was added and electrophoresis was performed as described by Morris and Smith (16). Gels were removed from the tubes, stained in dilute ethidium bromide (25 μg/ml) in 0.1 mM EDTA for 15 min., destained for 15 minutes in 0.1 mM

EDTA (20), and viewed on a short-wave UV light (Model C-63, Ultraviolet Products Inc., San Gabriel, CA 91778). Gels were photographed on Kodak Contrast Process Pan film with UV and orange filters.

Recovery of nucleic acids from gels. Gel slices containing individual bands were ground in a sterile mortar and pestle in 1 ml of buffer containing 0.05 M glycine and 0.03 M K_2HPO_4, pH 9.2 (16). The eluates were slash-inoculated into indicator plants using 10 slashes per plant (12).

Quantitation of CEV from gels. The relative amounts of CEV from extracts from infected tissue were determined by quantifying the UV absorbancy of the viroid band in gels at 260 nm. This was done by completing the base line on the gel scan under the peak, carefully cutting the peaks from the strip chart recording of the gel scans, and measuring the area with a Model LI-3000 LI-COR portable area meter (Lambda Instruments Corp., Lincoln, NB 68504). Three readings were taken for each peak and averaged.

RESULTS

Detection of CEV from citron by PAGE. The citrus exocortis viroid was detected consistently in concentrated extracts from CEV-infected plants using the PAGE procedure (fig. 1). Citron, tomato, and *G. aurantiaca* plants inoculated with eluates from gel slices containing the viroid band (fig. 1) developed typical CEV symptoms while eluates from other nucleic acid bands on the gel did not cause symptoms.

Mild (E10) or severe (E16B) isolates of CEV were detected reliably by the PAGE procedure from 20 g of roots harvested 2 weeks after plants were inoculated, or 20 g of leaves 3 weeks after plants were inoculated. Both isolates were reliably detected in all samples in plants tested weekly from 3 to 8 weeks after inoculation.

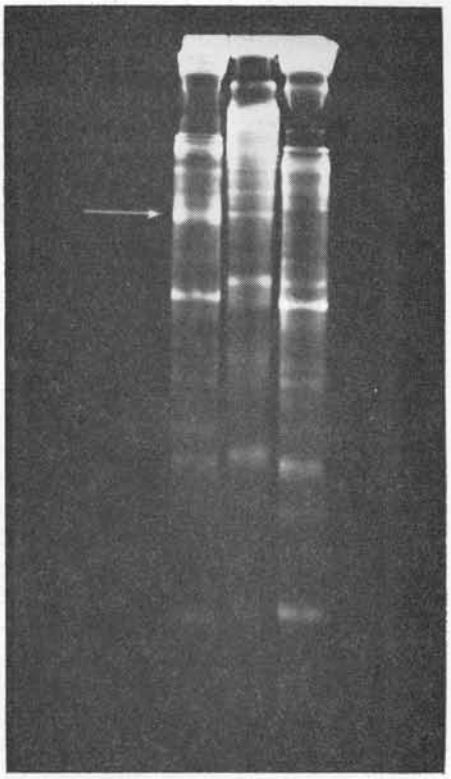

Fig. 1. Migration of nucleic acids extracted from (left) 20 g of citron leaves infected with a mild isolate (E10) of citrus exocortis viroid (CEV), (middle) 50 g of grapefruit leaves infected with a severe isolate (E9) of CEV, and (right) 50 g of healthy grapefruit leaves in 5% polyacrylamide gels. The gels were electrophoresed 5 hours, stained in dilute ethidium bromide, and photographed over UV light.

The PAGE procedure was tested for detection of additional mild CEV isolates from roots and leaves of citron plants 2 weeks after plants were inoculated. Mild CEV isolates (E6B, E7A, E14B, E30, and E32) and one severe isolate (E22) were used. Two weeks after plants were inoculated, CEV was detected in gels from all 18 extracts (three samples per isolate) of the 20-g root samples tested and in 15 of 18 of the 20-g leaf samples tested. Citrus exocortis viroid was not de-

tected in self-budded healthy citron plants. In another test, citron plants were inoculated with these same six isolates and were cut back immediately after inoculation. New leaves from the emerging flush tissue were assayed when they reached full expansion (about 3 weeks after plants were inoculated). All isolates of CEV were detected in all attempts (three samples per isolate) by the PAGE assay.

This PAGE procedure was tested to see if the time needed to detect CEV in field trees could be shortened by inoculating citron plants with buds collected from field trees. The viroid was detected by the PAGE assay from 20-g samples of citron roots collected 2 weeks after plants were inoculated with buds from CEV-infected, field-grown sweet orange, grapefruit and citron trees (table 1). Similarly, CEV was detected by the PAGE assay of 20-g samples of citron leaves 3 weeks after plants were inoculated but not from healthy, self-budded citron plants.

Detection of CEV from field trees. The PAGE assay was also used on tissue collected from field trees of sweet orange or grapefruit. Infections of CEV were detected in all 18 PAGE assays from 50-g samples of succulent, expanding leaf tissue collected in the summer. However, CEV usually was not detected from 20-g samples of similar young tissues, or from 50-g samples of older leaves or mature, fully expanded flush tissue. We detected CEV in only 5 of 12 attempts from flush tissue collected during January and February from CEV-infected field trees.

Detection of CEV from different types of tissues. The PAGE assay was compared for detecting CEV from young and old citron leaves. Mild (E10) and severe (E16B) isolates were detected in plants tested at 3-, 4-, 5- and 7-week intervals postinoculation from young citron leaves which developed after inoculation. However, CEV was not detected from mature leaves which already existed at the time of inoculation. A comparison of the amounts of a mild CEV isolate (E10) present in young, succulent, partly expanded leaves and in old, hardened, fully expanded leaves collected from systemically infected citron was made by measuring the area under the viroid band peak from absorbancy profiles (260 nm) of polyacrylamide gels after electrophoresis of extracted nucleic acids. The average area under the viroid peak from three replicates was 1.88 cm^2 for young leaves compared to 0.82 cm^2 for the old leaves. However, the differences were not significant by the *t* test.

The amount of CEV was also estimated from gel scans of electrophoresed extracts from symptomatic and nonsymptomatic leaves of E16B infected citron. There was a significantly higher amount of CEV in symptomatic leaves (mean of 1.59 cm^2) as compared to nonsymptomatic leaves (mean of 0.63 cm^2). Citrus exocortis viroid was detected in all attempts from 20-g samples of leaf blades and 20-g samples of combined midribs and stem bark from young growth flushes from CEV E10- and E16B-infected citron plants.

When the PAGE assay was run at weekly intervals using only 12-g leaf samples of citron or sweet orange tissue inoculated with CEV isolate 16B, CEV could be detected only at 2 and 3 weeks postinoculation in citron plants and not thereafter. Citrus exocortis viroid was not detected in any of the leaf samples from sweet orange.

Relative amounts of severe and mild CEV isolates in citron. The relative amounts of CEV isolates E10 (mild) and E16B (severe) extracted from 20-g leaf samples collected from systemically infect-

TABLE 1
DETECTION OF CITRUS EXOCORTIS VIROID FROM 20-g SAMPLES OF TISSUE BY POLYACRYLAMIDE GEL ELECTROPHORESIS FROM CITRON PLANTS INOCULATED WITH BUDS FROM INFECTED FIELD TREES

Weeks after inoculation	CEV isolate and donor									
	E9 Valencia		E11 Valencia		E10 Citron		E10 Marsh		Self-budded citron	
	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves
2	6/6*	4/6	6/6	4/5	6/6	5/6	7/7	8/8	0/3	0/3
3	6/6	6/6	6/6	5/5	6/6	6/6	7/7	8/8	0/3	0/3

*Number of samples from which citrus exocortis viroid was detected/number tested. Each sample was obtained from two citron plants.

ed citrons in the same greenhouse were compared by measuring the viroid peak area on gel scans (fig. 2). The peak from seven extractions for the severe isolate, E16B, ranged from 0.91 to 2.41 cm^2 with a mean of 1.55 cm^2 while the peak area for E10 ranged from 0.11 to 2.41 cm^2 with a mean of 0.97 cm^2.

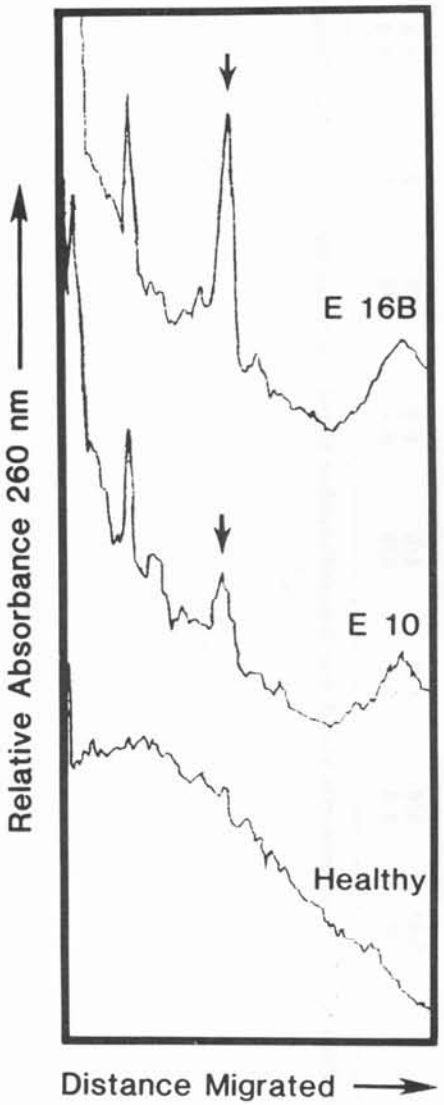

Fig. 2. Absorbance profiles of nucleic acids extracted from 20 g of citron leaves infected with a severe (E16B) isolate, a mild (E10) isolate of citrus exocortis viroid, and from healthy leaves after electrophoresis on 5% polyacrylamide gels. The arrows indicate the viroid peaks (determined by infectivity).

Although the differences in peak areas tended to be consistent, they were not significant by the *t* test.

The relative amounts of CEV (based on absorbancy) were also determined from extracts from citrons which had been cut back when inoculated with five mild and one severe CEV isolate. The new leaves were collected about 3 weeks postinoculation and used for PAGE assay. Leaves infected with the severe isolate E22 contained significantly more CEV than leaves infected with any of the five mild isolates (table 2). Leaves infected with CEV E22 had an average peak area of 3.51 cm^2 while the averages from leaves infected with the mild isolates ranged from 1.19-0.86 cm^2.

DISCUSSION

The PAGE procedure described permitted reliable and quick (one day) detection of CEV from 20-g citron leaf samples harvested 3 weeks after grafting the indicator plant with buds from the candidate test tree. The procedure also permitted reliable detection of CEV from 20-g samples of citron roots,

TABLE 2
RELATIVE AMOUNTS OF CITRUS EXOCORTIS VIROID EXTRACTED FROM 20-g SAMPLES OF CITRON LEAVES HARVESTED 3 WEEKS POSTINOCULATION*

Isolate	Peak area (cm^2)†
E22 (severe)	3.51 a‡
E7A (mild)	1.19 b
E6B (mild)	1.10 b
E30 (mild)	1.06 b
E32 (mild)	0.91 b
E14B (mild)	0.86 b

*Plants cut back at time of inoculation.
†Peak areas were were determined with an area meter on strip chart absorbance profiles (260 nm) of electrophoresed 5% polyacrylamide gels.
‡Treatments sharing a common letter do not differ significantly at the 95% confidence level by Duncan's multiple range test.

but this was not as convenient as leaf samples. The PAGE procedure allows for more effective use of citron plants and greenhouse space, provides faster detection than indexing based on observation of symptoms, and complements conventional indexing procedures.

To reliably and consistently detect CEV, the procedure of Morris and Dodds (15) had to be modified by extracting tissue at buffer ratio of 1:9, by including 0.28% bentonite in the extraction buffer and by using a 20% ethanol concentration in CF-11 cellulose chromatography. Singh and Sanger (26) have shown that the viroid-like RNA of PSTV and CEV eluted with both the 15% ethanol eluate and with buffer alone from CF-11 cellulose columns. Other studies (6, 24) indicate that the pattern of viroid elution from CF-11 cellulose is intermediate to that of double-stranded and single-stranded RNAs. A 20% ethanol concentration also binds some nonviroid single-stranded RNA to the CF-11 cellulose. Extensive washing of the CF-11 cellulose with large volumes (250 ml) of buffer after binding the viroid to the CF-11 cellulose apparently eliminates materials which interfere with electrophoresis and detection of CEV by ethidium bromide staining. Single-stranded RNAs are eluted from the CF-11 together with CEV, but to save time, the nucleic acids were not partitioned by 2.0 M lithium chloride to eliminate single-stranded RNAs. Consequently, there are several nonviroid bands on the gels (fig. 1). When the PAGE procedure is first performed, the viroid band must be identified through infectivity tests and by coelectrophoresis of a purified viroid standard. Subsequently, it may be located by its position on the gel, and in cases where necessary, eluted from the gel and assayed for infectivity. Identification of the viroid band may be made easier by staining the gel for 4-5 hours in a solution which contains 50 ng/ml ethidium bromide, 50 μg/ml ribonuclease (RNase 1A) and 0.3 M NaCl. This treatment eliminates single-stranded RNA but leaves double-stranded RNA and the CEV (15).

Isolates of CEV vary in severity from very severe to very mild (3, 8, 24, 30). Because the PAGE procedure depends on isolation and visualization of the viroid RNA and not on biological symptoms, it is well suited to detect mild CEV isolates which are difficult to detect by biological indexing. Under ideal conditions, some severe CEV isolates may be detected 3-4 weeks after inoculation by citron indexing. However, symptoms induced by mild CEV isolates may take several months to appear and, even then, the symptoms are difficult to discern. If no symptoms are apparent after at least 6 months, the diagnosis is usually considered negative (1). Potato spindle tuber viroid isolates also differ in severity, with some so mild that they are considered latent (27). Polyacrylamide gel electrophoresis was shown to be more reliable than the tomato bioassay to detect PSTV (21).

Based on quantitation of UV absorbancy, there is more viroid in young tissue than in older, hardened tissue. Using mild (E10) and severe (E16B) isolates of CEV and the PAGE assay, CEV was not detected in the mature leaves which were already on the citron plant at the time of inoculation. Also, young expanding leaves of citron systemically infected with mild CEV isolate E10 contained more viroid than old, fully expanded leaves. This observation is consistent with Semancik's (23) observation that viroid synthesis is favored by cell division or occurs in intimate association with mitotic

activity. Morris and Smith (16) have shown there is less PSTV in older potato tissue than in young, actively growing tissue.

The PAGE assay also indicated a higher titer of CEV in citron than in sweet orange or grapefruit tissues. The only consistent detection of CEV from sweet orange or grapefruit tissue was by using 50-g samples of very young, succulent leaf samples collected from field trees in the summer or from warm greenhouses.

The amount of CEV present in citron appears to be influenced by the severity of the CEV isolate. Severe CEV isolates were present in greater amounts in citron plants than the mild isolates tested.

The PAGE procedure to detect CEV will probably be most useful in situations where a relatively small number of plants must be indexed such as for checking budwood source trees in budwood certification programs. The disadvantage of the procedure for checking large numbers of samples is the cost of chemicals, the need for purification and electrophoresis equipment, and the requirement for technical labor. However, even for large-scale use, the procedure significantly reduces the time such citron plants need to be maintained.

The PAGE procedure was used successfully to directly detect CEV from field-grown sweet orange and grapefruit trees. The key was to use large (50 g) samples of succulent, expanding-flush tissue collected during the summer. Samples collected from field-grown trees in January and February did not give consistent detection of CEV in trees known to be CEV infected, presumably because lack of new growth and low temperatures do not favor CEV replication. Samples may be collected from candidate field trees during the summer and assayed immediately or stored frozen for subsequent processing. The application of the PAGE procedure to detect CEV should become more efficient when more information is obtained on CEV titers from different citrus cultivars and from samples collected during different times of the year.

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