Association of Viroids with a Graft-Transmissible Dwarfing Symptom in Australian Orange Trees

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ABSTRACT

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Orange trees (*Citrus sinensis* on *Poncirus trifoliata* rootstock) dwarfed by graft-inoculation in earlier field trials were tested to determine if viroids were associated with dwarfing symptoms. Uninoculated trees without dwarfing symptoms were used as controls. Trees dwarfed with any of three sources of inoculum (isolates) gave positive results in three types of test for viroids, namely, symptom development in citron (*C. medica*), gynura (*Gynura aurantiaca*), and tomato (*Lycopersicon esculentum*), hybridization with a nucleic acid probe specific for citrus exocortis viroid (CEV), and polyacrylamide gel electrophoresis of nucleic acid extracts. Four other isolates gave mild viroidlike symptoms in citron and some faint hybridization reactions, but negative results in the other tests. The results suggest that viroids are consistently associated with dwarfing, that CEV is present in at least some cases, and that viroids that are harder to detect than (and possibly distinct from) CEV are present in other cases.

It is commonly accepted that viroids and viruses other than those conferring mild strain cross-protection have no desirable effects in plants and should be eliminated wherever possible. However, field trials conducted by the New South Wales (N.S.W.) Department of Agriculture have shown that certain isolates of graft-transmissible dwarfing agents (GTD agents) reduce the size of selected orange scions (*Citrus sinensis* (L.) Osbeck) on trifoliate orange (*Poncirus trifoliata* (L.) Raf.) rootstock without deleterious effects (1). Such dwarfed trees are less expensive to spray and harvest than vigorous trees; they can be planted at a higher density, and thereby offer higher returns in the early years after planting and more efficient use of water and fertilizer (10). Graft-transmissible dwarfing (GTD) is therefore a host/pathogen interaction that can be used to advantage. Commercial application of GTD in Australia awaits the identification of the GTD agents so that the uniformity of inocula and likelihood of accidental transmission can be assessed.

The symptom of dwarfing in citrus trees on trifoliate orange rootstock has been attributed to citrus exocortis viroid (CEV) in the United States (9) and Australia (12,13,24). This is because exocortis (scaling of the rootstock bark) and dwarfing are usually associated and can be cotransmitted by grafting. However, dwarfed trees without scaling have been observed in Australia (4,12) and Italy (8), and graft inocula (buds) from the Australian trees reproduce the symptom of dwarfing without scaling. Most GTD isolates used in horticultural performance trials in N.S.W. are of the nonscaling type.

There is preliminary circumstantial evidence that CEV is associated with dwarfing in the case of nonscaling GTD isolates. Five of six isolates have given leaf-curling reactions in the CEV indicator citron (*C. medica* L.) (6,11), and two produce dwarfing of trees on a number of exocortis-susceptible rootstocks (5). CEV has been isolated and sequenced from single samples of leaves representing two of the nonscaling isolates (26). Citrus tristeza virus, psorosis 'virus', and xyloporosis 'virus' have been eliminated as likely dwarfing agents by indexing (6,11), but the possibility that viroids other than CEV are involved has not been examined previously.

Here, orange trees dwarfed with six nonscaling isolates and (for comparison) one scaling isolate were tested to find if viroids are consistently associated with dwarfing and if these viroids can be identified as CEV.

MATERIALS AND METHODS

Isolates of GTD agents. Each isolate was derived from buds of a single dwarfed tree. The nonscaling isolates, designated by the accession number of the source tree, were 3531, 3532, 3535, 3536, 3538, and 3539. The scaling isolate was 033. Isolates 3538 and 3539 were from Marsh grapefruit (*C. paradisi* Macf.) and the remainder from Washington navel orange source trees (11).

Orange trees. First-transmission trees, located in a trial at Somersby, N.S.W., were Bellamy navel orange on trifoliate orange rootstock, inoculated with buds from source trees in 1955 (11,13). Second-transmission trees, located at Yanco, N.S.W., were Bellamy navel and Newton (Keenan) Valencia orange on trifoliate orange rootstock, inoculated with buds from first transmission trees in 1964 (1). The trees sampled here were: first-transmission trees only for isolates 3535 and 3536, second-transmission trees only for 033, both first- and second-transmission trees for 3531, 3532, 3538, and 3539, and uninoculated (nondwarfed) control trees of equivalent age and variety in both trials. Isolates 3531, 3532, 3538, and 3539 gave highly reproducible dwarfing in both trials (1), but 3535 and 3536 were only transmitted to one tree each at Somersby.

Collection of rootstock and scion samples from orange trees. Bud sticks for graft-inoculation of citron were collected in December and February from four positions on the outside of the canopy. Leaf and bark samples used for extraction of nucleic acids were collected in March (late summer) unless stated otherwise.

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Rootstock and scion bark (two 2- × 10-cm patches of each) were cut from immediately above and below the graft union, scraped to remove outer dead bark, pared into thin shreds, crushed to a fine powder under liquid nitrogen, and stored at −80 C up to the time of extraction. Leaves (a mixture of young and mature leaves from all around the canopy) were crushed and stored as for bark.

Extraction of nucleic acids. The procedure for extraction of rootstock and scion samples was basically that of Morris and Smith (16), except for use of a less concentrated extraction buffer, a higher buffer:tissue ratio, and bentonite. Crushed, frozen leaf, or bark samples (5 g) were homogenized with a Sorvall Omnimixer or IKA Ultra-Turrax in the presence of 20 ml of water-saturated phenol, 20 ml of chloroform-pentanol (25:1 v/v), and 15 ml of glycine buffer (0.12 M glycine, 0.6 M Na_2HPO_4, 0.38 M NaCl, 0.09 M 2-mercaptoethanol, 0.38 mg/ml of bentonite, 0.64% sodium dodecylsulfate, adjusted to pH 9.3 with NaOH). Nucleic acids in the aqueous phase were enriched for low molecular weight nucleic acids with 2 M LiCl and dialyzed as described (16), and then precipitated with 2.5 volumes of ethanol, resuspended in 0.1 mM sodium ethylenediaminetetraacetate (EDTA), pH 8.0, rapidly frozen by immersion in liquid nitrogen and stored at −15 C. Nucleic acid concentrations were determined from UV spectra, assuming an extinction coefficient of 25 $(mg/ml)^{-1}$ cm^{-1} at 260 nm.

Citron and tomato leaves were also extracted by this procedure, except that fresh rather than frozen tissue was used.

Precautions against cross-contamination. Dwarfed trees were not hedged at any stage and indicator plants were arranged to prevent foliar contact. Secateurs used for sampling infected plants were dipped in 1% sodium hypochlorite solution (19) and razor blades, where used, were discarded between different inocula. Glassware and equipment used for preparing nucleic acid extracts were carefully washed with detergent, and then soaked in 1% sodium hypochlorite or 1 M KOH.

Inoculation and cultivation of indicator plants. Citron plants (seedling selection Arizona 861) were grown from cuttings and graft-inoculated in the summer of 1980–81. Four buds from each orange tree were tested (two buds on each of two citrons). The plants were maintained with a high nitrogen fertility level and cut back intermittently to promote new growth. Greenhouse temperature was subject to seasonal variation, but a daily maximum of 28 C or higher was recorded for 78% of the year. Mean maxima and minima were 35 and 19 C for January and 26 and 11 C for July.

Tomato (*Lycopersicon esculentum* Mill. 'Rutgers') and gynura (*Gynura aurantiaca* DC) plants were inoculated with leaf nucleic acid extracts from graft-inoculated citrons (1 mg of nucleic acids per milliliter of 0.05 M potassium phosphate buffer, pH 8.0). Inocula were rubbed onto Carborundum-dusted cotyledons of tomato seedlings, or introduced through 20 razor incisions on the stems of gynura cuttings. The plants were grown in an air-conditioned greenhouse with day and night temperatures set at 32 and 26 C, respectively.

CEV-A from South Australia was used as a reference isolate in inoculation experiments. This isolate has been positively identified as CEV by nucleic acid sequencing (25) and gives severe leaf-curling symptoms on citron (*unpublished*), but its symptoms in trees on trifoliate orange rootstock have never been recorded.

Hybridization assay. The dot-blot membrane hybridization procedure was as described by Barker et al (3). The cDNA probe (single-stranded, ^{32}P-labeled complementary deoxyribonucleic acid) was prepared by J. E. Visvader of the Biochemistry Department, University of Adelaide; a full-length, positive-sense DNA copy of CEV-A was cloned in phage vector M13mp93 (26) and used as a template for synthesis of insert-length cDNA with α-^{32}P-dATP and dCTP as label (3). The probe was specific in that it gave no significant hybridization with uninfected orange, citron, or tomato nucleic acids, but its specificity for CEV as opposed to other viroids was not tested.

Polyacrylamide gel electrophoresis (PAGE). Nucleic acid samples (25 μg) were analyzed on 150 × 130 × 0.7 mm slab gels containing 5% acrylamide and 0.25% N,N′-methylenebisacrylamide. The buffer system (40 mM Tris acetate, pH 7.2, 20 mM Na acetate, 2 mM EDTA) was that used by Loening (15) and electrophoresis was at 24 mA for 3.5 hr. Gels were stained with ethidium bromide (10 mg/ml of solution in water), destained briefly with three changes of distilled water, and photographed under ultraviolet transillumination.

RESULTS

Reactions on citron. All seven GTD isolates from dwarfed trees gave CEV-like symptoms in citron (Table 1). However, there were clear distinctions between isolates in terms of severity and time of symptom development. Scaling isolate 033 and nonscaling isolates 3535 and 3536 gave reactions typical for severe strains of CEV (7): strongly curled leaves, lesions on the abaxial surface of midribs, vertical cracking of stems, and stunting (Fig. 1A). Trees infected with 033, 3535, and 3536 induced symptoms on at least one citron plant within 10 mo of inoculation (Table 1), though individual citron plants showed symptoms as early as 60 days or as late as 12 mo. In contrast, nonscaling isolates 3531, 3532, 3538, and 3539 gave a milder type of symptom, namely, mild curling of leaves or bending at individual sites on the midrib (Fig. 1B), often confined to a few leaves of each plant. Most instances of this symptom developed at 27–29 mo after inoculation, and only one was observed at 10 mo (Table 1). Mild symptoms were not recorded for every tree tested, nor for every duplicate inoculated citron. On the basis of this symptomatology, isolates 033, 3535, and 3536 are called S-isolates (for their severe reaction on citron) and isolates 3531, 3532, 3538, and 3539 are called M-isolates (mild reaction).

None of the uninoculated control trees, which included representatives from both orange cultivars and both field trials, gave any symptoms in citron (Table 1).

Reactions on tomato and gynura. Extracts from citrons infected with the three S-isolates gave mild bunching of foliage of tomato and mild curling of leaves of gynura (Fig. 2). These symptoms were similar to, but less severe than, those of CEV-A (insets Fig. 2). None of eight extracts from citrons infected with M-isolates (three of 3531, two of 3532, two of 3539, and one of 3538) gave symptoms in tomato or gynura. Three controls derived from uninoculated trees also failed to produce symptoms.

Hybridization assay of nucleic acid extracts from orange trees.

TABLE 1. Reactions of citron to graft transmissible dwarfing isolates from orange trees

Isolate	Number of orange trees tested[a]	Number of trees for which a leaf-curling reaction was recorded in citron: 10-mo post-inoculation[b]	29-mo post-inoculation[b]	Reaction of citron
033	3	3	3	Severe
3535	1	1	1	Severe
3536	1	1	1	Severe
3531	6	0	5	Mild
3532	4	0	4[c]	Mild
3538	5	0	3[c]	Mild
3539	6	1	4	Mild
UI[d]	8	0	0	Nil

[a] Both first-transmission and second-transmission trees were included for all isolates except 033 (second-transmission only), and 3535 and 3536 (first-transmission only).

[b] A tree was recorded as giving a reaction when one or both of the duplicate inoculated citron plants showed symptoms.

[c] For two trees only (one 3532 and one 3538), reactions were first observed at 38 mo after inoculation.

[d] UI denotes uninoculated control trees.

Three series of extracts were tested in a single hybridization experiment, using a constant amount of CEV-specific probe and constant conditions for hybridization and exposure of X-ray film (Fig. 3).

The results for first-transmission and (for isolate 033 only) second-transmission Bellamy navel orange trees sampled in late summer are shown in Figure 3A. Each isolate was represented by one tree. The S-isolates gave positive results: hybridization spots that were moderately intense for leaves, intense for scion bark, and very intense for rootstock bark. M-isolates gave only faintly positive (3531 and 3532 scion bark) or negative results, even though five times as much total nucleic acid was assayed. The uninoculated control tree also gave negative results for the larger (5×) nucleic acid aliquot.

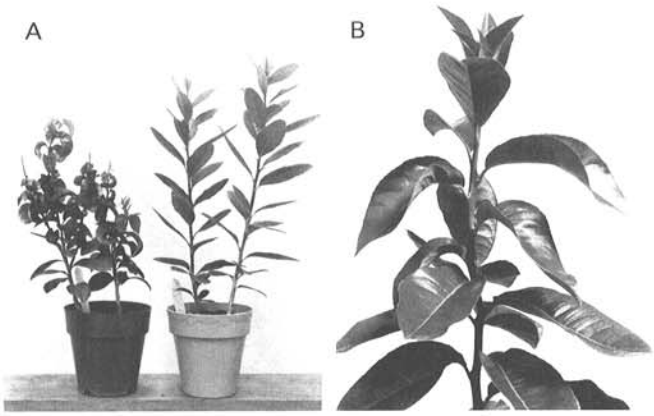

Fig. 1. Leaf-curling reactions on citron. **A,** Plant showing severe symptoms after inoculation with GTD isolate 033 (left) compared with a symptomless control plant, inoculated with buds from an uninoculated orange tree (right). **B,** Mild symptoms produced by isolate 3532.

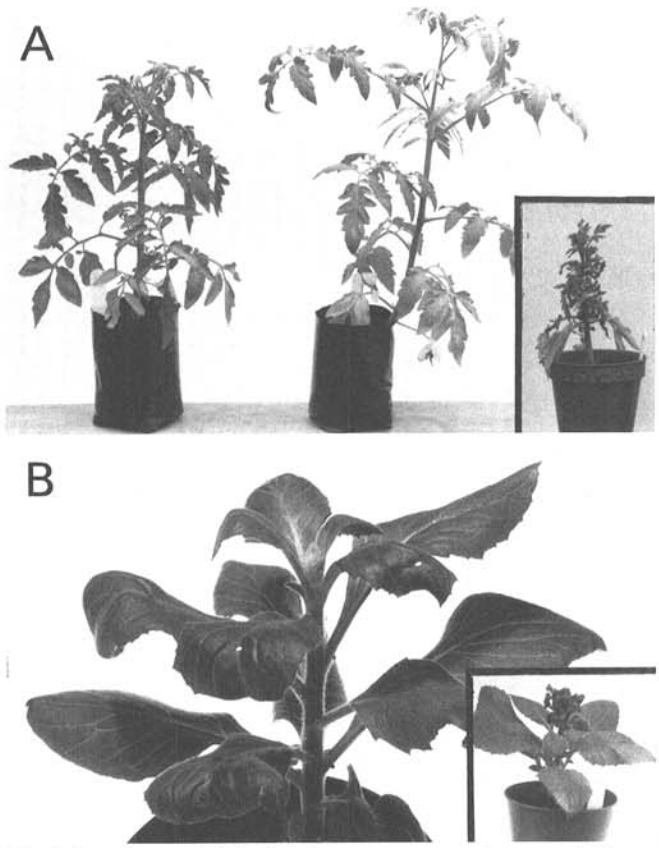

Fig. 2. Reactions typical for S-isolates on tomato and gynura. **A,** Tomato plant inoculated with isolate 033 and showing mild bunching symptoms (left) compared with an uninoculated plant (right). **B,** 033-inoculated gynura plant with mild leaf curling. The insets show reactions obtained with CEV-A under the same conditions.

Essentially the same results were obtained with second-transmission Newton Valencia orange trees sampled in late summer (Fig. 3B). An S-isolate (033) gave intense spots for scion and rootstock bark, whereas all four M-isolates and an uninoculated control did not give spots. The amount of nucleic acid assayed was constant in this series.

Another series of extracts was prepared from second-transmission Bellamy navel orange trees, sampled in early spring (September). Leaf, scion bark, and rootstock bark were included, and the amount of nucleic acid assayed was constant. The results (Fig. 3C) were similar to those of the first two series. However, relatively faint hybridization spots were obtained for 033-infected scion bark and rootstock bark, and no spots were seen for 033-infected leaves. A rootstock bark sample from the same 033-infected tree, but collected in late summer, gave intense spots with one fifth the amount of nucleic acid (heavy spot at far right of Fig. 3A). It therefore appeared that the amount of CEV relative to host nucleic acids (titer) varied considerably between seasons.

It was apparent from Figure 3A and C that both rootstock bark and scion bark contained much higher titers of CEV than did leaves.

Nucleic acids insoluble in 2 M lithium chloride, retained during preparation of the above nucleic acid samples, were also tested after redissolving in a minimal volume of buffer (results not shown). These samples gave only faint spots for S-isolates and no spots for M-isolates. Therefore, no substantial loss of CEV occurred during the LiCl fractionation step used for all extracts.

In other hybridization experiments, leaves of graft-inoculated citron plants gave positive results for all three S-isolates and negative results for all four M-isolates. Leaves from tomato plants

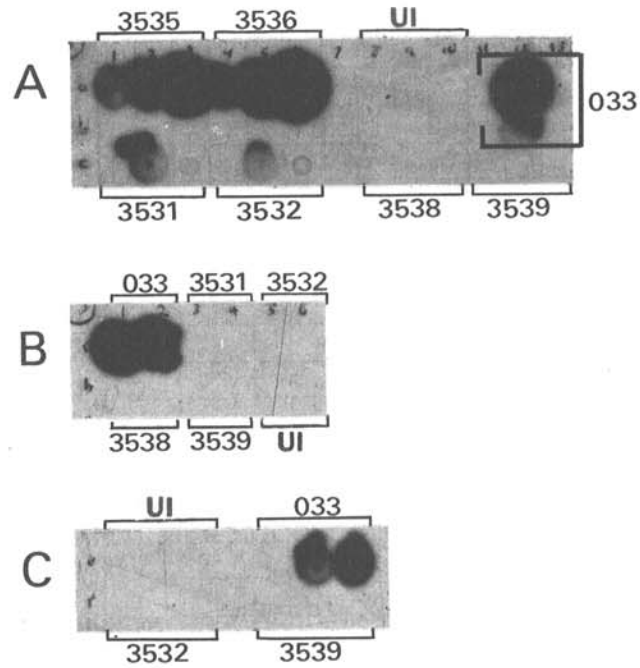

Fig. 3. Hybridization assay with leaf and bark nucleic acid extracts from orange trees. Each bracket defines the hybridization spots for one tree and the GTD isolate with which that tree was infected. UI denotes control extracts from uninoculated control trees. **A,** Extracts from first-transmission trees, sampled in late summer. The extracts within each bracketed set were (left to right) leaves, scion bark, and rootstock bark; the 033 extracts, from a second-transmission tree, were rootstock bark (upper spot) and leaves (lower spot). Positions a7, a11, and b1-11 on the membrane were blank. Amounts of nucleic acid spotted were 1 μg for isolates 3535, 3536, and 033, and 5 μg for 3531, 3532, 3538, and 3539. **B,** Extracts from second-transmission trees, sampled in late summer. The left and right hand spots within each bracket were scion bark and rootstock bark, and all spots contained 1 μg of nucleic acid. **C,** Extracts from second-transmission trees, sampled in early spring. The spots within each bracket were, from left to right, leaves, scion bark, and rootstock bark, and all spots contained 5 μg of nucleic acid. Positions e4 and f4 were blank.

inoculated with citron extracts gave the same results as the corresponding citrons, except that the positive spots for S-isolates were much stronger (indicating higher titers of CEV in tomato).

PAGE. The nucleic acid extracts used for the hybridization assay were further tested by PAGE. S-isolate 033 (in scion leaves, scion bark, rootstock bark, and citron leaves) gave at most a trace of a band in the position of CEV. M-isolates and uninoculated controls gave no infection-related bands. Typical results (for 033 and controls only) are shown in Figure 4, tracks A–E.

In extracts from inoculated tomato plants, S-isolates (e.g., 3536 and 033 in Figure 4, tracks J and K) and CEV-A (track L) gave a conspicuous pair of infection-related bands. The slower, more intense band was presumed to be CEV, as there was no band of corresponding mobility for uninoculated plants (track F). The faster band had the same mobility as a minor host band, and could represent either a viroid ribonucleic acid (RNA) or host RNA, which was amplified by CEV infection. Tomato plants inoculated with M-isolates (e.g., 3531 and 3539, tracks H and I) or uninoculated control extracts (track G) gave no infection-related bands.

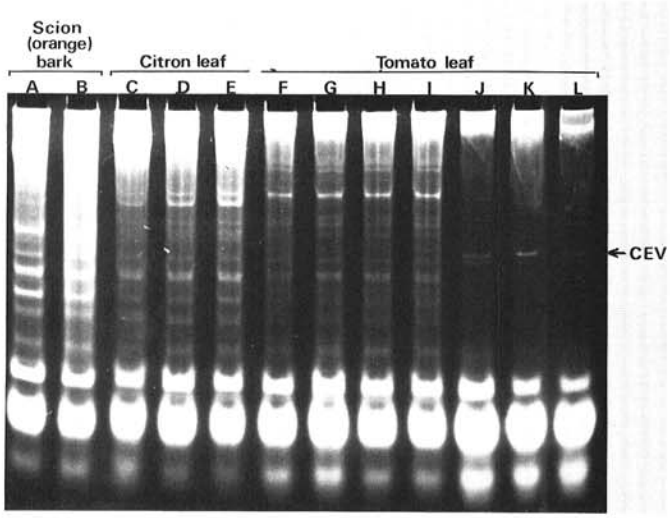

Fig. 4. Detection of infection-related nucleic acids by PAGE. Inoculations were: **A,** UI; **B,** isolate 033; **C,** uninoculated citron; **D,** UI; **E,** 033; **F,** uninoculated tomato; **G,** UI; **H,** isolate 3531; **I,** 3539; **J,** 3536; **K,** 033; **L,** CEV-A. UI denotes controls derived from uninoculated orange trees. The arrow indicates a pair of bands (lanes J, K, L), the slower of which is presumed to be CEV, the faster of which appears to be CEV-related.

DISCUSSION

The symptomatology, PAGE, and hybridization tests, although chosen here specifically for detecting CEV, are all capable of detecting other viroids as well. For example, citron reportedly gives leaf-curling symptoms for a citrus viroid distinct from CEV (22). Gynura shows curling and distortion of leaves in response to CEV, potato spindle tuber viroid (PSTV), and chrysanthemum stunt viroid, and tomato shows bunchy top symptoms of varying severity with CEV, PSTV, and cucumber pale fruit viroid (17). PAGE in 5% polyacrylamide gels can be used to detect many types of viroid (21), and we have found that a cDNA probe complementary to PSTV hybridizes strongly with CEV under our hybridization conditions while giving no reaction with control extracts (*unpublished*). A positive result in any one test can be considered indicative of a viroid of some type. Positive results in all tests provide corroborative evidence that the viroid is CEV.

All of our GTD isolates (one scaling and six nonscaling) in orange trees were found to give leaf-curling reactions in citron, whereas uninoculated control trees gave no reactions. This is evidence that viroids are associated with the dwarfing symptom. The failure to observe reactions for some trees and individual duplicate inoculated citrons is probably due to the absence of viroids in a portion of the buds from a given tree.

The scaling isolate (033) and two of the nonscaling isolates (3535 and 3536) gave typical CEV symptoms in citron, gynura, and tomato, strong hybridization with a CEV-specific probe, and a PAGE band with the same mobility as CEV (Table 2). Therefore, it is clear that these three isolates (S-isolates) contain CEV although their symptoms in gynura and tomato are milder than those of CEV-A.

Four nonscaling isolates (M-isolates: 3531, 3532, 3538, and 3539) gave mild leaf-curling reactions in citron, which were very late in developing compared with CEV reactions (Table 2). These isolates produced no symptoms in either gynura or tomato. Attempts to detect viroids by PAGE and hybridization assays were unsuccessful, apart from the faint, inconsistent hybridization spots with nucleic acid extracts from 3531- and 3532-infected scion bark. The observation that the M-isolates were indistinguishable from each other in all tests, yet so clearly distinct from the CEV-positive S-isolates, suggests that they contain either a mild form of CEV, which is particularly difficult to detect in nucleic acid extracts, or a viroid distinct from CEV. Nucleic acid sequencing is the only way to distinguish between these possibilities.

The existence of viroids in the M-isolates is questionable because of the negative PAGE results. However, precedents exist for a viroid that is extractable in only very low or undetectable amounts. There is overwhelming evidence for the existence of

TABLE 2. Summary of tests for viroids in different graft-transmissible dwarfing isolates

	Symptoms in orange trees on trifoliate orange rootstock		Results of test[a]				
					PAGE of nucleic acid extracts		Dot-blot assay of nucleic acid extracts
Isolate	Symptoms on rootstock bark	Dwarfing symptoms	Leaf-curling reaction in citron[b]	Viroidlike reaction in tomato and gynura	Orange, citron[c]	Tomato	Orange, citron[c]	Tomato
033	Scaling	Severe	Severe	+	f	+	+	+
3535	Nonscaling	Moderate	Severe	+	f	+	+	+
3536	Nonscaling	Moderate	Severe	+	f	+	+	+
3531	Nonscaling	Mild	Mild	–	–	–	f[d]	–
3532	Nonscaling	Mild	Mild	–	–	–	f[d]	–
3538	Nonscaling	Mild	Mild	–	–	–	–	–
3539	Nonscaling	Mild	Mild	–	–	–	–	–
UI[e]	Nonscaling	Nondwarf	Nil	–	–	–	–	–

[a] If any one of several trees dwarfed with a given isolate were positive, a positive result (+, f) is recorded in the table. 'f' denotes a faint PAGE band or dot-blot reaction. Minus (–) denotes negative results for all trees tested.
[b] 'Severe' denotes quickly-developing, severe leaf-curling and 'mild' denotes slowly-developing, mild leaf curling.
[c] The tissues used to prepare extracts were leaves, scion bark, and rootstock bark from orange trees, and leaves from graft-inoculated citrons.
[d] Faint hybridization spots were seen only for scion bark and only one tree.
[e] Uninoculated control trees.

chrysanthemum chlorotic mottle viroid, yet it apparently has never been visualized as a stained band in polyacrylamide gels (17,20). Mild strains of CEV (or more correctly, citrus viroids that give mild leaf-curling in citron) have only recently been shown to give a band in citron extracts, and the bands were faint compared with those for severe strains of CEV (2). There are no reports that mild CEV can be detected in extracts of herbaceous hosts, and attempts to demonstrate symptoms of mild strains in gynura have failed (14). Further evidence for the existence of viroids in three of our M-isolates, based on transmission to *Chrysanthemum morifolium* Ramat., is given elsewhere (23).

The results of the hybridization assay suggested that titers of CEV (isolate 033) in orange trees were higher in late summer than in early spring and higher in bark than in leaves. Baksh et al (2) have also noted seasonal fluctuations of CEV in orange and grapefruit leaves using PAGE as a detection method. Higher temperatures presumably are responsible for the higher CEV levels in late summer because high temperatures are reported to enhance expression of CEV symptoms in citron, trifoliate orange, and gynura (18) and give increased concentrations of PSTV in potato leaves (16). Season- and tissue-related fluctuations of viroid titers have important implications for indexing programs for citrus and other perennial crops.

With the possible exception of isolates 3535 and 3536, both of which are poorly characterized with respect to dwarfing symptoms, the severity of dwarfing is consistent with the severity of viroidlike reactions in citron for each isolate (Table 2). This strongly suggests that viroids are the cause of dwarfing. Inoculation of orange trees with purified viroids will be required to fulfil Koch's postulates. However, this will not be possible for the M-isolates until the viroids can be purified.

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