Detection of Viroids in Dwarfed Orange Trees by Transmission to Chrysanthemum

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ABSTRACT

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The viroid indicator *Chrysanthemum morifolium* was inoculated with four mild isolates of graft-transmissible dwarfing agents (M-isolates), which previously gave mild leaf-curling reactions in citron (*Citrus medica*) but negative results in other tests for viroids. One isolate failed to induce any symptoms in chrysanthemum; there was no evidence of viroid infection by hybridization with a ^{32}P-labeled complementary RNA probe specific for citrus exocortis viroid (CEV) or by polyacrylamide gel electrophesis (PAGE). Each of the other three isolates gave rise to two types of symptom: a severe reaction characteristic of CEV and a mild reaction the same as that reported for viroids other than CEV. The mild reaction consisted of large chlorotic leaf spots without the leaf distortion and stunting caused by CEV. Both types of reaction were observed during a second passage in chrysanthemum where plants with mild symptoms were used as the source of inoculum. Extracts of plants showing severe reactions gave strong hybridization with the CEV probe and an intense band with the same mobility as CEV as shown by PAGE. These plants clearly contained CEV. Plants with mild reactions gave weak hybridization with the CEV-probe and no band by PAGE. Therefore, the agent(s) responsible for mild reactions was either a mild form of CEV, which can be extracted only in extremely small amounts, or a type of viroid distinct from CEV. The results suggest that the three M-isolates that give symptoms on chrysanthemum are either mixtures of the mild viroid and CEV in which the latter is suppressed, or strains of the mild viroid that, when extracted and inoculated onto chrysanthemum, can mutate to give the typical (severe) form of CEV. The results, though inconclusive for the one M-isolate that failed to give symptoms, support our previous evidence that viroids are associated with dwarfing.

Seven isolates of graft-transmissible dwarfing agents (GTD agents) have been used in horticultural performance trials with orange trees (*Citrus sinensis* (L.) Osbeck) on trifoliate orange (*Poncirus trifoliata* (L.) Raf.) rootstock in New South Wales. Three isolates (S-isolates) clearly contain citrus exocortis viroid (CEV). This has been demonstrated in our previous paper (16) by symptomatology in citron (*C. medica* L.), gynura (*Gynura aurantiaca* DC), and tomato (*Lycopersicon esculentum* Mill.), nucleic acid hybridization with a CEV-specific probe, and polyacrylamide gel electrophoresis (PAGE) with CEV as a standard. However, for the remaining four isolates (M-isolates), the only evidence of viroids is mild leaf-curling symptoms in citron; the results for the hybridization and PAGE assays are essentially negative, and attempts at transmission to gynura and tomato have failed (16). On the basis of these experiments, the presence of viroids in the M-isolates is questionable.

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) gives characteristic symptoms and high extractable yields when mechanically inoculated with several types of viroid (10). Therefore, we used this host to test for and further characterize viroids in the M-isolates.

MATERIALS AND METHODS

Isolates of GTD agents. Each isolate was derived from a single dwarfed orange or grapefruit (*C. paradisi* Macf.) tree and propagated in orange trees by inoculation with buds (16). The four M-isolates were 3531, 3532, 3538, and 3539. Three S-isolates (033, 3535, and 3536) were used for comparison as these were known to contain CEV. The M- and S-isolate inocula used here were 2 M LiCl-soluble nucleic acids from infected orange trees and citron plants, prepared as described in our previous paper (16).

Growth and inoculation of chrysanthemum plants. All experiments with chrysanthemum (cultivar Bonnie Jean) were carried out in controlled environment growth rooms with day and night temperatures of 27 C and 25 C, respectively, and a photoperiod of 16 hr. Lighting, mounted at a vertical distance of 1 m from the soil surface, consisted of 215 W Gro-Lux fluorescent tubes (one per 5 cm of bench width) and 60 W incandescent bulbs (one per 0.26 m^2 of bench area).

Shoot tip cuttings were grown in John Innes potting compost (7) in 1-L black plastic bags and fertilized at 2-wk intervals with soluble fertilizer (NPK ratio 23:4:18) or ammonium nitrate (1 g/L) both supplemented with $MnSO_4$ (0.02 g/L) and iron chelate (0.02 g/L). Inoculation was carried out when the plants had grown to a height of 8–14 cm. A drop of inoculum (20 μl of nucleic acids at a concentration of 1 μg/μl in 0.05 M potassium phosphate, pH 8.0) was placed on a razor blade and introduced by means of 15–30 puncture wounds down the length of the stem. Shoot tips were removed at the time of inoculation and plants were cut back at intervals of 4–6 wk.

Extraction of nucleic acids. The following one-step procedure gave extracts in which CEV could be clearly detected by either the hybridization or PAGE assays. Fresh chrysanthemum leaf samples (0.5 g) were homogenized in 3 ml of phenol mixture (water-saturated phenol containing 0.8% 8-hydroxyquinoline) and 5.5 ml of the extraction buffer (0.5 M sodium acetate-acetic acid, pH 6.0, 10 mM $MgCl_2$, 20% (v/v) ethanol, and 3% (w/v) sodium dodecylsulfate) described by Laulhere and Rozier (6). The

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homogenate was shaken at 37 C for 10 min, mixed with 3 ml of chloroform-pentanol (25:1, v/v), and shaken for a further 10 min at room temperature. The aqueous phase was separated by centrifugation, and the nucleic acids were precipitated by addition of 2.5 volumes of ethanol, sedimented at 10,000 *g* for 15 min, dried in a freeze-drier, resuspended in 50 μl of 0.1 mM sodium ethylenediaminetetraacetate, and rapidly frozen by immersion in liquid nitrogen before storage at −20 C.

Crude homogenates. Chrysanthemum leaves (0.5 g) were ground in 1 ml of 0.05 M potassium phosphate buffer, pH 8.0, and centrifuged for 1 min in a microcentrifuge. The supernatant was stored at −80 C until it was used in the hybridization assay.

Other materials and methods. As previously described (16), extensive precautions were taken to prevent cross-contamination between isolates in the field, greenhouse, growth rooms, and laboratory. Inoculated chrysanthemum plants were spaced to prevent transmission by foliar contact.

The probe used for the hybridization assay was ^{32}P-labeled complementary ribonucleic acid (cRNA) prepared by J. E. Visvader of the Biochemistry Department, University of Adelaide. The complete sequence of CEV-A was cloned into the plasmid vector pSP6-4, which contains a promoter for phage SP6 RNA polymerase, and the insert was then transcribed to give single-stranded ^{32}P-RNA transcripts using α-^{32}P-GTP as label (3). The choice of the cRNA probe rather than the cDNA probe used in our earlier work (16) was based on the greater sensitivity of cRNA for detection of CEV sequences (J. Visvader, *personal communication*). The probe gave no hybridization with extracts from healthy plants, but the extent to which it might hybridize with viroids other than CEV was not tested.

The dot-blot hybridization assay and PAGE of nucleic acids were described previously (16). Staining of nucleic acids in polyacrylamide gels with silver (8) was done using a kit from Bio-Rad Laboratories, Richmond, CA.

RESULTS

Types of reaction in chrysanthemum. Two very distinct types of reaction were observed in transmission experiments with M-isolates. The first was a severe reaction typical of CEV in chrysanthemum (10,13). At the early stages of infection, severe reactions were recognized by yellowing along the veins and slight leaf distortion (Fig. 1A). Later, numerous chlorotic spots appeared in new leaves and leaf distortion became more pronounced (Fig. 1B). Leaves became progressively smaller and more distorted, the stems became brittle, and the plants appeared stunted (Fig. 2).

The second type of reaction was mild by comparison and virtually identical to reactions described for potato spindle tuber viroid (10), chrysanthemum stunt viroid (2,10,13), and cucumber pale fruit viroid (13) in chrysanthemum. The earliest signs of infection were minute chlorotic spots on the tips and margins of young leaves (leaves at far left of Fig. 3). Most of these spots subsequently disappeared and were replaced by large spots, bright yellow to pale green in color, which were scattered all over the lamina. In some cases (Fig. 3B), spots become necrotic or contained green islands. Leaves showed no obvious distortion or reduction in size and the plants were not stunted. Mild symptoms never developed into severe symptoms, even when the plants were cut back repeatedly for periods of up to 16 mo.

There were no obvious differences between mild reactions produced by different M-isolate inocula. Most of the severe reactions were indistinguishable from reactions caused by the S-isolates (Fig. 2A). Two, however, were atypical; one was very severe (Fig. 2B) and the other caused leaf distortion but relatively little spotting or chlorosis (Fig. 2C). These atypical symptoms were reproduced during a second passage in chrysanthemum.

Mild reactions were observed only in a growth room under conditions described in Materials and Methods and disappeared if

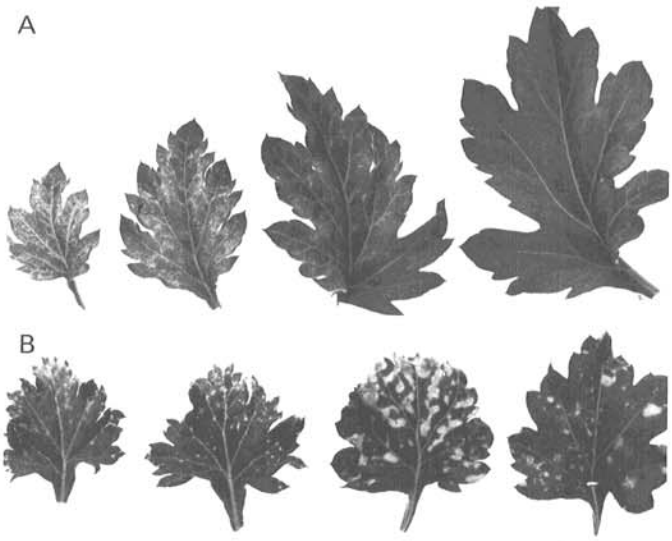

Fig. 1. Severe reactions in chrysanthemum leaves. **A,** Early symptoms, 40 days after inoculation. **B,** Late symptoms, 6 mo after inoculation. Each series of four leaves was from one shoot, and the youngest leaves are shown on the left.

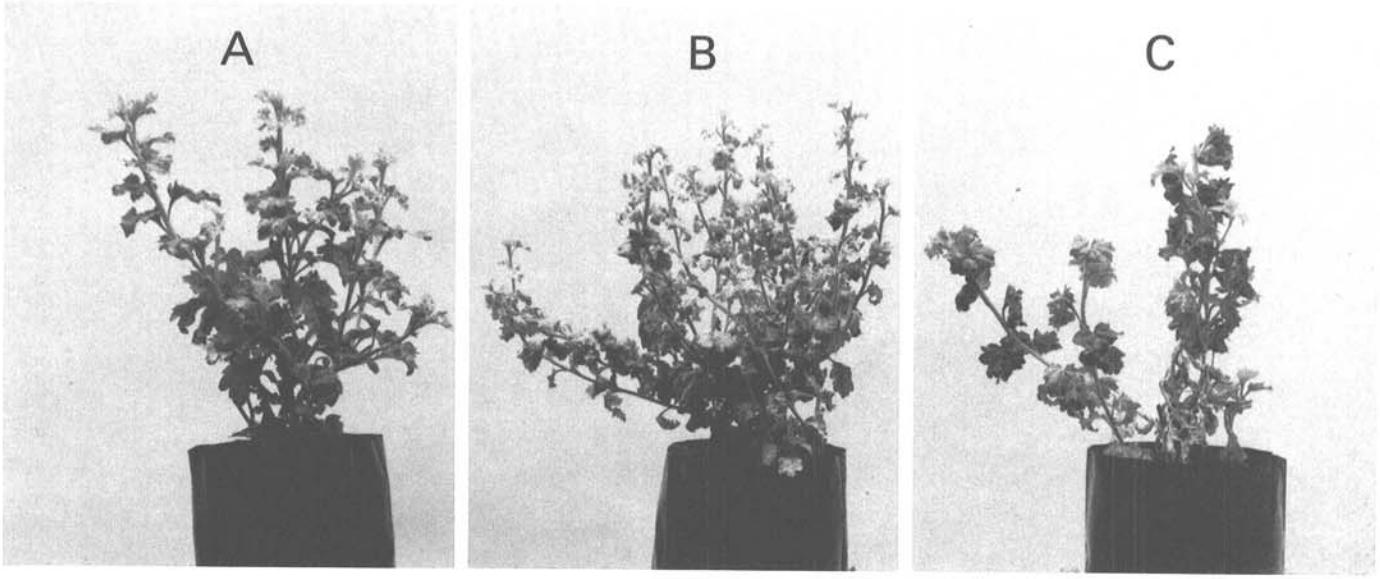

Fig. 2. Severe reactions in chrysanthemum plants. Plants were photographed 6 mo after inoculation. Plants **A** and **C** were cut back several times, whereas plant **B** was not cut back at any stage.

plants were moved to a greenhouse at a similar temperature. Severe reactions were observed in either the growth room or greenhouse.

Reactions produced by different isolates. Inoculation of chrysanthemum plants with nucleic acid extracts from infected orange trees and budded citrons gave the results shown in Tables 1 and 2. For citron extracts, reactions were ascribed to the particular orange tree used as bud-inoculum. One of the M-isolates (3538) failed to produce symptoms (Table 1). Each of the other three isolates 3531, 3532, and 3539) gave instances of both mild and severe reactions among the different trees tested. Two trees (one infected with 3531 and the other with 3532) gave both types of reaction. Other infected trees produced one or other of the reactions or, in some instances, no reaction (Table 1).

The mild reaction was the more frequent type in terms of both the number of trees that gave reactions (Table 1) and the number of chrysanthemum plants (replicate inoculations) that developed symptoms. Results for individual extracts from three trees are given as examples (Table 2). A total of 70 mild reactions was recorded compared with only seven severe reactions for the experiments summarized in Table 1. The three trees recorded as giving only severe reactions (one 3531 and two 3539) each produced symptoms in only one of the 4–12 inoculated plants.

The infectivity of the M-isolates was generally low. Of the inocula that produced reactions, only a few gave reactions in four out of four inoculated plants. Repeated freezing and thawing of the nucleic acid extracts appeared to cause a drop in infectivity. In contrast, the three S-isolates shown to contain CEV in our previous work had a high infectivity on chrysanthemum, which was not obviously affected by freezing and thawing. The reactions for the S-isolates (82 total) were all of the severe type.

For M-isolates, both severe and mild reactions were late in developing; 6–14 wk for severe reactions and 9–17 wk for mild reactions. In contrast, S-isolates produced symptoms (severe only) within 3–8 wk.

Uninoculated control plants were included in every experiment, and out of 46 such controls, only one developed symptoms.

Reactions produced by a second passage in chrysanthemum. Chrysanthemum plants showing mild and severe reactions to M-isolates were used to prepare nucleic acid extracts. The results of inoculation with these extracts are given in Table 3. Two of the four mild inocula gave rise to severe as well as mild reactions. In contrast, severe inocula gave rise to severe reactions only. The onset of severe reactions (2.5–6 wk) was more rapid here than when citrus tissues were used as inoculum.

Hybridization assay for CEV in inoculated chrysanthemum plants. Hybridization with a CEV-specific cRNA probe was used to examine if CEV was associated with severe and mild reactions in chrysanthemum. Figure 4A shows the results obtained with nucleic acid extracts from individual chrysanthemum plants. All plants with severe reactions derived from M-isolates (grid references a3–a8, Fig. 4A) gave intense hybridization spots that were similar to those produced by isolate 033 (grid references a1 and a2). Plants with well-developed mild reactions to isolates 3531 (b1–b4) and 3532 (c1–c7) gave spots that were relatively faint. Plants with indistinct mild reactions (d1–d4) gave faint spots or no spots, and symptomless plants (with one possible exception, grid e5) gave no spots. The symptomless plants (row e) included two inoculated plants and six uninoculated controls.

As it was conceivable that low levels of CEV might be lost in the nucleic acid extraction procedure, crude homogenates were prepared from the leaves of chrysanthemum plants and tested by the hybridization assay. Plants showing severe reactions again gave strong positive spots (Fig. 4B, row a). However, in contrast to the results with nucleic acid extracts (Fig. 4A), plants with mild reactions (Fig. 4B, rows b, c, and d) gave no spots.

PAGE test for CEV and other viroids in inoculated

TABLE 1. Reactions recorded in chrysanthemum for nucleic acid extracts of individual orange trees

Isolate	Number of orange trees tested[a]	Number of trees giving reactions in chrysanthemum: Severe reaction only	Mild reaction only	Severe and mild reactions	No reactions
3531	6	1	2	1	2
3532	6	0	5	1	0
3538	3	0	0	0	3
3539	5	2	1	0	2

[a] For each tree tested, nucleic acid extracts used to inoculate chrysanthemum plants were from one or more of the following tissues: leaves, scion (orange) bark, rootstock (trifoliate orange) bark, or leaves from bud-inoculated citron. Each extract was inoculated onto at least one group of four plants and all the reactions derived from each tree were recorded. The table is a summary of eight inoculation experiments.

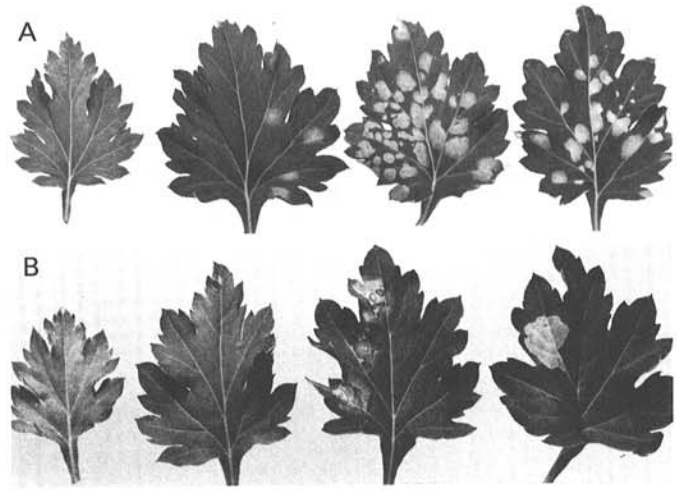

Fig. 3. Mild reactions in chrysanthemum leaves. **A,** Leaves showing typical chlorotic spots and no leaf distortion. **B,** Leaves showing large lesions and necrotic areas within spots. Each set of leaves was taken from one shoot and the youngest leaves are shown at left.

TABLE 2. Reactions recorded in chrysanthemum plants for individual nucleic acid extracts from orange trees

Isolate	Infected orange tree	Tissue used to prepare extract	Number of chrysanthemum plants inoculated	Number of chrysanthemum plants showing symptoms: Severe reaction	Mild reaction	No reaction
3531	2nd transmission Newton Valencia[a]	Scion bark	4	1	2	1
		Rootstock bark	8	1[b]	5	2
3532	1st transmission Bellamy navel[a]	Scion bark	16	2[c]	13	1
		Rootstock bark	4	0	1	3
3532	2nd transmission Bellamy navel	Mixture of scion & rootstock bark	40	0	29	11

[a] Trees listed in Table 1 as giving both severe and mild reactions.
[b] Plant showing atypical severe reaction (plant C, Fig. 2).
[c] One plant showed an atypical severe reaction (plant B, Fig. 2).

chrysanthemum plants. The nucleic acid extracts tested by the hybridization assay were further analyzed by electrophoresis in 5% polyacrylamide gels and staining with silver. Plants showing severe reactions gave an intense band with the same mobility as CEV (Fig. 5A). Plants with mild reactions (Fig. 5B) and symptomless plants (Fig. 5C) gave no trace of CEV (Fig. 5A) or any other band not also present in uninoculated plants (tracks 1–6, Fig. 5C). Use of ethidium bromide as stain also failed to reveal any trace of a viroid band in plants showing mild reactions.

DISCUSSION

Of the two types of reaction produced by M-isolates in chrysanthemum, the severe type clearly was caused by CEV. This was based on CEV-like symptoms, strong hybridization with a CEV-specific cRNA probe, and a symptom-related nucleic acid species with the same electrophoretic mobility as CEV in polyacrylamide gels. The finding of CEV in M-isolates is consistent with the work of Visvader and Symons (17) in which CEV was extracted from a 3532-infected orange leaf sample, transmitted to chrysanthemum, and positively identified by nucleic acid sequencing.

The agent responsible for the mild type of reaction could not definitely be identified as CEV, because it gave only faint hybridization with the CEV-probe and no symptom-related nucleic acid band. However, it was almost certainly a viroid for the following reasons: its symptoms were the same as those described for potato spindle tuber and other viroids (2,10,13); it was associated with three M-isolates that were previously observed (16) to give mild viroidlike symptoms in a completely unrelated host (citron); based on tests with chrysanthemum nucleic acid extracts (Fig. 4A), it hybridized to some extent with the CEV-probe; it was mechanically transmissible using 2 M LiCl-soluble nucleic acids from citrus tissues as inoculum; finally, it was unlikely to be a virus, because electron microscopy of negatively-stained homogenates and ultra-thin sections of chrysanthemum leaves failed to reveal any particles (*unpublished*).

The viroid responsible for mild reactions (hereafter called 'mild viroid') must be present at very low concentrations in chrysanthemum extracts, as it could not be detected by PAGE under conditions where CEV was strongly detected. There is evidence that it is present at low concentrations (relative to CEV) in citrus extracts as well, based on the following observations: the faint or undetectable hybridization of citrus extracts with a CEV probe (16); the low infectivity of M-isolates (which contain the mild viroid) compared with S-isolates (which appear to contain

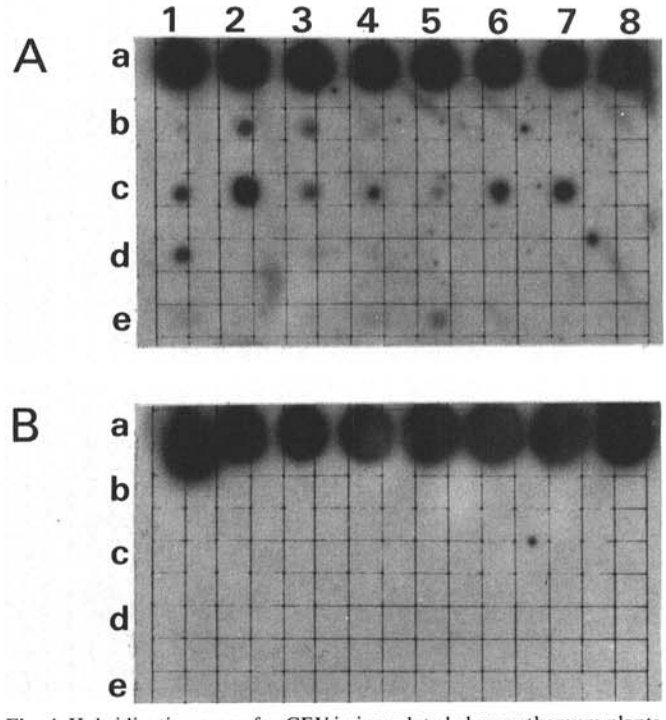

Fig. 4. Hybridization assay for CEV in inoculated chrysanthemum plants. **A,** Results for nucleic acid extracts. **B,** Replicate experiment with crude homogenates from the same plants. The extracts and homogenates, each from leaves of a single chrysanthemum plant, were spotted on the two nitrocellulose membranes according to the following plan: row a, plants with severe symptoms; row b (spots 1–4) and row c (spots 1–7), plants with mild symptoms; row d (spots 1–4), plants with mild symptoms, which were faint or indistinct; row e, symptomless plants. Positions b5-8, c8, and d5-8 were blank. The extracts used to inoculate chrysanthemum plants are listed below by the isolate and tissue extracted: SB (orange scion bark), RB (trifoliate orange rootstock bark), CL (leaf from bud-inoculated citron), and CHR (chrysanthemum inoculated with citrus extract). Grid reference a1, 033/SB; a2, 033/CHR; a3, 3531/SB; a4, 3531/RB; a5, 3531/CHR; a6, 3531/CL; a7, 3532/CHR; a8, 3539/SB; b1, 3531/SB; b2–4, 3531/CHR; c1, 3532/SB + RB; c2, 3532/CHR; c3–7, 3532/SB; d1, 3531/SB; d2, 3532/SB + RB; d3, 3538/CHR; d4, 3539/CHR; e1–6, uninoculated controls; e7, nondwarf/SB; e8, 3538/SB.

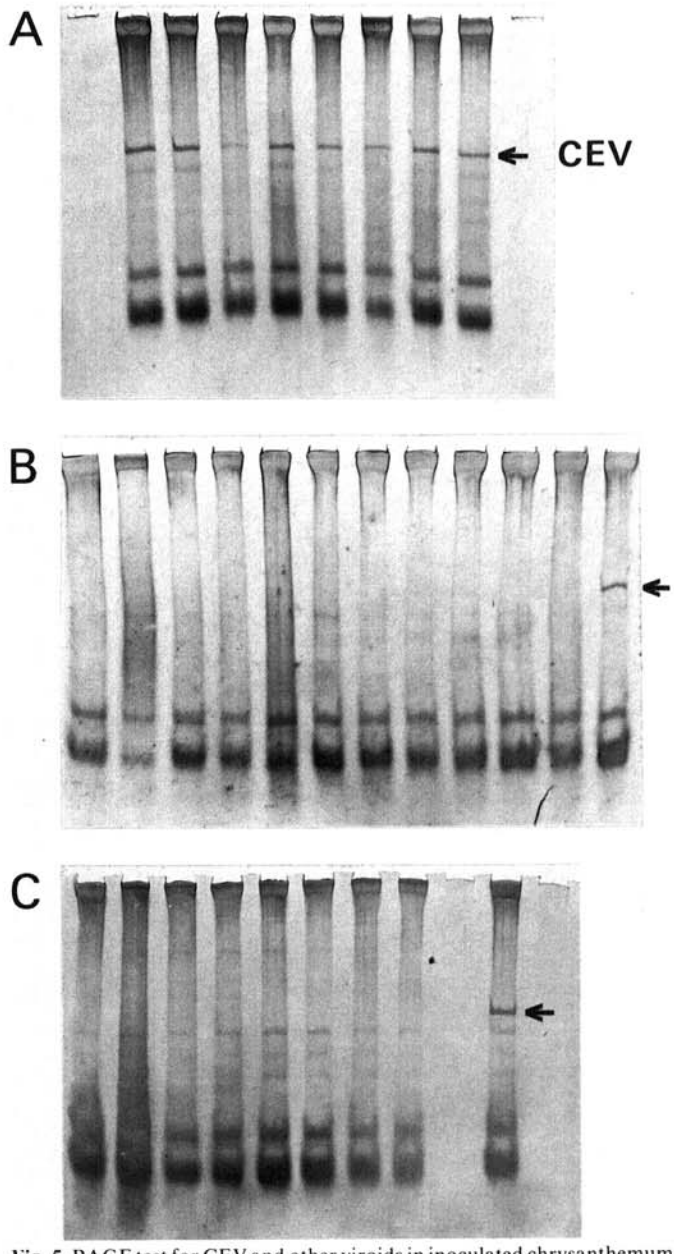

Fig. 5. PAGE test for CEV and other viroids in inoculated chrysanthemum plants. The nucleic acid extracts were the same ones used for the dot-blot tests shown in Fig. 4A. **A,** Plants with severe symptoms (row a, Fig. 4A). **B,** plants with mild symptoms (rows b and c, Fig. 4A) with CEV (033) control in the far right-hand track; **C,** extracts from symptomless plants (row e, Fig. 4A) with CEV control on the far right.

only CEV) in nucleic acid extracts when inoculated onto chrysanthemum; and the loss of infectivity of nucleic acid extracts with repeated freezing and thawing and the failure to obtain even weak hybridization reactions in crude extracts, both of which could reflect a critical concentration that is more readily affected by degradative processes. The low concentrations in extracts could result from either a low concentration in infected plants, or an unusual difficulty in extraction of the viroid.

As shown by the hybridization assay in our previous (16) and present work, CEV reached easily detectable concentrations in orange trees, citron plants, and chrysanthemum plants inoculated with S-isolates. However, it was not clearly detectable in extracts from orange trees and citron plants infected with M-isolates (16), even though a number of these extracts produced CEV reactions in chrysanthemum. Neither was it clearly detectable in extracts from chrysanthemum plants showing mild reactions to M-isolates, even though two of these plants (Table 3) gave rise to CEV in a second-passage inoculation; faint hybridization spots or no spots were observed in every case. Difficulties in the extraction of CEV cannot easily account for these observations, and it is probable that the mildly reacting citrus and chrysanthemum tissues all contained very low concentrations of CEV.

The results suggest that the three symptom-producing M-isolates are stable mixtures of CEV and the mild viroid. CEV is apparently suppressed in terms of both symptoms and concentration in infected tissue, and both viroids are present at much lower concentrations than those normally attained by CEV. There are precedents for mixtures of CEV species within an isolate (12,14,17). However, a persistent suppression of a severe viroid by a mild one would be a new phenomenon. Chrysanthemum stunt viroid, which gives mild symptoms, and mild strains of potato spindle tuber viroid (PSTV) have been found to give only temporary cross-protection against CEV and severe PSTV in tomato and chrysanthemum (1,9); if the plants are grown for extended periods and/or cut back several times, the latter, more severe viroids are eventually expressed. Mild isolates of CEV reportedly give no measurable cross-protection against severe isolates in citron (4), Rangpur lime (*C. limonia* Osbeck) or orange trees on Rangpur lime rootstock (14). Whereas mild isolates can be obtained from orange trees on Rangpur lime rootstock (14) and citrons (12) showing severe symptoms, the reverse has not been found.

There is a second explanation for the M-isolates that is consistent with our results. The mild viroids could be mild strains of CEV, which occasionally mutate to give the typical (severe) form. Mutation might only occur in connection with extraction and/or inoculation onto chrysanthemum; no graft-inoculated orange trees or citrons have ever developed severe symptoms or clearly detectable levels of CEV in response to M-isolates. In vivo mutation has not been reported for any viroid. However, naturally occurring severe isolates of PSTV differ from mild isolates by as few as two nucleotides (15), so that severe mutants might be expected to arise occasionally from mild strains.

Among plant viruses, there are precedents for our observations with M-isolate viroids. Mass inoculation of tomato plants with a symptomless mutant of tobacco mosaic virus was reported to give mosaic symptoms in about 3% of inoculated plants (11). Inoculation of small lesion isolates of cucumber mosaic virus (CMV) onto cowpea (*Vigna unguiculata* ssp. *cylindrica* 'Catjang') gives rise to large lesions at a rate of 0.53% (5). There is strong evidence that the large lesions arise by mutation of CMV and that, in contrast to other hosts, Catjang cowpea selects to some degree for large lesion mutants (5). Chrysanthemum may be analogous to cowpea in selecting for severe forms of CEV.

In summary, the results are strong evidence that three of the M-isolates (3531, 3532, and 3539) contain viroids. CEV is present in these three isolates, but an unidentified mild viroid (possibly a mild form of CEV) predominates. The failure of some trees to transmit viroid symptoms (Table 1) is probably due to very low concentrations of viroids in the inocula and/or the small number of chrysanthemum plants inoculated. The results are inconclusive for one isolate (3538) that failed to give symptoms; its mild leaf-curling symptom on citron (16) provides evidence of a viroid, but it appears to be incapable of infecting chrysanthemum under our conditions. The finding of a mild viroid in three M-isolates (but not in S-isolates) is consistent with the mild symptoms of M-isolates in citrus: mild leaf curling in citron and mild dwarfing of orange trees on trifoliate orange rootstock. The present work therefore supports our previous evidence (16) that viroids are associated with dwarfing.

It is noteworthy that sensitive biochemical tests (nucleic acid hybridization and PAGE) have proven ineffective for detecting viroids in orange trees and citron plants infected with the M-isolates. Even highly sensitive hosts (orange trees on trifoliate orange rootstock, citron, and chrysanthemum) give mild symptoms which take months or years to develop. The extreme difficulty of detecting these viroids, apparently symptomless in all but a few plants, suggests that similar mild viroids may be more widespread in horticultural crops than is presently realized.

TABLE 3. Reactions produced by a second passage in chrysanthemum

Isolate	Reaction type of first passage plant	Second passage reactions[a]		
		Severe	Mild	No reaction
3531[b]	Mild	1	3	0
3531	Mild	1	2	1
3531	Mild	0	2	2
3532[d]	Mild	0	2	2
3531[b]	Severe	4	0	0
3532[c]	Severe	4	0	0
3532[c]	Severe	4	0	0

[a] The inocula for the second passage were nucleic acid extracts from leaves of individual chrysanthemum plants. Each inoculum was used on four plants.

[b,c,d] The sources of first-passage inoculum were, respectively, the same trees listed from top to bottom in Table 2.

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