Additional Hosts and an Efficient Purification Procedure for Four Viroids

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ABSTRACT

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Additional hosts of potato spindle tuber viroid (PSTV), citrus exocortis viroid (CEV), chrysanthemum stunt viroid (CSV), and chrysanthemum chlorotic mottle viroid (ChCMV) are reported. *Chrysanthemum morifolium* is a new host of PSTV (both mild and severe strains) and CEV. Both viroids reach high concentrations in chrysanthemum. Tomato (*Lycopersicon esculentum* 'Rutgers') and *Gynura aurantiaca* are new hosts of CSV; and *Chrysanthemum zawadskii* is a new host of ChCMV. PSTV and CEV were purified from these hosts, labeled in vitro with ^{125}I and identified by RNA fingerprinting analysis. Comparisons of fingerprints of these viroids from several hosts confirmed a previous report that the nucleotide sequence of viroid RNA is not determined by the host plant. An efficient purification procedure for PSTV, CEV, and CSV is described. Yields of 10–50 times greater than those of published procedures for PSTV were obtained. Additional evidence for the viroid nature of ChCMV and its symptomless strain were obtained by demonstrating infectivity following fractionation in 5–15% polyacrylamide gels.

Viroids are unique plant pathogens. They are comprised of single RNA species with molecular weights of 100,000–130,000 (11,30,34) and contain no capsid proteins (11,34). Apparently, viroid RNA does not code for any proteins (3,32,43) and at least for PSTV may not contain an AUG initiation codon for translation (16). Six different viroids (potato spindle tuber viroid [PSTV] [13], citrus exocortis viroid [CEV] [36], chrysanthemum stunt viroid [CSV] [12,18], cucumber pale fruit viroid [CPFV] [42], chrysanthemum chlorotic mottle viroid [ChCMV] [29], and cadang-cadang viroid [CCV] [26]) have been described, and there is recent evidence that the agent that causes the hop stunt disease also may be a viroid (31).

Several viroids infect and cause similar symptoms in the same hosts. For example, PSTV, CEV, CSV, and CPFV infect tomato (*Lycopersicon esculentum* Mill. 'Rutgers'). All except CSV cause stunting, epinasty, and bunchy-top symptoms (23,27,38). Likewise, PSTV, CEV, and CSV all cause symptoms in *Gynura aurantiaca* DC (1,37,40). On the basis of similar symptoms and physical properties, it has been suggested that PSTV and CEV might be the same entity (33,38,41). However, subsequent application of in vitro labeling and RNA fingerprinting techniques have demonstrated that PSTV, CEV, CSV, CPFV, and CCV are all distinctly different RNA species (4,8,17). Even strains of PSTV which differ in pathogenicity have been differentiated by their RNA fingerprints (6). The present study was undertaken to identify a common host of PSTV, CEV, CSV, and ChCMV which would permit individual identification on the basis of symptom expression. In addition, we have developed a more efficient method for viroid purification, have obtained evidence for the viroid nature of ChCMV and its nonsymptomatic strain (ChCMV-NS), and have extended earlier studies by Dickson et al (5) which demonstrated that the nucleotide sequence of viroid RNA is not significantly altered by the host plant.

MATERIALS AND METHODS

Source and propagation of viroids. Inoculum of severe and mild strains of PSTV (Designated PS and PM, respectively) was kindly provided by K. H. Fernow, and inoculum of CEV was kindly provided by J. S. Semancik. These viroids were propagated in either tomato (*L. esculentum* 'Rutgers') or chrysanthemum (*Chrysanthemum morifolium* Ramat. 'Bonnie Jean'). The Cornell University isolates of CSV, ChCMV, and ChCMV-NS were used. CSV was propagated in chrysanthemum cultivar Bonnie Jean, and both strains of ChCMV were propagated in chrysanthemum cultivar Velvet Ridge. Inoculum of PS, PM, and CEV was prepared by grinding 1 g of infected leaflets per milliliter of 0.04 M potassium phosphate buffer, pH 8.0, containing 0.3% 2-mercaptoethanol in a mortar and pestle. Inoculum was applied with a cotton swab to Carborundum-dusted leaves of tomato plants at the three- to four-leaf stage. Plants were maintained in greenhouses at 22–28 C, with supplemental fluorescent light in winter (12-hr photoperiod). At 60–75 days postinoculation (PI) all symptomatic leaflets were harvested, ground in liquid N_2, and stored at −80 C.

Three procedures were used to inoculate chrysanthemum plants. For PSTV, CEV, and CSV inoculations were made with nucleic acid concentrates (2- to 20-fold on a tissue weight basis) prepared by the procedure of Semancik and Weathers (37). A drop of this inoculum was placed midway on the stem of a 10- to 15-cm-tall rooted cutting, and inoculation was accomplished by making 25 deep puncture wounds through the drop along the stem with a No. 11 scalpel blade. Inoculations with ChCMV strains were made similarly with 20-fold nucleic acid concentrates or by the tissue implantation method of Dimock et al (14) or by the borate buffer–homogenization and leaf-rubbing method of Romaine and Horst (29). Infected chrysanthemum plants were maintained in greenhouses at 22–28 C with supplemental fluorescent lighting (12-hr photoperiod). Infected leaves of chrysanthemum were harvested fresh when needed. The nonsymptomatic nature of ChCMV-NS necessitated the use of a cross-protection test for its detection (19). The use of cross-protection was necessary since there is no differential host for ChCMV-NS. That is, plants purportedly infected with ChCMV-NS were challenge-inoculated with ChCMV. Those not developing ChCMV symptoms were considered to be infected with ChCMV-NS.

Host range studies. PS, PM, CEV, CSV, and ChCMV were inoculated to Rutgers tomato by rubbing the leaves, and to *G.*

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aurantiaca and Bonnie Jean and Velvet Ridge chrysanthemums by the stem puncture procedure. Bioassays of symptomless plants were performed on appropriate hosts 30–60 days PI. Forty-six different species were inoculated with ChCMV by either the leaf-rubbing method (29) or the tissue implantation method (14). Comparable inoculations of cultivar Deep Ridge chrysanthemums were always performed to establish the efficacy of the inoculum and the suitability of environmental conditions. Plants were observed for at least 30 days PI for symptom development. Both inoculated and uninoculated leaves of symptomless plants were bioassayed on 'Deep Ridge' chrysanthemum by the leaf-rubbing method.

Viroid purification. The purification procedures of Diener (9,10), Semancik et al (35,37), and Singh and Sänger (39) were tested with PSTV-infected tomato and CSV-infected chrysanthemum tissues. These procedures were found to be laborious, expensive, and inefficient, and a new, more rapid and efficient procedure was developed that utilizes the essential steps of the above, but modifies and changes their sequence. In our purification procedure 600 g of frozen tomato tissue (N_2-frozen then stored at −80 C) or of freshly N_2-frozen chrysanthemum tissue were ground for 3 min in a gallon-size Waring Blendor containing 600 ml buffer (0.10 M tris-HCl, 0.01 M EDTA, 0.10 M NaCl, 1% SDS, pH 8.9), 1,200 ml water-saturated phenol (containing 0.1% 8-hydroxyquinoline), 3 ml 2-mercaptoethanol, and 12 g Wyoming bentonite. The emulsion was poured into 250-ml centrifuge bottles and centrifuged 10 min at 6,000 *g*. Upper, aqueous phases were pooled and re-extracted with an equal volume of phenol by vigorous magnetic stirring for 60–90 min at 4 C. The centrifugation was repeated and aqueous phases were mixed with 2.5 volumes of cold 95% ethanol. After storage overnight at −20 C, the mixture was centrifuged for 10 min at 8,000 *g*. The crude nucleic acid pellets were dried under N_2 to remove the residual ethanol and then transferred to a 150-ml beaker with a spatula. The centrifuge bottles were rinsed with a total of 60 ml of TKM buffer (0.01 M tris-HCl, 0.01 M KCl and 0.0001 M $MgCl_2$, pH 7.4), which was then added to the pellets. The pellets were broken up with a spatula and 1.2 ml of 0.10 M $MgCl_2$ was added. Then 0.72 ml of RNase-free DNase (250 ug/ml in 0.15 M NaCl) were added and the mixture was incubated at room temperature for 60–90 min. The mixture was then dialyzed overnight at 4 C in 2 L of STE buffer (0.05 M tris-HCl, 0.001 M EDTA, 0.10 M NaCl, pH 7.2). The dialyzed preparation was placed in a 250-ml centrifuge bottle. One-fourth volume of 10 M LiCl was added, and the mixture was stored overnight at 4 C. The mixture was centrifuged 15 min at 8,000 *g*, and the pellet, (primarily rRNAs) was discarded. The supernatant (low-molecular-weight RNAs) was made to 28% ethanol (v/v), and 33 g of CF-11 cellulose powder (Whatman) were stirred into approximately 250 ml of liquid in a 1-L beaker. The preparation was covered and stirred slowly overnight at 4 C. The CF-11 was allowed to settle out and the supernatant was decanted several times until the CF-11 had been rinsed with 1–2 L of STE + 28% ethanol or until the supernatant was colorless. The slurry was then poured into eight 50-ml centrifuge tubes and the CF-11 was pelleted by centrifugation for 5 min at maximum speed in a clinical centrifuge. The pellets were washed 2–3 times by adding STE + 28% ethanol, stirring with a glass rod and centrifuging. The RNA was eluted from the CF-11 by rinsing it six times with 8 ml of STE, stirring well and centrifuging each time. All rinses of each centrifuge tube (48 ml) were poured into separate 250-ml centrifuge bottles, and the nucleic acids were precipitated by addition of 150 ml of cold 95% ethanol and storage overnight at −20 C. The nucleic acids were pelleted by centrifugation for 20 min at 15,000 *g* and the eight pellets were dried under N_2. Pellets were then dissolved in a total of 10 ml of sterile deionized water by transferring the water from bottle to bottle.

Viroids were further purified and yields quantitated by polyacrylamide gel electrophoresis (22). Cylindrical gels (0.6 × 9 cm) composed of 5% acrylamide and 0.25% bisacrylamide (w/v) were loaded with samples of up to 0.5 ml. The samples were mixed with one-tenth volume of a solution containing 40% sucrose, and 0.015% bromphenol blue, and 0.015% xylene cyanole FF. Electrophoresis was performed in Plexiglas tubes for 3–4 hr (6 ma/tube) at room temperature or at 4 C, in the buffer of Loening (21) (0.04 M Tris-acetate, 0.02 M sodium acetate, 0.002 M EDTA, pH 7.5). Gels were prerun for 15–30 min at 6 ma/tube. Following electrophoresis, the gels were removed from the tubes, and scanned at 260 nm for quantitation. Gels were then stained for 15 min in 1 mM EDTA containing 20 mg ethidium bromide per liter and destained for 15 min in 1 mM EDTA. Viroid bands were located under UV light and excised with a razor blade. The RNA was extracted from the gel and contaminating acrylamide removed as described by Dickson et al (5). Purified viroid was stored at −80 C.

RNA labeling and fingerprinting. Viroid RNA was labeled with ^{125}I by the procedure of Prensky (25), as described previously (7) and fingerprinted by conventional methods (2,28). In the fingerprinting procedure the labeled RNA is digested with either RNase T_1 or RNase A. The products are then separated successively by high voltage electrophoresis and homochromatography. The oligonucleotides assume unique positions, which are revealed by autoradiography. In our experiments fingerprinting was used to obtain positive identification of viroids purified from their new hosts and to confirm that cross-contamination of viroid inocula had not occurred.

RESULTS AND DISCUSSION

In our host range experiments new hosts were identified for each viroid tested. It is now possible to differentiate four viroids and two viroid strains biologically by using three species of host plants (Table 1). As reported, PM caused mild symptoms on tomato (Fig. 1D) (15) and no symptoms on *Gynura* (40), but bioassays indicated that *Gynura* was infected (40). The new host for PM was chrysanthemum; Bonnie Jean developed the mild chlorotic spotting shown in Fig. 3 C, and a height reduction of about 20%. The identity of the PM purified from Bonnie Jean was confirmed by fingerprinting (Fig. 4C). Chrysanthemum thus expresses obvious symptoms and can be used for bioassays. This would eliminate the uncertainty involved in the cross-protection test in tomato, which previously was used to detect PM (15).

Typical PS symptoms (15,27,38) developed on tomato (Fig. 1B), but this severe strain of PSTV did not cause symptoms on *Gynura* (40). On Bonnie Jean chrysanthemum, PS induced the same chlorotic spotting as PM, but the spots were more intense (Fig. 3D) and the plants were more stunted than were those treated with PM. The identity of the PS purified from Bonnie Jean was confirmed by fingerprinting (Fig. 4B). Previously, Diener and Smith (cited in [5]) suggested that chrysanthemum was a host for their strain of PSTV. This was further indicated by Owens et al (24) who hybridized an in vitro synthesized DNA complementary to

TABLE 1. Differentiation of selected viroids and their strains by plant reactions

	Plant and reaction[b]		
Viroid or strain[a]	*Gynura aurantiaca*	Rutgers Tomato	*Chrysanthemum morifolium*[c]
PS	MiEp or NS	SeRu, SeEp, SeSt	ChSp, St
PM	NS	MiEp	MiChSp, MiSt
CEV	SeRu, SeEp, SeSt	SeRu, SeEp, SeSt	SeRu, SeEp, SeSt
CSV	MiEp	NS	SeChSp
ChCMV	NI	NI	ChMtl
ChCMV-NS	NI	NI	NS

[a] Viroids or strains: PS and PM = severe and mild strains of potato spindle tuber viroid, respectively; CEV = citrus exocortis viroid; CSV = chrysanthemum stunt viroid; ChCMV and ChCMV-NS = the chlorotic mottling and nonsymptomatic strains of chrysanthemum chlorotic mottle viroid, respectively.

[b] Plant reactions: Mi = mild, Se = severe, NS = infected, but no visible symptoms, NI = not infected, Ep = epinasty, Ru = rugosity, St = stunting, Ch = chlorotic, Sp = spots, Mtl = mottle.

[c] Cultivars Bonnie Jean or Velvet Ridge may be used for CEV, but Bonnie Jean is the best indicator for PS, PM, and CSV and Velvet Ridge is best for ChCMV.

PSTV to a low molecular weight RNA fraction from chrysanthemum which had been inoculated with PSTV. We have demonstrated that chrysanthemum is a host of both a mild and a severe strain of PSTV by purifying the progeny viroids and fingerprinting them.

Symptoms typical of CSV (large, bright yellow spots and leaf distortion) were expressed on Bonnie Jean chrysanthemum (Fig. 3E). No symptoms were expressed on Rutgers tomato, but tomato was shown to be infected by bioassay on chrysanthemum. Mild leaf distortion and epinasty were observed on *Gynura* inoculated with CSV (Fig. 2B). Infection of tomato by CSV also has been indicated by Owens (*personal communication*) and the infection of *Gynura* by CSV has been indicated by Bachelier et al (1). Polyacrylamide gel electrophoretic analysis of RNA extracted from CSV-infected *Gynura* and tomato plants failed to yield a detectable viroid band. Thus, RNA fingerprinting could not be carried out in these cases. On the other hand, these RNA extracts, when used to inoculate chrysanthemum plants, produced disease symptoms typical of CSV. Therefore, it appears that CSV can infect tomato and *Gynura* plants, but may replicate to a lesser extent than PSTV and CEV in these plants.

Symptoms typical of CEV were observed on both tomato (Fig. 1A) and *Gynura* (Fig. 2C). Generally, the symptoms of CEV in tomato are comparable in severity to those of PS (Fig. 1B). However, the symptoms of CEV in *Gynura* are much more severe than those caused by CSV (Fig. 2B). The CEV-infected *Gynura* are stunted, distorted, rosetted, and show pronounced rugosity (Fig. 2C); whereas the CSV-infected *Gynura* are barely stunted and show mild rugosity (Fig. 2B). The new host for CEV is chrysanthemum. Following inoculation with CEV, both Bonnie

Fig. 1. Rutgers tomato plants infected with (left to right): citrus exocortis viroid; potato spindle tuber viroid (PSTV) (severe strain); PSTV (intermediate strain from T. O. Diener); and PSTV (mild strain); and uninfected control plant at right.

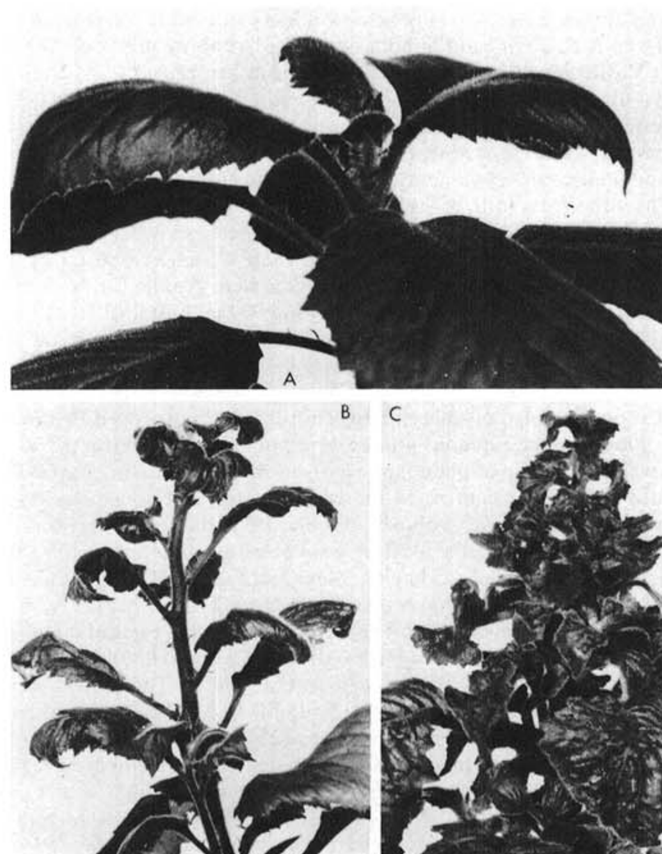

Fig. 2. Symptoms caused in *Gynura aurantiaca* plants by selected viroids: **A,** uninfected control; **B,** chrysanthemum stunt viroid (36 days postinoculation); and **C,** citrus exocortis viroid (45 days postinoculation).

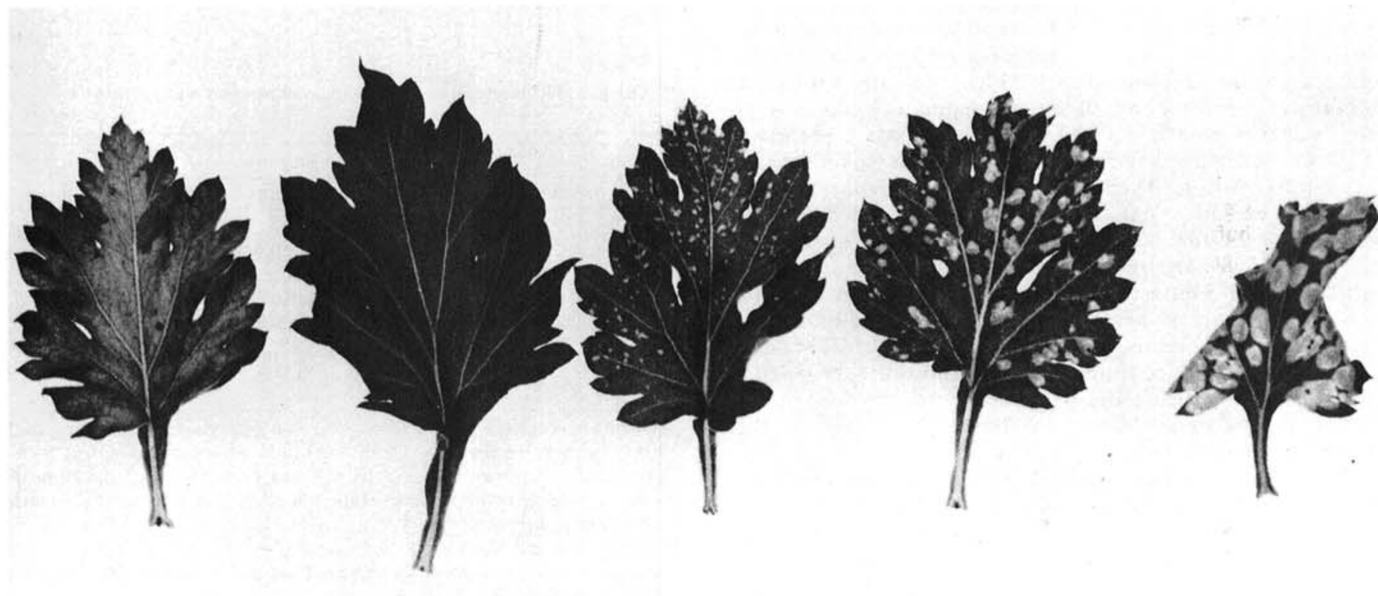

Fig. 3. Symptoms caused in chrysanthemum (cultivar Bonnie Jean) leaves by selected viroids (left to right): chrysanthemum chlorotic mottle viroid; uninfected control; potato spindle tuber viroid (PSTV) (mild strain); PSTV (severe strain); and chrysanthemum stunt viroid.

Jean and Velvet Ridge chrysanthemum expressed pronounced vein clearing and distortion of the expanding leaves of the apex and the lateral branches (Fig. 5). These symptoms were specific for CEV and were unlike those caused in chrysanthemum by any other viroid. Infected plants did not recover, but expressed increasingly more severe symptoms until the leaves were only 10–20% of normal size and the plants were severely stunted (Fig. 5). The stems of such plants were very brittle and the plants often died if subjected to heat or water stress. The identity of CEV purified from Bonnie Jean was confirmed by fingerprinting (Fig. 4A).

Symptoms typical of ChCMV were expressed on Bonnie Jean (Fig. 3A) and Velvet Ridge chrysanthemum. Under experimental conditions in which chrysanthemum was readily infected no symptoms were observed on Rutgers tomato or *Gynura* and "recovery" inoculation bioassays to chrysanthemum from inoculated tomato and *Gynura* all were negative for ChCMV. In the extended host range studies with ChCMV only *C. zawadskii* var. *latilobum* became infected with ChCMV, and it was a symptomless host. The following plants did not express symptoms and all "recovery" inoculations from these plants to chrysanthemum were negative: *Ageratum houstonianum* Mill., *Arctotis stoechadifolia* Bergius., *Artemisia douglasiana* Bess., *A. lactiflora* Wallich ex DC., *Aster* sp., *Bellis perennis* L., *Calendula officinalis* L., *Callistephus chinensis* (L.) Nees, *Centaurea cyanus* L., *Chrysanthemum arcticum* L., *C. carinatum* Schausb., *C. frutescens* L., *C. multicaule* Desf., *C. nivellei* Br.-Bl. & Marie, *C. parthenium* (L.) Bernh., *C. segetum* L., *Cichorium endiva* L., *C. intybus* L., *Coreopsis auriculata* L., *Cosmos sulphureus* Cav., *Cotula barbata* DC., *Cyamopsis tetragonoloba* (L.) Taub., *Cynara cardunculus* L., *Dahlia pinnata* Cav., *Encelia farinosa* A. Gray, *Gaillardia aristata* Pursh., *Gazania longiscapa* DC., *Helianthus decapetalus* L., *Helichrysum bracteautum* (Venten.) Andr., *Lactuca sativa* L., *Momordica balsamina* L., *Rudbeckia hirta* L., *R,. triloba* L., *Scopolia sinensis* Hemsl., *Senecio cruentus* (Masson) DC., *Sesbania exaltata* (Raf.) V. L. Cory, *Solanum tuberosum* L., *Tagetes patula* L., *Taraxacum officinale* Wiggers, *Tithonia rotundifolia* (Mill.) S. F. Blake, *Tragopogon porrifolius* L., and *Zinnia elegans* Jacq. Thus, the host range of ChCMV appears at present to be restricted to the two species of chrysanthemum. The host range of ChCMV-NS was not determined, and we have studied this viroid only in *C. morifolium* due to the difficulty of its bioassay.

However, we have partially purified both ChCMV and ChCMV-NS and recovered the infectious agents from 5, 10, and 15% polyacrylamide gels. The bulk of the infectivity was associated with or near the 7–9S host RNA band, but unlike the other viroids, the infectivity of ChCMV or ChCMV-NS could not be associated with a UV-absorbing RNA band. We found in 5% polyacrylamide gels (containing 0.25% bisacrylamide) that xylene cyanole FF migrated at the same rate as the 7–9S RNA band (and slightly faster than CEV, CSV, and PSTV) and that bromphenol blue migrated at the same rate as the 4S RNA. This observation simplified the timing of electrophoresis runs and facilitated the locating and excision of the RNA bands for bioassays. However, we were unable to purify adequate amounts of ChCMV for fingerprinting analysis. Thus, ChCMV and ChCMV-NS possess some of the characteristics associated with other viroids; they are composed of low-molecular-weight RNA, infectivity persists in phenol and other organic solvents, and no virus-like particles can be associated with the diseases they cause. ChCMV and ChCMV-NS are distinct from the other viroids in their narrow host range and that, thus far, are intractable to purification by the procedures we describe, and many variations of that procedure. However, Langowski et al (20), reported physical properties of ChCMV, but gave no detailed purification procedure.

The purification procedure developed during these investigations was more rapid, more efficient, and more economical than those described previously (9,10,35,37,39). Fewer steps and manipulations were performed with the RNA and smaller volumes of caustic and expensive reagents were used. Yields presented are based on the A_{260} readings in 5% polyacrylamide gels. The spectrophotometer was calibrated with identical gels containing measured amounts of 4S RNA. The yields given are averages of many experiments and differed only slightly from final RNA yields, because the RNA extraction procedure of Dickson et al (5) was 70–100% efficient. Viroid yields using our modified procedure were compared with published viroid yields as described by other workers for their individual purification procedures. From PS-infected tomato plants (infected 70–90 days) we routinely obtained yields of 700–1,000 μg/kg of tissue, compared to the 40–80

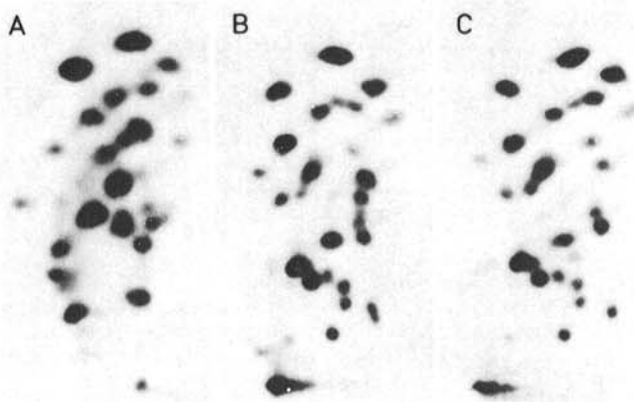

Fig. 4. RNA fingerprints of ^{125}I-labeled **A,** citrus exocortis viroid, **B,** severe potato spindle tuber viroid, and **C,** mild potato spindle tuber viroid—all purified from Bonnie Jean chrysanthemum. Fingerprinting was carried out as described previously (2,28): first dimensions (right to left) was high-voltage electrophoresis on cellulose acetate at pH 3.5; second dimension (bottom to top) was ascending RNA homochromatography on thin layers of DEAE-cellulose.

Fig. 5. Symptoms caused by citrus exocortis viroid on Bonnie Jean chrysanthemum. **A,** (left to right): uninfected plant, 28 and 88 days postinoculation, respectively. **B** and **C**: close-ups of plants 28 and 45 days postinoculation, respectively.

μg/kg obtained by Diener (10). From PM-infected tomato plants the yields were 300–350 μg/kg. From PS-infected chrysanthemum (infected 60–90 days) we obtained yields of 1,500–1,900 μg/kg. Purification of CSV from Bonnie Jean chrysanthemum (infected 60–120 days) by this procedure yielded 1,200 μg/kg. When CEV was purified from *Gynura,* tomato, and Bonnie Jean chrysanthemum by this procedure yields of 90, 330, and 670 μg/kg, respectively, were obtained. This compares with a CEV yield from *Gynura* of 20–40 μg/kg reported by Semancik et al (35). Viroid yields were not reported by Sänger et al (30), but Mühlbach and Sänger (23) report yields of 110 and 70 μg/kg for PSTV and CEV, respectively, from Rutgers tomato. Thus, PSTV yields from tomato by our purification procedure are 10–50 times greater than by other procedures. For CEV, our yields are 2–4 times greater from *Gynura* and three times greater from tomato. With each of PS, PM, CSV, and CEV the yields from chrysanthemum were always about twice that from tomato. We now routinely propagate these viroids in chrysanthemum because of the greater yield and because chrysanthemum is not a host of tobacco mosaic virus, a frequent greenhouse contaminant.

RNA fingerprints of three of the viroids purified from different hosts were compared to one another and to previously published fingerprints (5,6,8) to determine whether or not the propagation host alters the sequence of the viroid RNA. The viroid and host combinations examined were: CEV (from tomato, chrysanthemum, and *Gynura*), PS (from tomato and chrysanthemum), and PM (from tomato and chrysanthemum). In all cases, each viroid retained its characteristic fingerprint pattern regardless of the host from which it had been purified. These results extend the results of Dickson et al (5) which show that viroids undergo no major sequence changes during replication in different hosts. Thus, despite the limited amount of genetic information contained in the viroid RNA and the apparent absence of an AUG initiation codon (16), viroids appear to code for, or regulate, their biosynthesis with great fidelilty regardless of the host in which they are propagated.

On the basis of our results it is now possible to biologically differentiate several viroids and their strains. Thus, for many types of experiments, it is no longer necessary to purify and fingerprint these viroids to assure their identity. However, if viroid purification is necessary we have determined suitable hosts and developed a purification procedure that is efficient for all the viroids investigated except ChCMV.

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