Occurrence of Small RNAs in Severely Diseased Vegetable Crops

C. FAGOAGA, J. A. PINA, and N. DURAN-VILA, Instituto Valenciano de Investigaciones Agrarias (IVIA), Apartado Oficial, 46113 Moncada, Valencia, Spain

ABSTRACT

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Vegetable crops in the eastern part of Spain have been shown to be affected by a number of virus and viruslike diseases. A complex of viruses has been considered to be the cause of the severe symptoms especially observed during the summer in tomato. The results of a survey conducted to evaluate the incidence of viroidlike RNAs in vegetable crops revealed a surprisingly high frequency of small RNAs of nonhost origin present in a wide range of host tissues. A series of CMV-related satRNAs have been positively identified by molecular hybridization. A new viroidlike RNA, tentatively named eggplant latent viroid (ELVd), was found in eggplant.

Vegetable crops in the eastern and southern parts of Spain are being affected by a number of virus and viruslike agents. The symptoms observed during the summer, probably caused by a complex of vector-transmitted viruses, are very severe and limit production in some areas. Viruses identified include cucumber mosaic (CMV), tomato spotted wilt (TSWV), tomato mosaic (ToMV), potato virus Y (PVY), potato leafroll (PLRV), watermelon mosaic (WMV-II), zucchini yellow mosaic (ZYMV), squash mosaic (SqMV), and muskmelon necrotic spot (MNSV) (9,11).

Although viroids are not frequently associated with epidemics in vegetable crops, a number of viroid-induced diseases, including cucumber pale fruit (CPFVd) (27), tomato planta macho (TPMVd) (4), tomato apical stunt (TASVd) (28), and tomato bunchy top (3,16), have been reported. Here we report the results of a survey of viroid and viroidlike agents in severely affected vegetable crop species.

MATERIALS AND METHODS

Plant material sources. Tissue samples were collected from field plots of the Servicio de Plantas de Vivero. These plots are located in a vegetable-growing area and include species affected by the major diseases of the area. Samples were collected from symptomatic and asymptomatic plants. The tissue was either extracted immediately after collection or kept at −20 C until processed. The species and number of samples tested are listed in Table 1.

Nucleic acid extraction. Unless otherwise stated, tissue samples (5 g each) of young leaves and stems were homogenized in extraction medium EM-1 (0.4 M Tris-HCl, pH 8.9; 10 g/L SDS; 5 mM EDTA, pH 7.0; 4% mercaptoethanol) containing water-saturated phenol, essentially as described earlier (24,25). The total nucleic acid preparations were partitioned in 2 M LiCl, and the soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer (10 mM Tris-HCl, 10 mM KCl, 0.1 mM $MgCl_2$, pH 7.4).

In order to improve the quality of the viroid-containing preparations from eggplant tissue, the original protocol was altered by: 1) using a modified extraction medium EM-2 (0.5 M Na_2SO_3, 10 g/L SDS, 4% mercaptoethanol) as suggested for grapevines and tissues containing a high concentration of phenols and acidic compounds (26); 2) supplementing EM-1 and EM-2 with 10% polyvinylpyrrolidone (PVP); 3) using preparative CF-11 cellulose chromatography as recommended for the removal of DNA and other pigmented components (23); and 4) using different resuspension media, i.e., TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0), TNE (10 mM Tris-HCl, pH 8.0; 5 mM NaCl, 0.1 mM EDTA, pH 8.0), RM-1 (TKM supplemented with KCl up to 20 mM), RM-2 (TKM supplemented with $MgCl_2$ up to 10 mM), and ddH_2O.

Aliquots (20 μl) of the final preparation equivalent to 300 mg of fresh weight tissue were always analyzed by 5% (w/v) polyacrylamide gel electrophoresis (PAGE) and/or used for infectivity assays.

Gel electrophoresis. Small RNAs were detected by 5% PAGE (18). The nucleic acid preparations were subjected to 60 mA for 2.5 hr, then stained with ethidium bromide.

Viroids were identified by sequential PAGE (sPAGE). The first electrophoresis was performed as described above. After ethidium bromide staining, the segment of the gel defined by citrus exocortis viroid (CEVd) and avocado sunblotch viroid (ASBVd) used as standards was excised and subjected to a second 5% PAGE (containing 8 M urea) at 16 mA for 4 hr (22). When a high definition in the region of the circular forms of the viroids was desired, the second gel was polymerized as described by Rivera-Bustamante et al (20). The viroids were viewed following silver staining (8). The ASBVd, CEVd, and citrus viroids Ia and Ib (CVd-Ia and CVd-Ib) used as standards were purified from the viroid collection maintained at Instituto Valenciano de Investigaciones Agrarias (IVIA). *Nicotiana glauca* Graham infected with CMV was provided by Pedro Moreno of IVIA.

Nuclease treatments. Aliquots (40 μl) of the nucleic acid preparations were incubated for 20 min at 37 C with either RNAse (20 units in 20 mM Tris ClH, pH 7.5) or DNAse (2 units in 40 mM Tris HCl, pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$). DNAse- and RNAse-treated samples were phenol-extracted, precipitated with ethanol, resuspended in 20 μl of TNE, and analyzed by sPAGE.

Molecular hybridization. The cRNA probes were synthesized by a transcription reaction in the presence of ^{32}P-labeled UTP (>400 Ci/μmol) using cloned plasmid DNA. The CMV satRNA probe was transcribed with T7 RNA-polymerase after linearizing the plasmid psK B260 (kindly provided by F. García-Arenal, Universidad Politécnica, Madrid), which contained the sequence of the attenuating CMV-satRNA, B2-satRNA. The apple scar skin viroid (ASSVd) probe was transcribed with SP6 RNA-polymerase after linearizing the plasmid pUAS14 (kindly provided by A. Hadidi, USDA Bioscience Service Center, Beltsville, MD), which contained a 274-bp-long cDNA derived from residues 296-228 of the ASSVd sequence. The ASBVd, CEVd, and hop stunt viroid (HSVd) probes were transcribed with T7 RNA-polymerase with plasmids pASBVd (kindly provided by J. S. Semancik, University of California, Riverside) and pCEVd and pHSVd

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(kindly provided by H. L. Sänger, Max Planck Institut, München). Plasmid pASBVd contained a partial cDNA derived from the ASBVd sequence inserted in the pGEM II vector, and plasmids pCEVd and pHSVd contained dimeric cDNA clones of CEVd and HSVd as described by Puchta et al (19). Nucleic acids were electrotransferred or slot-blotted to polyvinylidene difluoride (PVDF)-based membranes (Immobilon-N, Millipore). Electotransfer was performed at 80 V for 1 hr, directly from the urea-containing gel obtained after sPAGE, using TBE (40 mM Tris, 40 mM boric acid, and 1 mM EDTA, pH 8.3) as a blotting buffer. The membranes were baked at 80 C for 2 hr. Prehybridization and hybridization were performed under high stringency conditions in 50% formamide and 5× SSPE (0.6 M NaCl, 75 mM trisodium citrate, 65 mM NaH_2PO_4, pH 6.5, 2 mM EDTA) at 42 C for 24 hr as described by Maniatis et al (15). Unless otherwise stated, the membranes were washed twice in 2× SSC, 0.1% SDS at room temperature for 15 min, followed by two washes in 0.1× SSC, 0.1% SDS for 45–60 min at 68 C.

Infectivity assays. Aliquots of viroid-containing nucleic acid preparations were used for mechanical inoculation of the following viroid hosts: eggplant (*Solanum melongena* L. 'Sonja'), tomato (*Lycopersicon esculentum* Mill. 'Rutgers'), cucumber (*Cucumis sativus* L. 'Suyo'), crysanthemum (*Chrysanthemum* × *morifolium* Ramat. 'Bonnie Jean'), and citron (*Citrus medica* L. 'Etrog'). Eggplant, tomato, and cucumber were propagated as seedlings, chrysanthemum as rooted cuttings, and citron by grafting onto rough lemon (*Citrus jambhiri* Lush.) rootstock. Tomato and cucumber were inoculated by stem puncture, and eggplant, chrysanthemum, and citron were inoculated by slashes with a razor blade dipped in the nucleic acid preparation. Inoculated plants were kept at 28–32 C, and artificial light was provided during the winter to achieve a photoperiod with 16 hr of light. Plants were periodically pruned and the tissue analyzed by nucleic acid extraction and sPAGE.

Table 1. Identification of small RNAs of nonhost origin isolated from several vegetable crop species

Species	Small nonhost RNAs[a]	
	Satellites	Viroids
Broad bean (*Vicia faba* L.)	0/5	0/5
Carrot (*Daucus carota* L.)	0/2	0/2
Cauliflower (*Brassica oleracea* L. var. *botrytis* L.)	0/3	0/3
Cucumber (*Cucumis sativus* L.)	0/4	0/4
Eggplant (*Solanum melongena* L.)	5/24	2/24
Beet (*Beta vulgaris* L. subsp. *vulgaris*)	1/1	0/1
Muskmelon (*Cucumis melo* L.)	1/3	0/3
Potato (*Solanum tuberosum* L.)	0/6	0/6
Squash (*Cucurbita pepo* L.)	5/10	0/10
Tomato (*Lycopersicon esculentum* Mill.)	14/18	0/18
Turnip (*Brassica napus* L.)	0/3	0/3
Watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai)	2/3	0/3

[a] Number of samples with small nonhost RNAs/number of samples surveyed.

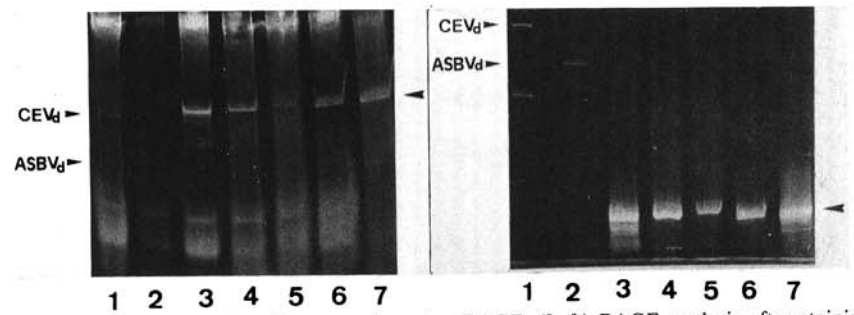

Fig. 1. Analysis of nucleic acid preparations by sPAGE: (Left) PAGE analysis after staining with ethidium bromide and (right) sPAGE under 8 M urea after staining with ethidium bromide. Lane 1, CEVd-infected citron; lane 2, ASBVd-infected avocado; lane 3, CMV satRNA-infected *Nicotiana glauca*; and lanes 4–7, field sources of squash, watermelon, eggplant, and tomato, respectively.

Fig. 2. (Left) Analysis of nucleic acid preparations by sPAGE and ethidium bromide staining and (right) molecular hybridization against a CMV satRNA probe. Lane 1, CMV-infected *Nicotiana glauca* containing a satRNA used as a positive control; lanes 2–5, field sources of squash, watermelon, eggplant, and tomato, respectively, containing small RNAs; and lane 6, virus-free tomato used as a negative control.

RESULTS

Detection of small nonhost RNAs. When the nucleic acid preparations were subjected to 5% PAGE, a prominent nucleic acid band with an electrophoretic mobility slightly slower than CEV was observed in 28 of the 72 samples surveyed, representing six of the 12 vegetable species studied (Table 1). The relative concentrations of this nucleic acid varied from very high, as in eggplant, tomato, muskmelon, and squash, to very low, as in beet and watermelon (Fig. 1, left; *data not shown*). When the preparations were subjected to sPAGE, these molecules moved toward the bottom of the urea-containing gel (Fig. 1, right). Molecular hybridization of the electroblotted samples against a cRNA probe from CMV satRNA showed a positive reaction. The reaction was identical to that observed in preparations of *N. glauca* used as positive controls (Fig. 2). This demonstrated the widespread occurrence of CMV satRNA in tomato, eggplant, beet, muskmelon, squash, and watermelon.

Identification of viroids in eggplant. Analysis of nucleic acid preparations by sPAGE and silver staining showed that two eggplant samples that did not show any small RNAs by ethidium bromide staining (Fig. 3A) presented a band with the mobility of the circular forms of viroids in the urea-containing gel after silver staining (Fig. 3B). This band was identified as a viroidlike RNA. No viroidlike RNAs were detected in the remaining samples (Table 1). When preparations containing viroidlike RNAs were subjected to sPAGE, circular and linear forms were also detected (Fig. 3B; *data not shown*). The electrophoretic mobility of this viroidlike RNA was compared with other known viroids by subjecting artificial viroid mixtures to sPAGE (Fig. 3C). The results revealed a close migration with chrysanthemum stunt viroid (CSVd) and CVd-Ia and CVd-Ib from citrus.

When eggplant preparations were subjected to RNAse and DNAse treatments, the nucleic acid band was sensitive to RNAse but not to DNAse. These results (Fig. 4A) indicated that the nucleic acid band identified in eggplant was RNA. When eggplant seedlings were inoculated with preparations containing this viroid-like RNA, no specific symptoms were observed in the inoculated plants (Fig. 4B). However, 2 mo after inoculation,

viroidlike molecules that were not present in the uninoculated controls were detected in three of the four inoculated seedlings (Fig. 4C). The viroidlike RNA was tentatively designated as eggplant latent viroid (ELVd) and has been mechanically transmitted by razor blade slash with 100% efficiency. ELVd was detected only in cv. Sonja, whereas satellite RNAs were identified in all eggplant cultivars tested (Avan, Baluroi, Bonica, and Sonja).

Other properties of ELVd. Attempts to transmit ELVd to tomato, cucumber, chrysanthemum, and citron on infectivity assays gave negative results.

Unlike other viroids, ELVd was less stable through the processes of extraction and purification and during storage at −20 C. In order to obtain better yields of highly stable viroid-containing preparations, several modifications of the original protocol were tested. Utilization of $NaSO_3$ and preparative CF-11 cellulose resulted in cleaner extracts than those obtained by using EM-1 , whereas supplementation with PVP had no effect. The resuspension medium TNE gave slightly better preparations than TE, RM-1, RM-2, and ddH_2O. Therefore, the standard method adopted for extraction of ELVd was use of the medium EM-2, followed by preparative CF-11 cellulose and partitioning in 2 M LiCl, with final resuspension on TNE buffer.

The hybridization of ELVd-containing nucleic acid extracts against probes representing the major viroid groups was negative (*data not shown*). Similarly, when the membranes were washed at 55 C instead of 68 C, no positive reaction was found (Fig. 5).

DISCUSSION

The results of the survey reported here illustrate the widespread occurrence of nonhost small RNAs in several vegetable crop species in Spain. On the basis of molecular hybridization analysis, the small RNAs identified in eggplant, beet, muskmelon, squash, tomato, and watermelon have been characterized as CMV satRNAs. An epidemic of tomato necrosis induced by CMV plus satRNA that started in 1986 (10) has apparently progressed to most tomato-growing areas. Our results indicate that this pathogen combination is also affecting other crops.

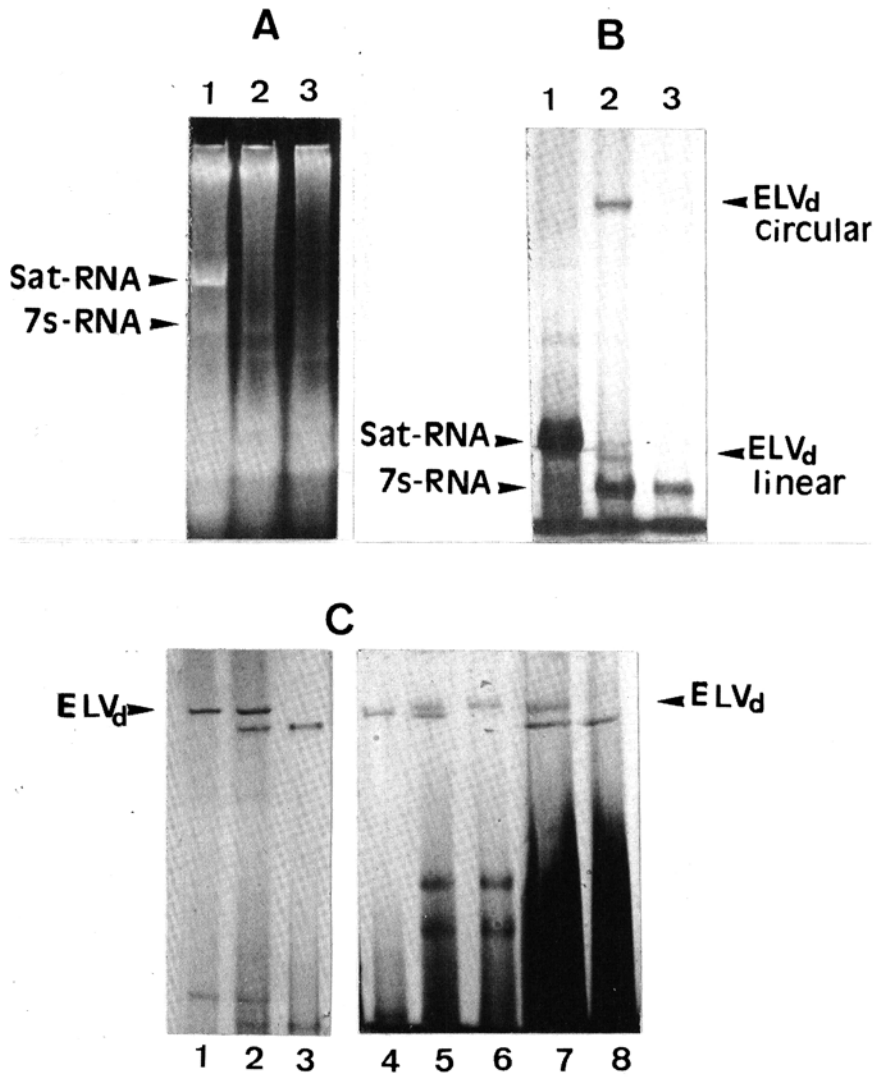

Fig. 3. Analysis of nucleic acid preparations from eggplant cv. Sonja by sPAGE: **(A)** First gel stained with ethidium bromide. **(B)** Urea containing gel stained with silver. Lane 1, sample containing satRNA; lane 2, sample containing viroidlike RNA; and lane 3, sample free of nonhost small RNAs. **(C)** Comparison of the electrophoretic mobility after silver staining of ELVd with other viroids. Lane 1, ELVd; lane 2, artificial mixture of ELVd and CSVd; lane 3, CSVd; lane 4, CVd-Ia; lane 5, artificial mixture of ELVd and CVd-Ia; lane 6, ELVd; lane 7, artificial mixture of ELVd and CVd-Ib; and lane 8, CV-Ib.

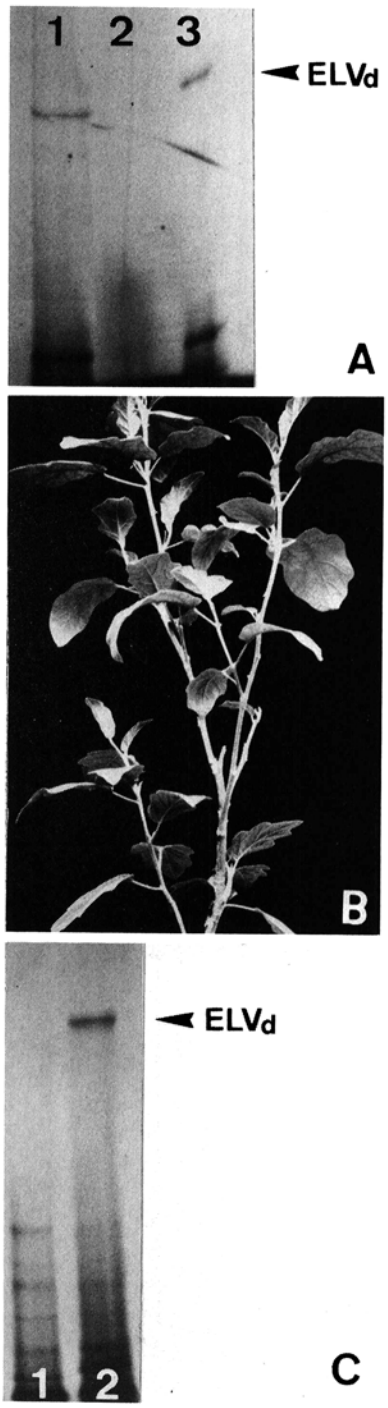

Fig. 4. (A) Lane 1, analysis of samples containing the viroidlike RNA; lane 2, samples that had been incubated with RNAse; lane 3, samples that had been incubated with DNAse. **(B)** Eggplant inoculated with ELVd. **(C)** Nucleic acid extraction and sPAGE: Lane 1, uninoculated controls; and lane 2, inoculated eggplants seedlings.

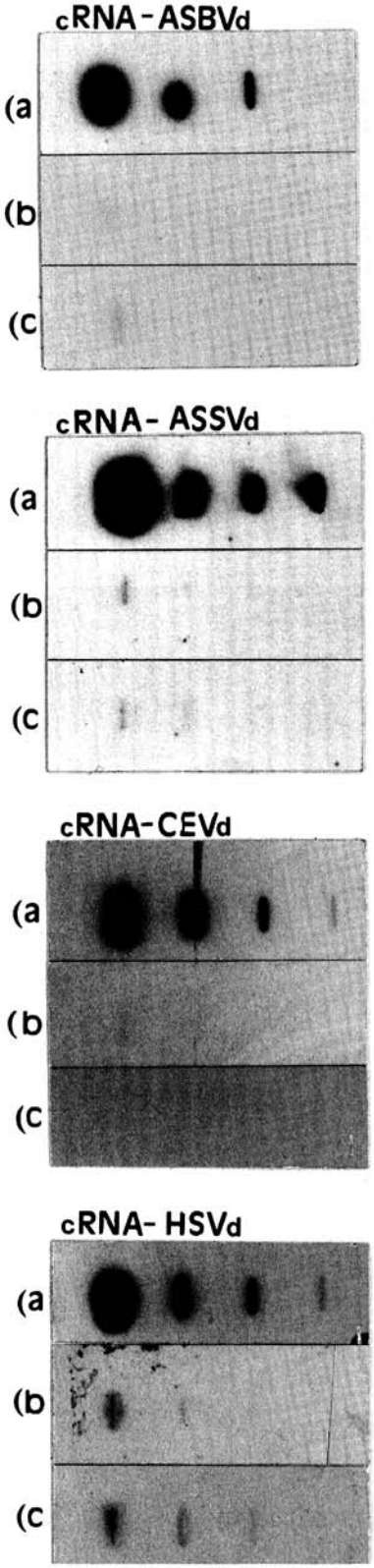

Fig. 5. Autoradiograph of slot blots containing: row a, positive controls; row b, nucleic acid preparations from ELVd-infected eggplants; and row c, healthy eggplants after hybridization with (top to bottom) ASBVd, ASSVd, CEVd, and HSVd probes. The membranes were hybridized under high stringency conditions and washed at 55 C.

Although the origin of satRNAs is uncertain (21), an increasing number of reports illustrate the occurrence of this kind of pathogenic RNAs infecting agricultural crops (5,7,12,13,30). The CMV satRNAs already characterized show enormous heterogeneity in sequence and biological activity (1,2,6,17,29). Moreover, satRNA populations have been shown to change rapidly in response to change in host species (14). Therefore, the widespread occurrence of naturally occurring CMV satRNAs in different agricultural species will probably result in greater heterogeneity of the satellite RNA populations already present in our agricultural environment. Since the source plants studied here were probably severely affected by one or more viruses, the real impact of the CMV strains and their satellite RNAs on the disease syndrome observed in these crops cannot be evaluated.

A small RNA identified in the eggplant cultivar Sonja has been demonstrated to be a viroidlike RNA. Although a virusoid nature cannot be discounted, the high specific infectivity results achieved with ELVd preparations point toward a viroidlike agent. Under our experimental conditions, no symptoms were observed on inoculated eggplants. Therefore, the viroidlike RNA has been tentatively identified as eggplant latent viroid (ELVd). In order to verify the possible role of ELVd on the poor behavior of the local cultivar Sonja, further agronomic characterization is needed. Some characteristics of ELVd are: 1) identification in a seed-propagated annual crop, 2) lack of transmission to other hosts of known viroids (tomato, cucumber, chrysanthemum, and citron), and 3) unusually low stability for a viroidlike RNA.

Electrophoretic comparison of ELVd with other viroids indicated a close but not identical mobility to CSVd and CVd-I group from citrus. Hybridization against viroid-specific probes indicated that ELVd has little or no sequence homology with other known viroids. In addition, the low stability of ELVd under purification and storage conditions suitable for viroids indicates that ELVd may have some unusual structural properties. The availability of better isolation conditions will allow further characterization of the ELVd molecule.

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