VIROIDS

Effects of Sequence Variation on Symptom Induction by Citrus Viroid III

R. A. Owens, S. M. Thompson, P. A. Feldstein, and S. M. Garnsey

ABSTRACT. Interest in the use of viroids as potential citrus dwarfing agents goes back more than 30 yr, and infection of Valencia sweet orange scions growing on trifoliate orange rootstocks by citrus viroids Ia, IIa, or IIIb was recently shown to result in a dwarfed phenotype and enhanced fruit production. Viroids exist *in vivo* as populations of closely related sequence variants, and we are interested in developing variants of citrus viroid III as dwarfing agents specifically adapted for use with certain citrus cultivars. Toward that goal, we have constructed an expression cassette that releases a precisely full length, potentially infectious CVd-III RNA of known sequence from an RNA transcript synthesized *in vitro*. The viroid RNA is released through the action of a ribozyme sequence located at the 3' terminus of the transcript. RNAs derived from wild-type CVd-IIIb, as well as four other naturally-occurring sequence variants, were infectious when slash-inoculated into Etrog citron, and most of the sequence changes present in these variants were stably maintained in their respective progeny. In particular, two changes in the putative "pathogenicity domain" of CVd-III were associated with a marked reduction in symptom expression in Etrog citron.

Key words. Viroids, citrus dwarfing, symptom expression.

Viroids, the smallest known agents of infectious disease, are small (246 to 399 nucleotides), highly structured, circular, single-stranded RNA molecules lacking both a protein capsid and detectable messenger RNA activity. As described by Duran-Vila et al. (5), a single field-grown citrus tree may harbor as many as 4 to 5 different species of viroids. Some, such as citrus exocortis viroid (CEVd), cause specific disease symptoms; while the presence of others (e.g., citrus viroid III) results in a "dwarf" phenotype (9). The use of dwarf trees offers many potential economic and environmental benefits to the grower; indeed, several studies of the horticultural effects of so-called "graft transmissible dwarfing factors" (8, 15, 18) predate the demonstration that citrus exocortis is a viroid disease (27). More recently, Semancik et al. (28) have described the application of selected viroids for dwarfing and enhancing production of Valencia orange. To date, all dwarfing studies have been carried out with natural viroid isolates that may contain complex populations of sequence variants (31).

We are interested in the development of improved citrus viroid dwarfing agents, especially in the development of dwarfing agents specifically adapted to certain rootstock-scion combinations. Recent advances in understanding the role of "quasispecies" in the rapid evolution of RNA genomes (14) suggest that it may be possible to adapt methods developed for the selection and amplification of rare functional nucleic acids *in vitro* (see 16) for use with viroids *in vivo*. Here, we describe initial results from experiments designed to isolate sequence variants of citrus viroid III that induce symptoms of differing severity in Etrog citron, an indicator host.

MATERIALS AND METHODS

Construction of potentially infectious citrus viroid III cDNA clones. Construction of an expression cassette designed to permit

synthesis of a highly infectious, precisely-full-length potato spindle tuber viroid RNA in which the viroid cDNA is flanked by specially modified versions of the hammerhead and paperclip ribozymes from satellite tobacco ringspot virus RNA has been described elsewhere (6, 19). As shown in Fig. 1, a DNA fragment containing a paperclip ribozyme sequence specifically modified to cleave CVd-III RNA transcripts between residues G_{150} and G_{151} was added to the 3'-terminus of a full-length CVd-III cDNA by ligation at compatible *Nhe*I and *Spe*I sites. A promoter for T7 RNA polymerase was also fused to the 5'-terminus of the CVd-III cDNA. Details of the construction strategy have been published elsewhere (20).

Starting material for synthesis of full-length CVd-III cDNA was a preparation of total nucleic acids derived from sweet orange source tree E14b (33). The original (i.e., 1970) viroid isolation was made from a somewhat stunted, but thrifty looking 3- to 4-yr-old Hamlin sweet orange tree growing on trifoliate orange rootstock in a commercial citrus planting in Lake County, FL. No bark scaling was observed on the trifoliate orange rootstock of this tree, and indexing on

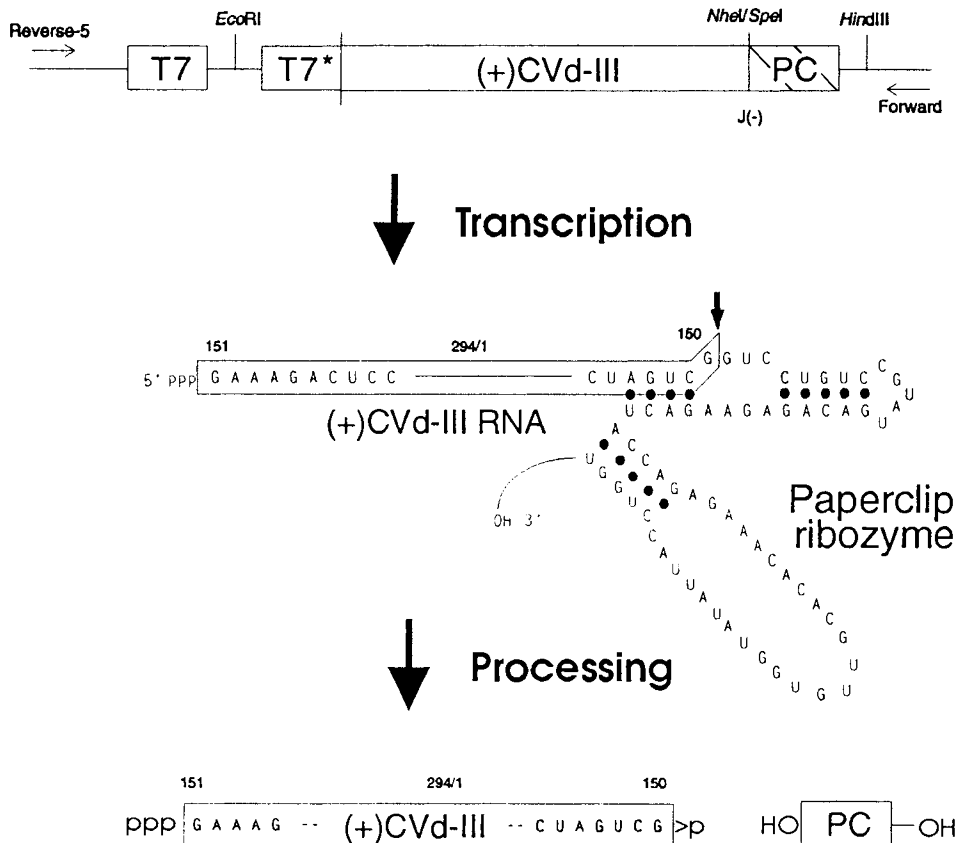


Fig. 1. Ribozyme-mediated synthesis of precisely-full-length CVd-III RNA. Schematic diagram shows the expression cassette and *in vitro* processing pathway for (+)CVd-III RNA transcripts. T7 and T7*, promoter sites for bacteriophage T7 RNA polymerase; (+)CVd-III, full-length CVd-III cDNA; J(-), cleavage site for a specially modified paperclip (PC) ribozyme from (-)sTRSV RNA; Reverse-5 and Forward, binding sites for M13 sequencing primers. The site of ribozyme cleavage (between positions 150 and 151) is indicated by a bold arrow.

Etrog citron produced a moderate response. Isolate E14b contains only CVd-III and has been maintained in sweet orange since its original isolation. Full-length CVd-III cDNAs were synthesized by RT-PCR using antisense primer C2 (5'-ACTCTCCG-TCTTTACTCCA-3', nucleotides 120 to 138) and sense primer H2 (5'-CTC-CGCTAGTCGGAAAGACTCCGC-3', homologous to nucleotides 139 to 162) essentially as previously described (24, 33). The CVd-III cDNA inserts in recombinant plasmids were examined for the presence of sequence changes by automated sequence analysis using an Applied Biosystems Model 373A sequencer and dye-labeled M13 forward and reverse primers.

Synthesis and bioassay of RNA transcripts. DNA templates for the synthesis of precisely-full-length CVd-III RNA were generated in PCR containing plasmid DNA and M13 forward (5'-GTAAAACGACGGCCA-GT-3') and reverse-5 (5'-CAGGAAA-CAGCTAT-GA-3') primers. *In vitro* RNA transcription reactions (50 µl) containing approximately 250 ng of DNA template and 75 units of T7 RNA polymerase were incubated overnight at 37°C as described by the manufacturer (Promega) except that the concentration of $MgCl_2$ was increased to 15 mM. After digestion of the DNA templates with RNase-free DNase, CVd-III RNA transcripts were recovered by phenol-chloroform extraction and ethanol precipitation. The ability of individual CVd-III RNA transcripts to undergo self-cleavage was assessed by polyacrylamide gel electrophoresis of ^{32}P-labeled RNAs under denaturing conditions.

Aliquots of unlabeled inoculum containing approximately 200 ng of fully-processed CVd-III RNA transcripts in 100 µl of 20 mM Na phosphate (pH 7.0) were slash-inoculated into the stems of Etrog citron growing on one of several different rootstocks (i.e., rough lemon, trifoliate orange, or Alemow). Inoculated plants were kept in a greenhouse under conditions favoring viroid replication (i.e., high light-warm temperature) and periodically cut back to encourage symptom development. In one experiment, plants were inoculated by grafting rather than slash-inoculation. Buds were excised from plants inoculated by slash-inoculation, and after transfer to viroid-free citron (one bud/plant, three plants/treatment), the stems of newly-inoculated plants were wrapped with parafilm for three weeks to prevent desiccation.

Characterization of viroid progeny. Viroid accumulation in inoculated plants was measured by dot-blot hybridization analysis using a full-length, digoxygenin-labeled probe specific for CVd-III. Beginning 3 to 4 mo post inoculation, freshly collected samples of green bark tissue (0.2 g) were homogenized for 30 sec in 3 ml of chilled extraction medium [100 mM Tris-HCl, pH 8.0-500 mM NaCl-50 mM EDTA-10 mM β-mercaptoethanol] using a Klecko apparatus. Aliquots (750 µl) of the resulting homogenate were transferred to microfuge tubes, and, after addition of 100 µl of 100 mg/ml SDS, the mixture was vortexed and then incubated at 65°C for 30 min. Following addition of 250 ml of 5 M potassium acetate, the contents of each tube were incubated for 20 min on ice before centrifugation. An aliquot (400 µl) of the resulting supernatant was transferred to a fresh microfuge tube, 40 µl of 3 M Na acetate and 800 µl of 95% ethanol were added, and total cellular nucleic acids allowed to precipitate at -20°C for at least two hr. Nucleic acids were collected by centrifugation, redissolved in 100 µl water and extracted with 50 µl phenol + 50 µl chloroform, and recovered by ethanol precipitation. After washing with 70% ethanol, pellets were briefly dried before being redissolved in water and subjected to dot blot hybridization analysis (21).

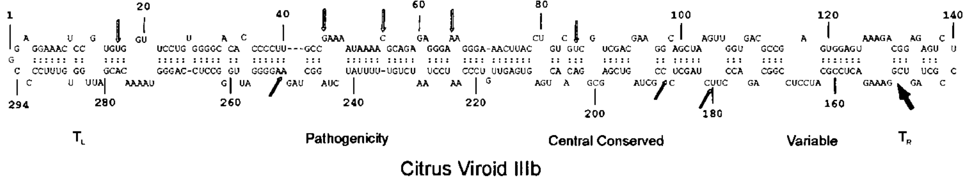
In addition to dot-blot hybridization, selected preparations of CVd-III progeny were further characterized by RT-PCR and nucleotide sequence analysis of the resulting enzymatically amplified viroid cDNAs. Antisense primer C2' (5'-ACTCTCCGTCTTTACTCCAC-3', positions 138 to 119] and sense primer H2' (5'-CTCCGCTAGTCGGAAAGACT-3', positions 139 to 158) are very similar in sequence to the primer set C2 + H2 described by Rakowski et al. (24). Primer lengths have been adjusted in order to achieve a better match between their calculated T_m values, however. Automated sequence analysis of the uncloned double-stranded PCR products was carried out as described above using a dye-terminator protocol and unlabeled primers C2' and H2'.

RESULTS

Identification of CVd-III sequence variants and synthesis of infectious RNA transcripts. Viroid replication proceeds via a rolling circle mechanism (2), and several studies have shown that multimeric viroid cDNAs (4) and their corresponding RNA transcripts (30) are much more infectious than their "monomeric" counterparts. Genetic manipulation of viroids, in contrast, is most easily carried out using monomeric cDNAs. One solution to this dilemma involves fusion of (i) promoter sequences for T7 RNA polymerase and (ii) an appropriately modified version of the paperclip ribozyme derived from sTRSV to, respectively, the 5' and 3' termini of a monomeric viroid cDNA. As shown in Fig. 1, spontaneous self-cleavage of the resulting RNA transcript releases a precisely-full-length, highly infectious linear viroid RNA (6, 25).

As first described by Visvader and Symons (31) for CEVd, host plants infected with a single viroid species may contain a complex mixture of sequence variants. Seven independent CVd-III cDNA clones derived from source tree E14 were characterized by nucleotide sequence analysis as well as electrophoresis of the corresponding RNA transcripts under denaturing conditions. Data summarized in Fig. 2 showed that all seven cDNAs contained 294 nt; only clones 1 and 2, however, were identical in sequence to CVd-IIIb (24, 29). Of the remaining clones, three contained single nucleotide substitutions, and two contained multiple substitutions. Interestingly, the changes at positions 44 and 54 present in both clones 31 and 33 are located in a portion of the molecule that corresponds to the "pathogenicity domain" of PSTVd and related viroids (17). The total of eight sequence changes present in clones 3 to 34 are distributed rather evenly throughout the overall rodlike secondary structure of CVd-IIIb.

To examine the infectivity of ribozyme-derived CVd-III RNAs, a group of six young Etrog citron plants were inoculated with a mixture of RNA transcripts from clones 1 to 3 and 31 to 33 using a stem slashing protocol in order to directly introduce the inoculum into the vascular system. As shown in Fig. 2, the termini of these RNAs are located in the right terminal loop of the viroid. Approximately 3 mo post inoculation, symptoms characteristic of CVd-III infection (e.g., leaf "droop" as well as a necrotic reaction involving leaf midveins and petioles) were visible, and hybridization analysis of young stem and petiole tissue revealed that all six plants had become infected with CVd-III (data not shown). Six mo post inoculation, the nucleotide sequence of the resulting progeny was determined by analysis of enzymatically amplified cDNAs and found to be identical to that of CVd-III clone 2 (i.e., wild-type CVd-IIIb). If CVd-III variants 3 and 31 to 33 were viable, their progeny appeared to be unable to successfully compete with the wild-type viroid.



Clone	Location of mutation(s) *								Total Changes
	$U_{16} \rightarrow G$	$G_{44} \rightarrow A$	$C_{54} \rightarrow U$	$A_{66} \rightarrow G$	$U_{85} \rightarrow C$	$U_{180} \rightarrow A$	$+G_{187A}$	$A_{251} \rightarrow U$	
1	-	-	-	-	-	-	-	-	0
2	-	-	-	-	-	-	-	-	0
3	-	-	-	-	-	-	-	+	1
31	-	+	+	+	-	+	+	-	5
32	-	-	-	-	+	-	-	-	1
33	-	+	+	-	-	-	-	-	2
34	+	-	-	-	-	-	-	-	1

Fig. 2. CVd-III sequence variants recovered from source tree E14. Locations of sequence changes in cDNA clones 3 and 31 to 34 are indicated by small arrows within the context of the overall rod-like secondary structure of CVd-IIIb (294 nt). Bold arrow, location of the 5' and 3' termini of the linear, ribozyme-derived CVd-III RNA synthesized *in vitro* (see Fig. 1).

Biological properties of individual CVd-III sequence variants. Having established that precisely-full-length CVd-III RNAs are potentially infectious, a second bioassay was carried out in order to examine the infectivity of individual sequence variants. Of particular interest were the properties of CVd-III clones 31 and 33, both of which contain mutations in the putative pathogenicity domain of CVd-III. Inoculated plants were tested for the presence of viroid progeny for six months post inoculation, and the assay results are summarized in Table 1.

Four of the five CVd-III sequence variants tested proved to be infectious. Inoculation with variants containing mutations in the left terminal loop (position 16), pathogenicity domain (positions 44, 54, 66, and 251), or variable domain (positions 180 and 187a) was followed by the appearance of viroid progeny. Even RNA transcripts from clone 34 which contained an additional 84 non-viroid nucleotides at their 5' termini were weakly infectious. The only mutation that appeared to abolish infectivity (i.e., a U→C substitution at position 85) is located in a portion of the molecule believed to play an important role in viroid replication (1).

Extensive studies with both laboratory-derived (23) and naturally-occurring variants of PSTVd (12) have shown that not all viroid sequence variants are stably maintained *in vivo*. Sequence analysis of uncloned PCR products derived from CVd-III variants 3 and 34 six mo post inoculation revealed that the mutations initially present at position 251 and 16 had reverted to wild-type. For clones 33 and 31, changes at positions 44 and 54 or 44, 54, 180, and 187a were stably maintained; only the A→G transition at position 66 of clone 31 was absent from the progeny. Of particular interest in light of the stable nature of the sequence changes at positions 44 and 54 was the fact that the leaf "droop" and veinal necrosis induced by clones 31 and 33

TABLE 1
INFECTIVITY OF INDIVIDUAL CVd-III VARIANTS

	Infectivity[z]					
Days post inoculation[Y]	Clone 2	Clone 3	Clone 31[y]	Clone 32[y]	Clone 33	Clone 34
79 (65)	-	+	+	-	-	-
119 (105)	2/3	2/3	+	-	+	-
185 (171)	2/3	2/3	3/3	-	3/3	1/3

[z]For pooled samples, infectivity reported as either positive (+) or negative (-). Where plants were tested individually, data expressed as no. infected plants / no. inoculated plants.
[y]Infectivity trials with CVd-III clones 31 and 32 were begun 14 d after those with clones 2, 3, 33, and 34. Sampling times for clones 31 and 32 are shown in parentheses.

appeared to be considerably milder than that induced by wild-type CVd-IIIb (i.e., clone 2).

To further examine this apparent difference in symptom expression, buds were excised from plants slash inoculated with CVd-III clones 2, 31, and 33 and grafted onto groups of three viroid-free citrons. Two mo post inoculation, symptoms had begun to appear in at least one plant from each treatment; four mo post inoculation, all nine inoculated plants contained readily detectable levels of viroid progeny. Shortly thereafter, the extent of necrosis affecting leaf petioles, midveins, and lateral veins was evaluated, and the plants were cut back to monitor possible differences in leaf droop and/or growth habit. Data presented in Fig. 3 illustrate the general reduction in symptom severity associated with sequences changes in the pathogenicity and variable domains of CVd-IIIb.

At the whole plant level (Fig. 3A), the presence of these sequence changes was clearly correlated with an overall reduction in the degree of leaf droop associated with CVd-III infection. Examination of the leaves and petioles of infected plants revealed a similar reduction in the severity of certain necrotic responses. For example, comparison of Figs. 3B to 3E shows that the "splitting" of leaf midveins so prominent among plants infected with wild-type CVd-III was virtually absent in those infected with clones 31 and 33. Consistent reductions were also observed in necrotic reactions associated with lateral veins, petioles, and portions of the leaf lamina adjacent to the midvein. Overall, symptom severity decreased in the order clone 2 (wild-type CVd-IIIb) >> clone 31 > clone 33. No differences were observed in the wrinkled appearance of petioles from the different viroid-infected plants.

Studies with PSTVd sequence variants (19) indicate that, even though mild and severe variants eventually reach similar titers in infected tissues, mild variants replicate more slowly. Thus, we decided to compare the titers of wild-type CVd-IIIb progeny with those arising from clones 31 and 33 at various times following slash inoculation. As shown in Fig. 4, comparison of pooled nucleic acid samples collected between 79 and 303 d post inoculation revealed that titers of wild-type progeny were consistently higher than those of either mild variant. Although the progeny from clone 33 appeared to replicate and/or move systemically more rapidly than those from clone 31, the significance of this finding remains to be determined. As described above, the symptoms observed on plants infected with variant 33 were less (not more) severe than those induced by variant 31.

DISCUSSION

Sequence analysis of cloned viroid cDNAs have shown that indi-

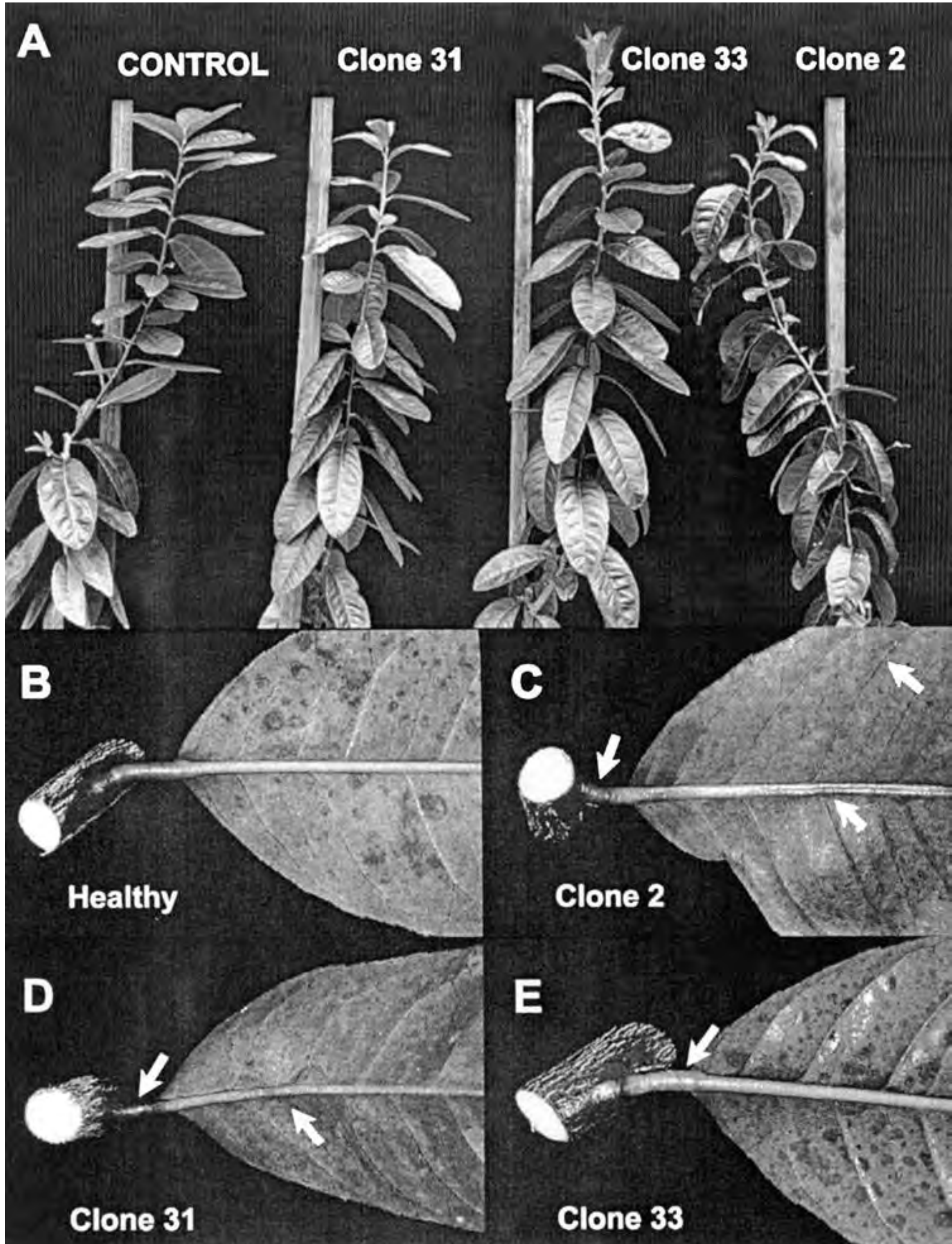


Fig. 3. Suppression of symptom production by sequence changes in the pathogenicity and variable domains of CVd-IIIb. (A) Comparison of epinasty and leaf droop in the upper portions of control (uninoculated) and viroid-infected Etrog citron approximately 9 mo post inoculation. (B to E) Comparison of necrotic reactions involving leaf petioles, midveins, and lateral veins. Note the absence of vein "splitting" in the midveins of leaves from plants infected with variants 31 (panel D) and 33 (panel E) as well as a general reduction in the intensity of the necrotic response (arrows).

vidual infected plants often contain complex mixtures of closely-related sequence variants (10, 22, 23, 31). As expected for members of an RNA "quasispecies", some of these variants appear to replicate stably *in*

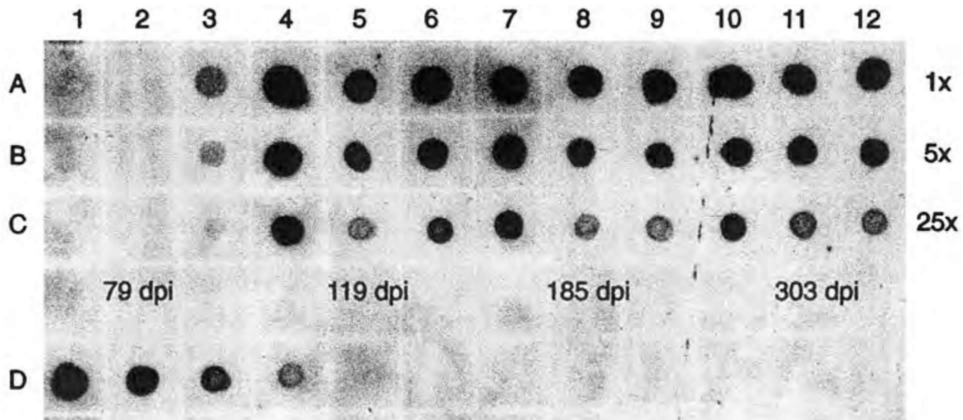


Fig. 4. Accumulation of CVd-III in systemically infected Etrog citron. Total nucleic acids were extracted from bark tissue collected at various times after slash-inoculation with CVd-III RNA transcripts, and the relative concentration of viroid progeny was estimated by dot blot hybridization. Columns 1, 4, 7, and 10, wild-type CVd-IIIb (i.e., clone 2); columns 2, 5, 8, and 11, clone 31; columns 3, 6, 9, and 12, clone 33. Rows A-C contain undiluted, 5-fold, and 25-fold dilutions of the various samples. Row D contains a similar dilution series of CVd-III RNA transcripts. dpi, days post inoculation.

vivo; others are rapidly replaced (i.e., outcompeted) by better adapted variants. Previous studies of the effect of CVd-III infection on tree dwarfing and fruit production (28) have used naturally-derived viroid isolates. Our data demonstrate (i) the infectious nature of several naturally-occurring sequence variants of CVd-III and (ii) the ability of many of the sequence changes distinguishing these variants to persist in their respective progeny. Thus, the basic requirements for future attempts to genetically manipulate CVd-III have been met.

Using any of several PCR-based mutagenesis strategies (3, 13), it is possible to introduce virtually any mutation or combination of mutations into our CVd-III expression cassette. Deciding which portion(s) of the viroid to mutagenize and how to select variants having the desired biological properties from the thousands of potential mutants remain to be determined, however. To date, the nucleotide sequences of four CVd-III variants have been published; i.e., CVd-IIIa CVd-IIIb, CVd-IIIb (Australia), and CVd-IIIc (24, 28, 29). As shown in Fig. 5, sequence variation among these three isolates is confined largely to the pathogenicity domain and central conserved region (defined by the presence of inverted repeats at positions 72 to 83 and 96 to 107). Firm conclusions about the extent of natural variation, however, require sequence information from a much larger number of CVd-III isolates; thus, we have recently examined the CVd-III component of several viroid complexes isolated from citrus sources in Israel and Costa Rica. Although variation was observed at a number of new positions (indicated by solid circles in Fig. 5), the overall distribution of sequence variation remained largely unchanged.

Previous studies with potato spindle tuber and related viroids have shown that nucleotides located within the pathogenicity domain play an important role in modulating symptom expression (11, 26, 32). We are most encouraged that several sequence changes in the corresponding portion of CVd-III had similar effects. It is important to note, however, that there may be no correlation between the ability of CVd-III infection to induce epinasty and

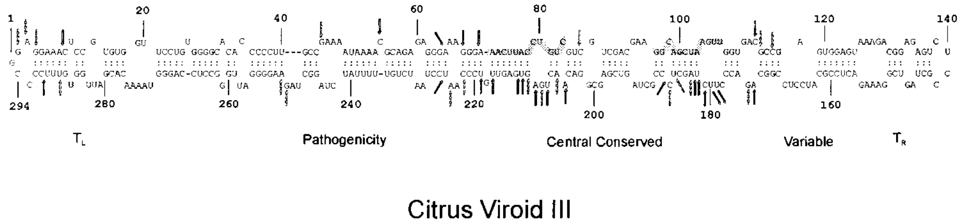


Fig. 5. Sequence variation among naturally-occurring isolates of CVd-III. Dark arrows, variation among isolates CVd-IIIa-c (24, 28, 29); light arrows, additional variation detected among CVd-III isolates from Israel and Costa Rica (R. A. Owens, R.W. Hammond, and M. Bar-Joseph, unpublished data). Because the primers used for RT-PCR (i.e., C2 + H2) bind to positions 120 to 162, no information about possible sequence variability in the right terminal loop was obtained.

necrosis in the leaves and petioles of an indicator host (i.e., Etrog citron) and the production of a dwarf phenotype in commercial citrus rootstock-scion combinations. Citrus exocortis viroid infection results in a reduced rate of root initiation in *Gynura aurantiaca* (7), and the stunting symptoms visible in the shoots of many viroid-infected plants is often accompanied by a reduction in root mass (R. W. Hammond, personal communication). Thus far, preliminary attempts to detect any effect of CVd-III infection on root initiation/growth have been unsuccessful. In light of the length of time required to evaluate citrus dwarfing agents under field conditions (i.e., 8 to 10 yr (28)), development of a bioassay suitable for the rapid selection and characterization of promising CVd-III variants under laboratory or greenhouse conditions is essential if we are to achieve our ultimate goal—the identification of viroid dwarfing agents specifically adapted to use with specific citrus varieties.

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