

## Limited Sequence Randomization: Testing a Strategy to Produce Improved Viroid Dwarfing Agents

R. A. Owens, S. M. Thompson, P. J. Sieburth, and S. M. Garnsey

**ABSTRACT.** Close planting of viroid-infected citrus growing on trifoliolate orange or trifoliolate hybrid rootstocks has been shown to increase fruit yield and lower production costs. We would like to adapt *Citrus viroid III* (CvD-III) for use with other rootstocks and, toward that end, have examined the extent of its natural sequence variability. Mutations at positions 44 and 54 of CvD-IIIb were shown to greatly reduce symptom expression on the indicator host Etrog citron. Here, we report the isolation of several novel CvD-III variants following inoculation of citron with a “library” of RNA transcripts in which five positions opposing positions 44 through 54 had been randomized. Individual trees tested 6 mo to 1 yr post-inoculation contained complex mixtures of sequence variants, some containing multiple spontaneous changes. Distribution of viroids and viruses in woody hosts is often uneven; thus, buds removed from three viroid-infected trees 6 mo post-inoculation were used to inoculate viroid-free citrons. The resulting infected trees were also examined for possible sequence differences among the viroid progeny. In some trees, progeny sequences were continuing to evolve as much as 2 yr post-inoculation. The effects of selected mutations on the secondary structure and biological properties of CvD-III are described. Sequence randomization, followed by *in vivo* selection, provides a promising method to isolate viroid dwarfing agents with specific biological properties.

*Index words:* Viroids, citrus dwarfing, *Citrus viroid III*.

Viroids, the smallest known agents of infectious disease, are small (246-399 nucleotides), highly structured, circular single-stranded RNA molecules lacking both a protein capsid and detectable messenger RNA activity (8, 14). As described by Duran-Vila et al. (5), single field-grown citrus trees may harbor as many as four to five different species of viroids. Some, such as *Citrus exocortis viroid* (CEVd) or *Citrus viroid IIb* (CvD-IIb) cause specific disease symptoms; the presence of others such as *Citrus viroid III* (CvD-III) (19, 26) may result in a “dwarf” phenotype. The use of dwarfed trees offers many potential economic and environmental benefits to the grower (1, 12, 27).

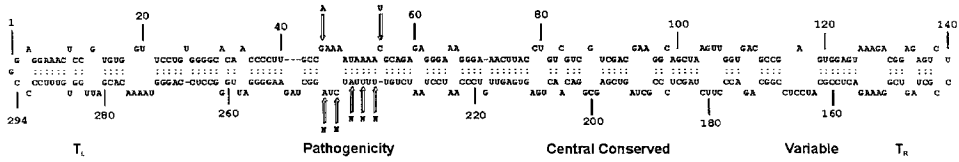
To date, all dwarfing studies have been carried out with natural viroid isolates that often contain complex populations of sequence variants (2, 28). We are interested in the development of improved viroid dwarfing agents, particularly agents adapted to specific rootstock-scion combinations. Toward that end, we have i) identified sequence variants

of CvD-III that induce distinct responses in Etrog citron (15) and ii) examined CvD-III variability in several Israeli dwarfing isolates (16). Here, we report initial results from experiments designed to generate “libraries” of CvD-III variants from which we will attempt to select improved viroid dwarfing agents.

### MATERIALS AND METHODS

**Mutagenesis and synthesis of infectious CvD-III RNA transcripts.** Construction of an expression cassette designed to permit synthesis of highly infectious, precisely-full-length CvD-IIIb RNA transcripts by ribozyme cleavage between G residues at positions 150 and 151 has been described elsewhere (15). As shown in Fig. 1, mutations were introduced at positions 44, 54, 236, 238, 240, 242, and 244 of CvD-IIIb via the polymerase chain reaction (PCR) using an overlap extension strategy (11).

In the first step, two pairs of primers [i.e., M13 reverse-5 (5'-CAGGAAACAGCTATGACC-3') +



**Fig. 1. Mutagenesis of CVd-IIIb.** Arrows denote G/A and C/U substitutions at positions 44 and 54 as well as five opposing positions randomized by oligonucleotide-directed mutagenesis.

CVdIII-54/44 (5'-CCCTCTCTGCATTTATTTTGGCAAGGGG-3') and CVdIII-44/54 (5'-CCCCCTTGCCAA-AAATAAAATGCAGAGAGGG-3') + M13 forward (5'-GTAAAACGACG-GCCAGTG-3')] were used to divide the 579 bp expression cassette into overlapping 278 and 332 bp fragments. After re-assembly [94°, 42°, 72° (1 min each) - 30 cycles], the cassette was again divided into a second set of overlapping 380 and 228 bp fragments using primer pairs M13 reverse-5 + CVdIII-254/226 (5'-CCTTCTAGCCNANANANACAGATTAGG-3') and CVdIII-226/254 (5'-CCTAATCTGTNTNTNTNTNGGCTAGAAGG-3') + M13 forward]. Mutagenized positions are denoted by underlined italics (N = equimolar mixtures of all four deoxynucleotides). Following final reassembly, the mixture of mutagenized CVd-III cDNAs was transcribed with T7 RNA polymerase as previously described (14). Mutagenesis efficiency was monitored by automated sequence analysis of the reassembled CVd-III cDNAs prior to transcription. All analyses were carried out on uncloned PCR products using Perkin-Elmer/Applied Biosystem's AmpliTaq-FS DNA polymerase and Big Dye terminators with dITP. Expression cassettes containing unmutagenized CVd-IIIb cDNA served as a control.

**Bioassay and characterization of viroid progeny.** Aliquots of RNA transcripts containing approximately 200 ng fully-processed CVd-III RNA in 100 µl 20 mM Na phosphate (pH 7.0) were slash-inoculated into the stems of Etrog citron (clone RMA

861-S1) scions growing on rough lemon rootstocks. Inoculated plants were kept in a greenhouse under high light-warm temperature conditions favoring viroid replication and periodically cut back to encourage symptom development. Starting 3 mo post-inoculation (p.i.), viroid accumulation in inoculated plants was monitored by petiole-print hybridization (18) using a full-length, digoxigenin-labeled RNA probe specific for CVd-III. To propagate progeny viroids, buds were taken from plants infected by slash-inoculation and transferred to viroid-free citrons (two plants/treatment).

Selected preparations of CVd-III progeny were further characterized by RT-PCR and sequence analysis of the resulting uncloned, enzymatically amplified viroid cDNAs using primers C2' (5'-ACTCTCCGTCTTTACTCCAC-3') and H2' (5'-CTCCGCTAGTCGAAAGACT-3') as previously described (15, 29). Multiple sequence alignments were generated with the Clustal V program implemented through the Megalign function of the Lasergene software package (Version 4.0 - DNASTAR). Minor adjustments were manually introduced in the final alignment to maximize sequence homology. Structural calculations were performed with *mfold* version 3.1 as implemented on the *mfold* server [http://bioinfo.math.rpi.edu/~mfold/rna/form1.cgi] (30).

## RESULTS

**Preparation of CVd-III mutant libraries.** As shown in Fig. 1, randomization of CVd-IIIb positions

236, 238, 240, 242, and 244 is expected to result in a mixture of more than 1,000 variants (i.e.,  $4^5 = 1024$ ), one of which will be the starting sequence. In the event that none of the new variants would be able to successfully compete with CVd-IIIb, a second mutagenesis was carried in which G>A and C>U substitutions were first introduced at positions 44 and 54. These two mutations were previously shown to cause a reduction in symptom severity and progeny accumulation (15).

**Bioassay of randomized CVd-III RNA transcripts.** The effect of sequence randomization on CVd-III infectivity was examined in two independent infectivity trials in which groups of seven to eight trees were slash-inoculated with serial dilutions of RNA transcripts. Unmutagenized CVd-IIIb RNA transcripts were included as a positive control in each trial. The resulting infectivity data are summarized in Table 1.

In the first trial, the starting material for mutagenesis was the wild-type viroid, CVd-IIIb. CVd-IIIb RNA transcripts appeared to be less infectious as a result of the randomization of positions 236-244, but a majority of the plants inoculated with 5,000 pg/μl RNA transcripts (i.e., 5/7) had become infected by 5 mo p.i. Sequence analysis of the

viroid progeny present in each of these plants, as well as one of two plants that became infected after inoculation with a 10-fold lower concentration of transcripts, revealed that all were wild-type CVd-IIIb (results not shown).

As shown in the lower portion of Table 1, mutagenesis of a CVd-IIIb variant containing substitutions at positions 44 and 54 appeared to have a more dramatic effect on infectivity. Although a majority of inoculated plants eventually became infected, the incubation period was much longer; i.e., 21 mo vs. 5 mo in the previous experiment. In order to monitor possible sequence evolution within viroid populations present in individual trees, buds were removed from three infected trees 13 mo p.i. and transferred to uninfected citrons.

**Characterization of CVd-III variants arising *in vivo*.** As shown in Fig. 2, the progeny arising from our second mutagenesis experiment contained a complex mixture of targeted and spontaneous sequence changes. Progeny isolated from trees 3, 5, and 8 soon after they had become infected contained only the G>A and C>U substitutions present at positions 44 and 54 in each inoculum molecule. In each case, later re-sampling revealed a tendency for one or both changes to revert to wild-type.

TABLE 1  
INFECTIVITY OF RANDOMIZED CVd-III RNA TRANSCRIPTS

Starting sequence	Infectivity*			
	3-3.5 mo	5 mo	10-11 mo	21 mo
Experiment 1				
CVd-IIIb - 5,000 pg/μl	4/7	5/7	nt	nt
500	1/7	nt	2/7	nt
50	0/7	0/7	nt	nt
Unmutagenized CVd-IIIb (500 pg/μl)	6/7	nt	nt	nt'
Experiment 2				
G <sub>44</sub> A + C <sub>54</sub> U - 10,000 pg/μl	1/8	2/8	4/8	5/8
1,000	0/7	0/7	nt	nt
Unmutagenized CVd-IIIb (1,000 pg/μl)	3/3	nt	nt	nt

\*Expressed as no. trees infected/no. trees inoculated. Infection status determined by post-inoculation petiole print hybridization (17).  
nt, not tested.

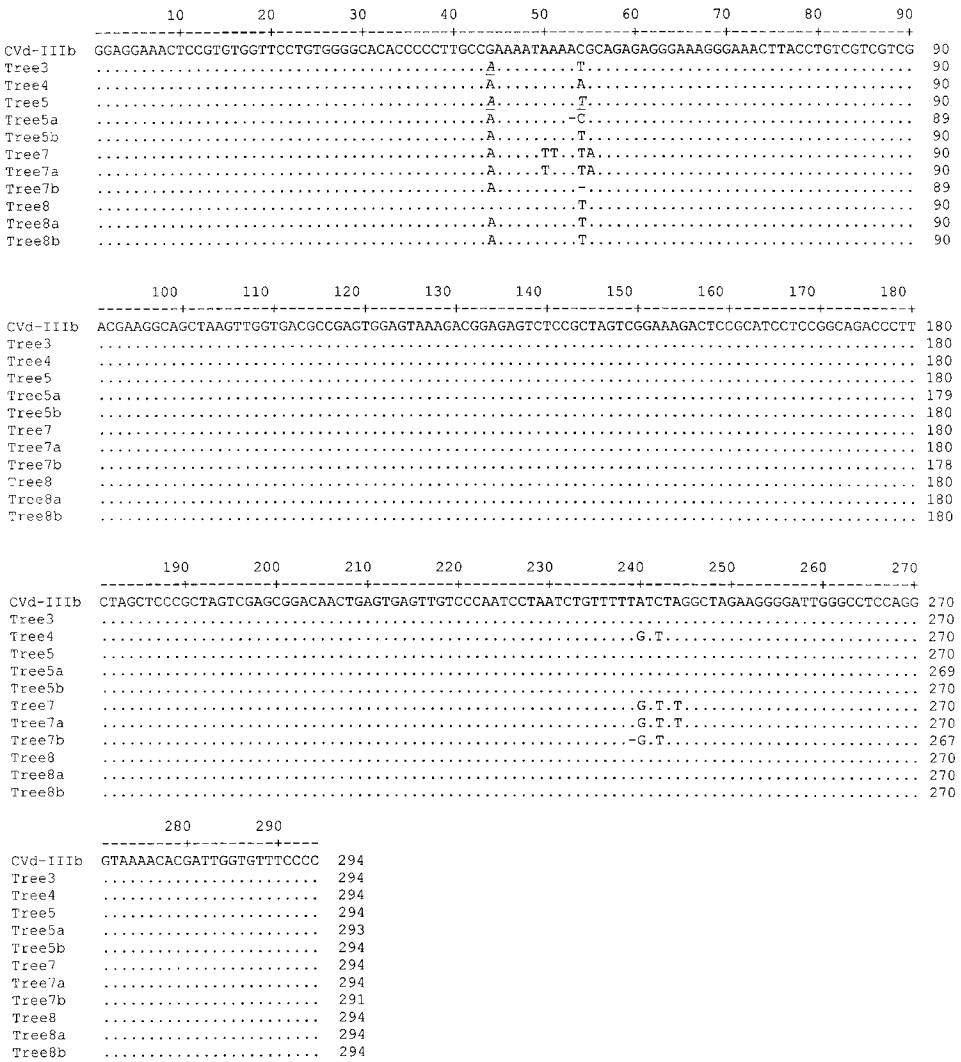


Fig 2. Sequence variants recovered after limited randomization of CvD-IIIb. Sequence changes not stably maintained *in vivo* are shown in *italics*. Graft inoculated trees are denoted by the suffixes “a” and “b”.

Eleven months p.i., tree 7 had also become infected. Sequence analysis indicated that this tree contained a mixture of sequence variants. As shown in Fig. 2, one of these variants eventually came to dominate the population. This novel CvD-III variant contains three directed (positions 240, 242, and 244) and three spontaneous changes (positions 50, 51, and 55). During the later stages of the assay, a fifth tree (i.e., tree 4) also became infected.

The progeny isolated from this tree contained four changes, all of which were present in the inoculum.

The distribution of viruses and viroids in woody hosts is often uneven (6, 10). Thus, one might expect that transfer of one or two buds from an infected tree to each of several viroid-free trees would result in the appearance of different CvD-III variants in each graft recipient. Data presented in Fig. 2 shows that this was what was observed. Although obviously

related in sequence, the viroids isolated from trees 5a and 5b or 7a and 7b were not identical.

Structural calculations indicate that certain combinations of changes may have significant effects on base-pairing within the pathogenicity domain. Such effects were most obvious for three variants derived from tree 7. The predicted lowest free energy structures for the pathogenicity domains of these variants are shown in Fig. 3, and the symptoms they induce in Etrog citron are compared in Fig. 4. While it is not yet possible to link specific sequence and/or structural changes with changes in symptom severity in Etrog citron, note that the changes present in the variants from trees 7 and 7b appear to intensify symptoms, while those from tree 7a have the opposite effect. The two changes present in the variant from tree 5b, a G>A change at position 44 and a C>U change at position 54, are predicted to have only minimal structural effects. As shown in Fig. 4, these changes resulted in a marked reduction in symptom expression.

### DISCUSSION

Dwarfing trials with gel-purified inocula not subjected to molecular cloning have shown that four different viroids (i.e., CEVd, CVD-Ia, CVD-

II, and CVD-IIIb) can be used to control tree size in citrus growing on trifoliolate orange or citrange rootstocks (27). For trees inoculated in the field 1 yr after transplantation, the dwarfing response takes several years to become apparent (12). Many currently used dwarfing isolates contain several different viroids, and segregation/exclusion of one or more viroids from such complexes can lead to marked variation in tree size (6, 10, 17). The use of viroids for tree size control is not risk-free, but more than 40 yr of field experience have failed to reveal any association between CVD-IIIb infection and disease in the combinations tested (12). Potential concerns include (i) mutation of a dwarfing strain to a virulent form and (ii) interaction with other agents to induce a new disease (22, 23). For example, the RG1 strain of *Potato spindle tuber viroid* (PSTVd) appeared spontaneously during serial passage of PSTVd-Intermediate and contains only three nucleotide substitutions. RG1 induces much more severe symptoms than PSTVd-Intermediate and overgrows the parental strain when the two variants are inoculated as a mixture (9).

Genetic recombination is a major force driving the evolution of viroids and viruses (3, 13, 24). Soong et al. (25) have shown that the *in vitro* process of PCR-mediated DNA shuf-

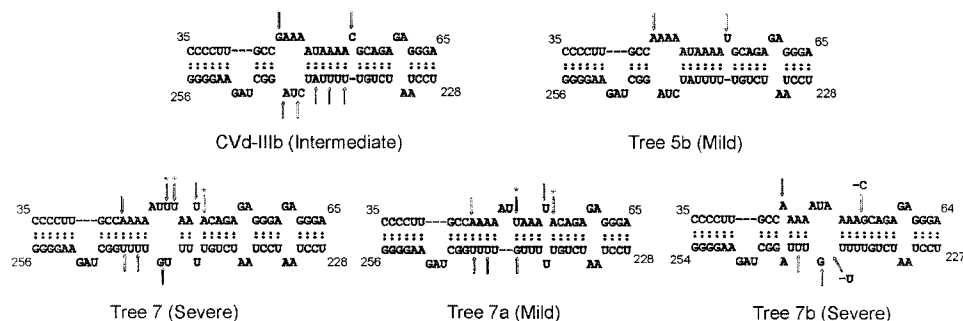


Fig. 3. Mutation-induced structural rearrangements of the CVD-III pathogenicity domain. Arrows, mutations present in original RNA transcript library; asterisks, spontaneous mutations arising *in vivo*. An overall assessment of symptom severity is given in parentheses.





**Fig. 4. Effect of sequence changes on CVd-III symptom expression in Etrog citron.** (A) Comparison of symptoms induced by CVd-IIIb (center, relatively severe epinasty) with those induced by variant 5b (left, mild epinasty) and variant 7 (right, mild epinasty but moderate stunting). (B) Variation in symptom severity arising after passage via bud grafting. Note that variant 7a (center) induces much milder symptoms than either variant 7 (left, the parental sequence) or variant 7b (right).

fling (molecular breeding) mimics this mechanism on a vastly parallel and accelerated scale. A single round of shuffling envelope sequence from six murine leukemia viruses followed by selection resulted in a chimeric virus with a completely new ability to replicate in Chinese hamster ovary cells. GenBank now contains 48 non-redundant CVd-III sequences, but the small genome size (approx. 300 bp) makes DNA shuffling difficult to apply. To limit the number of variants in this library and confine mutagenesis to the pathogenicity domain of CVd-III, we used a strategy known as “combinatorial cassette mutagenesis” (21) rather than DNA shuffling to create pools of CVd-III variants that we hoped would contain one or more variants differing in biological properties from CVd-IIIb. A key ingredient in the ultimate success of this or any other strategy is an efficient strategy to select variants having the desired biological properties.

The starting material in our first mutagenesis experiment was CVd-

IIIb, and no attempt was made to remove wild-type viroid from the resulting mixture of more than 1,000 CVd-III variants. Not surprisingly, this experiment failed to yield any novel variants. Only a small number of trees were inoculated, and previous analyses of naturally occurring citrus viroid isolates have shown that CVd-IIIb is one of the most common (and, therefore, presumably the most competitive) variants present (16). Quite a different result was observed in our second trial when the starting material for mutagenesis was an attenuated variant. Here again, less than 10 citron plants were slashed inoculated with an equally complex mixture of CVd-III RNA transcripts, but a total of five novel variants were recovered; i.e., three variants from the slash-inoculated plants and two more from trees subsequently inoculated via bud grafting.

These experiments need now to be repeated with trifoliate orange, citrange, and other viroid-sensitive rootstocks. It is possible that multiple sequence changes will be

required to produce a significant increase in dwarfing, as previously observed for the induction of cachexia and/or xyloporosis by citrus viroid II (20). Sequence randomization can be thought of as a means to “seed” the evolutionary pathway between the clusters of variants with different biological or structural properties that comprise an RNA quasispecies (7). In the case of human influenza A virus, identification of 18 codons in the viral hemagglutinin that are subject to positive selection *in vivo* opens the possibility of identifying the most fit viral strains and thereby predicting the future course of virus evolution (4).

Our previous analysis of CVd-III sequence diversity indicated that there may be as many as four fitness peaks (16). With the recent addition of 14 CVd-III sequences from Japan (GenBank Accession numbers AB054619 - 32), it should be possible to construct a much higher resolution portrait of the CVd-III quasispecies to guide future mutagenesis experiments. Among the important questions to be answered: How close is the genetic and functional linkage between different regions of the CVd-III genome? Can sequence changes introduced into one region induce spontaneous changes in another region, with unanticipated consequences for the biological properties of CVd-III? The outcome of the selection process might also be greatly influenced by inherent differences in the response of different sensitive rootstock/tolerant scion

combinations to CVd-III infection. Answers to these and other questions are required to properly assess the long-term stability of improved viroid dwarfing agents. To be statistically valid, such studies will require the use of much larger numbers of plants than those used in these “proof of principle” studies; to be useful, these assays will have to be extended to dwarfing trials in citrus groves.

One important challenge that we have not yet addressed is the development of a suitable screening assay to assess the dwarfing properties of newly isolated CVd-III variants. As described by Hutton et al. (12), viroid dwarfing on trifoliolate orange and citrange rootstocks occurs primarily through a reduction in the major summer growth flush. At the molecular level, how viroid infection affects interaction(s) between rootstock and scion is completely unknown. Field trials take 6-8 yr for initial evaluation, and, thus, development of a faster greenhouse-based assay suitable for screening purposes is essential. As we begin to refine our mutagenesis strategy, development of such a screening assay has become a top priority.

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