Host-directed processing of *Citrus exocortis viroid*

J. A. Szychowski, G. Vidalakis and J. S. Semancik

Department of Plant Pathology, University of California, Riverside, CA 92521, USA

Prolonged infection of tomato hybrid (*Lycopersicon esculentum* × *Lycopersicon peruvianum*) by *Citrus exocortis viroid* (CEVd) resulted in viroid-like enlarged structures, detected by gel electrophoresis. This population included two new enlarged variants or D-variants, D-87 and D-76, and three transient species or D-forms, D-38, D-40 and D-43. Sequence analyses exposed a locus near the terminal repeat region where major changes appeared consistently. In transmission tests to CEVd hosts, a variety of progeny populations were recovered, including progeny enlargements of and reversions to CEVd, as well as sequence fidelity to the inoculum. Transmission tests to citrus hosts of the genera *Citrus*, *Poncirus* or *Fortunella* were unsuccessful. The importance of host specificity to the recovery and processing of the various CEVd-related structures, as well as the temporal variability of progeny populations, was demonstrated.

Viroids are the smallest known plant pathogens, consisting of a non-translated, single-stranded, circular RNA of 246–475 nt, which is replicated autonomously by enlisting host-encoded proteins (Flores *et al.*, 1997), mediated through specific conformations (Owens *et al.*, 1986; Visvader *et al.*, 1985). *Citrus exocortis viroid* (CEVd) was first described as an agent causing bark scaling on *Poncirus trifoliata* (Fawcett & Klotz, 1948). Unusual variants of CEVd from a hybrid tomato (*Lycopersicon esculentum* Mill. × *Lycopersicon peruvianum*), CEVd D-92 and D-104 (Semancik *et al.*, 1994; Semancik & Duran-Vila, 1999) and, more recently, from eggplant (Fadda *et al.*, 2003), have been shown to display terminal repeats similar to naturally occurring variants of *Coconut cadang-cadang viroid* (CCCvd) (Haseloff *et al.*, 1982). Transmission of CEVd D-92 and D-104 to *Gynura aurantiaca* demonstrated that other plant species were also receptive to the processing and replication of these structures. Two additional enlarged CEVd variants and three transient forms from hybrid tomato are reported here.

Hybrid tomato (*L. esculentum* Mill. × *L. peruvianum* line 741505-45; supplied by Dr D. Pratt, University of California, Davis, CA, USA) was infected with partially purified CEVd from gynura. Initially, progeny were identical to the infecting CEVd. However, 6–12 months post-inoculation, CEVd-related variants larger than the inoculum viroid were detected by sequential 5 % PAGE (sPAGE) (Rivera-Bustamante *et al.*, 1986). These forms were intermediate between CEVd and CEVd D-92 (Semancik *et al.*, 1994) (Fig. 1).

RT-PCR products from CEVd-infected hybrid tomato were generated by utilizing specific CEVd primers complementary to residues 80–98 and identical to residues 99–117 (Gross *et al.*, 1982). Reactions were performed as described previously (Semancik *et al.*, 1994) with the exception that 1·5 mM MgCl₂ was used, together with an annealing temperature of 50 °C and 35 PCR cycles. Amplified products were purified by using an Ultrafree-DA kit (Millipore) and ligated into the vector pUC18. After sequencing, monomeric cDNA clones were subcloned into the vector pDP-19 for 
in vitro RNA transcription (MEGAscript; Ambion) and inoculation of 4–12 plants of the herbaceous hosts chrysanthemum (*Dendranthema grandiflora*), datura (*Datura stramonium*), eggplant (*Solanum melongena*), gynura (*G. aurantiaca*), tomato (L. esculentum) and the hybrid tomato, as well as the woody plants citrus (*Citrus medica*), sweet orange (*Citrus sinensis*), trifoliate orange (*P. trifoliata*) and Meiwa kumquat (*Fortunella spp.*). Inoculations were repeated at least three times on all herbaceous hosts and citrus, but only once with kumquat and trifoliate orange. Progeny were monitored within single plants. Herbaceous hosts were assayed 2–12 months post-inoculation (Igloi, 1983; Semancik *et al.*, 1988), whilst woody cultivars were assayed for 2–3 years. Viroid sequences were aligned by using CLUSTAL W (Thompson *et al.*, 1994) after manual adjustments to maximize similarity. Secondary structure was predicted by Mfold version 2.3 (Zuker, 1989).

Of the 18 CEVd-related cDNA clones that were sequenced, six different molecules were recovered. The majority of clones (83 %) were CEVd, as well as two previously unreported larger D-variants, D-87 and D-76. Three additional molecules, D-43, D-40 and D-38, were designated ‘D-forms’, as replication could not be confirmed. However, biological activity of the D-forms was demonstrated by the synthesis of CEVd-related progeny. As additions to the CEVd genome contained regions of terminal repeats in the

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variable (V) and terminal (T2) domains (Keese & Symons, 1985) similar to CEVd D-92 (Semancik \textit{et al.}, 1994), the 'D' nomenclature, indicating the number of duplicated nucleotides, was adopted.

The CEVd-related molecules were intermediate in size between CEVd (372 nt) and CEVd D-104 (475 nt). Fig. 2(a) shows the structure of the D-variants and their relationship to the U2 and L2 sequences of CEVd D-92. The four clones of CEVd D-87 (Fig. 2a) all displayed the same changes at the initiation position of the 92 nt repeat of D-92. Changes found were as follows: G\textsuperscript{180} \rightarrow A; deletion of 18\textsuperscript{2} UC\textsuperscript{184} in the upper strand of the molecule (U1); and deletion of A\textsuperscript{260} and A\textsuperscript{273} \rightarrow U in the lower strand (L1). The following changes were also observed in individual clones: A\textsuperscript{20} \rightarrow G; addition of C\textsuperscript{148}; deletion of G\textsuperscript{178} and G\textsuperscript{221}; C\textsuperscript{206} \rightarrow U; and G\textsuperscript{425} \rightarrow A.

The five clones of D-76 all displayed a 7 nt deletion from the upper and lower strands of CEVd D-92, 172 AACAAGG\textsuperscript{178} in U1 and 274 UUCCUUU\textsuperscript{280} in L1 (Fig. 2a). These deletions are juxtaposed on the upper and lower strands of the molecule, close to the initiation position for additions to CEVd for synthesis of CEVd D-104. Additional changes close to the initiation site were the addition of G\textsuperscript{176} (U1), the deletion of A\textsuperscript{269} and U\textsuperscript{271} \rightarrow G (L1). Two changes that were found in single clones, A\textsuperscript{50} \rightarrow U and C\textsuperscript{411} \rightarrow U, are not shown in Fig. 2(a).

Clones of the three CEVd-related forms with major asymmetric deletions were recovered, but were represented as symmetrical structures with the addition of a quasicomplementary structure from D-92 (Fig. 2b). Both D-43 and D-38 had 46 nt deletions from D-92 at the same U2 location. CEVd D-40, however, lacked almost the entire L2 region. Interestingly, D-40 and D-38 were also missing the same 7 nt in U1 and L1, respectively, as D-76 (Fig. 2a). Changes found in the pathogenic (P) domain (A\textsuperscript{95} \rightarrow U, U\textsuperscript{412} \rightarrow C and A\textsuperscript{413} \rightarrow U) of D-87 were observed in all three D-forms. CEVd D-40 had three mutations and two additions in the terminal 1 (T1) domain.

Specific processing of CEVd-related molecules in alternative hosts was examined. Considering the asymmetric structure of the D-forms, replication was not anticipated. Nevertheless, as the complete CEVd genome was retained, tests for synthesis of CEVd-related structures were conducted. Tomato was the most receptive host to infection by the D-variants. Successful transmission in the herbaceous hosts varied from 2 months in tomato to 6–12 months in datura and chrysanthemum. Transmission of the D-variants was not successful to citrus species of the genera \textit{Citrus}, \textit{Poncirus} or \textit{Fortunella}, in agreement with the loss of transmission to citrus by CEVd D-92.

Symptoms ranged from severe epinasty and rugosity in tomatoes and chrysanthemum to no symptoms in datura and eggplant. Gynura symptoms were most variable, displaying mild rugosity developing into severe epinasty as CEVd emerged as the principal progeny. Segregation of CEVd variants within a single plant was observed, with a severe shoot containing CEVd and D-variants from leaves with bent-leaf symptoms.

CEVd-related progeny found in herbaceous hosts after inoculation with RNA transcripts of D-variants and D-forms are presented in Table 1. None of the D-forms replicated with fidelity, however, were biologically active for synthesis of CEVd. In contrast, the D-variants, especially CEVd D-92 and D-87, replicated with fidelity, particularly in datura and eggplant (Table 1). With prolonged infection, a reduction of the genome to CEVd was observed following inoculation with D-104 and D-87.

Chrysanthemum was most recalcitrant to inoculum replication, with only CEVd D-92 recovered as pure progeny. This may be more reflective of the structure of D-92 than of the chrysanthemum replication process, as fidelity of D-92 was maintained in all hosts.
The observation of reciprocal progeny following infection from CEVd was apparently the result of two factors: (i) a significant to viroid evolution. The generation of CEVd D-92 replication might now be viewed as perhaps being significant in providing a ‘repair’ function in the hosts, resulting in an enlarged and biologically active structure. CEVd was recovered from the mixed progeny population of D-43, whilst D-40 and D-38 reverted only to CEVd in gynura.

As viroids are non-translated and rely on the host for replication, it is critical to characterize not only the sequence and conformation of the viroid genome, but also the biological interactions of a specific viroid with a particular host. Genome enlargement is an infrequent observation with D-87 and D-43 suggested a specific and temporal relationship between the specific D-variant and D-form as possible progenitors of D-92 as a stable structure. This is not to subordinate the importance of the specific host in supporting replication of both structures. Only Rutgers tomato supported an enlarged genome when inoculated with D-40.

Tomato species supported variable populations of progeny following D-variant inoculation. Initial inoculation of CEVd D-104 was successful; however, after 6 months only CEVd was detected. In Rutgers tomato, D-87 inoculation resulted in stable progeny, whilst the hybrid tomato necessitated cloning for 7/17 D-87 and 10/17 CEVd. With D-40, most progeny from tomato species reverted to CEVd. The asymmetric D-40 inoculum was processed in Rutgers into D-87 progeny by completion of the 7 nt deletion in U1 and the large 45 nt block in L1–L2 (Fig. 2b).

Mixed (+) and transient (→) populations are indicated.

<table>
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<tr>
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<th>D-variants</th>
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<tr>
<td></td>
<td>D-104</td>
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<td>D-87*</td>
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<td>D-92</td>
<td>D-87 (6/16) + D-43 (1/16) + CEVd (9/16)</td>
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<td>D-92</td>
<td>D-87</td>
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<td>Tomato hybrid</td>
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<td>D-92</td>
<td>D-87 (7/17) + CEVd (10/17)</td>
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*Numbers in parentheses indicate no. viroid cDNA clones/total no. sequenced.

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Table 1. CEVd-related progeny after inoculation with RNA transcripts of D-variants and D-forms

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Of particular interest to the characterization of biological activity of D-variants was the fact that transmission to any citrus cultivar was unsuccessful, even though all contained the CEVd genome. From this, it may be inferred that the irregular orientation and/or enlargement of the CEVd genome seriously interfered with the biology of the D-variants in citrus species. This was unexpected, as CEVd is of economic importance to the citrus rootstock, *P. trifoliata*, whereas sweet orange and citron are reservoir hosts.

A recent report (Fadda et al., 2003) identified a CEVd D-96 variant in eggplant 5 years after CEVd inoculation. The present study suggests that not only tomato and eggplant, but also chrysanthemum, datura and gynura, have the ability to support the synthesis of enlarged CEVd genomes. Although D-variants and D-forms have previously only been generated by infection in the hybrid tomato and eggplant, gynura might have the ability to synthesize larger variants from a CEVd inoculation, as evidenced by the D-87 progeny recovered after D-43 infection.

Viroid structure and conformation is essential in replication and hairpin II (HPII) is important in providing a binding site for polymerase II and/or transcription factors (Loss et al., 1991; Steger & Riesner, 2003). The AGCU tetrads found in CEVd D-92 were suggested as possible sites for the involvement of a ‘jumping polymerase’ as an explanation for the duplicated regions. Two AGCU tetrads were located within the loop of the HPII structure, as well as in both the D-variants and D-forms.

This family of CEVd-related molecules also shows a
structural similarity to a non-infectious 350 nt Potato spindle tuber viroid (PSTVd) cDNA in a tobacco genome, which resulted in a replicating 341 nt PSTVd in planta (Wassenegger et al., 1994). The in vivo 9 nt deletion in PSTVd was located opposite the long truncated in vitro deletion mutant, similar to the loops and deletions observed in D-38 and D-40. It has been hypothesized that evolution of the truncated PSTVd occurred as a result of either the polymerase II being forced to skip over the bulged-out loop and template switching, favoured by base pairing, or the bulged-out loop being cut out and religated.

Multiple binding sites have been reported between PSTVd RNA and a tomato protein (Gozmanova et al., 2003). This RNA-binding motif, which is responsible for interaction of PSTVd and the tomato protein Virp1, was also found in CEVd directly adjacent to the AGCU tetrad in the U1 region. Thus, a complex binding reaction, possibly involving up to four proteins per RNA molecule, may be involved in the processing of the enlarged genomes. As AGCU is a palindrome, albeit small, these tetrads have the potential to base pair to any of the three strands – donor, nascent or acceptor. In addition, given the proposed molecular conformation of the CEVd D-forms, containing large asymmetric deletions, a jumping polymerase could be envisioned. At this time, it is only conjecture whether the properties of genome enlargement as described here indicate a general process for viroid synthesis and/or recombination.

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References


