Sequence and structure of defective interfering RNAs associated with cucumber necrosis virus infections

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The presence of symptom-attenuating defective interfering (DI) RNAs in a laboratory culture of cucumber necrosis tombusvirus (CNV) was confirmed. Sequencing of cDNA clones of these DI RNAs revealed that CNV DI RNAs retained sequences from the CNV 5'-untranslated and 3'-terminal regions as well as a portion of the coding region for the 92K protein. Similar sequence arrangements were also observed in symptom-attenuating DI RNAs generated de novo from synthetic wild-type CNV transcripts. A comparison of the sequences and biological activities of these CNV DI RNAs is presented. In co-infections of synthetic wild-type CNV and CNV DI RNAs, prominent RNA species of a higher Mr than the DI RNA used in the co-infection were found. The possible nature of these RNA species is discussed.

Cucumber necrosis tombusvirus (CNV) (Rochon & Tremaine, 1988) is an icosahedral virus with a monopartite genome of positive-sense ssRNA approximately 4.7 kb in length (Rochon & Tremaine, 1989). The only known natural systemic host for CNV is the cucumber; however, the experimental host range of CNV is wide and diversified (McKeen, 1959). Viral transmission can occur naturally via the zoospores of the fungus Olpidium radicale (Dias, 1970). The CNV genome has been completely sequenced (Rochon & Tremaine, 1989) and is proposed to consist of at least five open reading frames (ORFs) (see top of Fig. 1b) which give rise to protein products of 33K, 92K, 41K, 21K and 20K. A small ORF in the extreme 3' end of the genome may encode a sixth protein (Boyko & Karasev, 1992). The 92K protein could arise from readthrough of the amber codon that terminates the ORF for the 33K protein. The 92K protein is presumed to be a component of the virus-encoded replicase based on amino acid sequence similarity with the putative polymerases of related viruses (Rochon & Tremaine, 1989). The function of the 33K protein is not known. The 41K protein is the viral coat protein (Rochon & Tremaine, 1989) and is expressed from a 2.1 kb subgenomic mRNA (Johnston & Rochon, 1990). The 21K and 20K proteins are expressed from extensively overlapping but distinct ORFs on a single, bifunctional 0.9 kb subgenomic mRNA (Rochon & Johnston, 1991). The functions of these two proteins are not known, but the 20K protein is suspected to play a role in CNV RNA replication based on the observation that a CNV mutant that does not express this protein rapidly generates defective interfering (DI) RNAs (Rochon, 1991).

DI RNAs are deletion mutants of the viral genome which are unable to replicate in the absence of helper virus. DI RNAs interfere with helper virus replication probably through competition for replication and encapsidation proteins (Roux et al., 1991). In model systems, it has been demonstrated that DI particles influence disease processes; however, whether they play a role in natural infections remains to be firmly established (Roux et al., 1991). Defective RNAs occur ubiquitously among animal RNA viruses and have also been described in association with the dsRNA killer virus of yeast (Kane et al., 1979; Bruenn, 1980) and many plant RNA viruses including the cherry strain of tomato bushy stunt tombusvirus (TBSV-Ch) (Hillman et al., 1987), cymbidium ringspot tombusvirus (CyRSV) (Burgyan et al., 1989), turnip crinkle carmovirus (TCV) (Li et al., 1989), clover yellow mosaic potexvirus (White et al., 1991) and tomato spotted wilt tospovirus (Resende et al., 1992). It is believed that DI RNAs arise as a result of the viral replicase, in complex with its nascent daughter strand, falling off its template strand at regions of secondary structure and then reinitiating RNA synthesis at a different site either on the same or a different RNA template (Lazzarini et al., 1981). Spontaneous generation of DI RNAs from viral genomic RNA templates (de novo generation) has been demonstrated for TBSV-Ch (Knorr et al., 1991), CyRSV...
It was previously reported that RNA extracts of plants infected with a laboratory culture of CNV (CNV-Lc) contained high levels of a low Mr (approximately 400 nucleotides) virus-related RNA species. In addition, plants infected with CNV-Lc displayed attenuated symptoms in comparison with plants inoculated with synthetic wild-type (wt) CNV transcripts (Johnston & Rochon, 1990). These observations taken in conjunction with the fact that synthetic CNV mutants that do not express the 20K protein rapidly generate DI RNAs de novo (Rochon, 1991) suggested that the 400 nucleotide RNAs associated with CNV-Lc infection may be DI RNAs and further that the wt CNV genome, like the 20K mutant genome, is capable of generating DI RNAs de novo. In this paper we demonstrate that the 400 nucleotide RNAs are in fact DI RNAs and are likely to be responsible for the symptom attenuation associated with CNV-Lc.

In order to generate DI RNAs de novo from wt inoculum, synthetic transcripts were produced from a full-length CNV cDNA clone (pK2/MS) as described previously (Rochon & Johnston, 1991) and rub-inoculated onto *Nicotiana clevelandii* plants. The use of synthetic transcripts for inoculation ensured that the starting material was DI RNA-free. Serial high m.o.i. passages were carried out as described previously (Rochon, 1991). After 14 high m.o.i. passages, large amounts of low Mr RNAs were detected in two out of six plants examined. These two plants also displayed attenuated symptoms and persistent infection typical of CNV-Lc-inoculated plants. This was distinct from the severe systemic necrosis observed in the remaining four plants which became desiccated and died 2 weeks post-infection. Leaf RNA from two such plants was extracted as described previously (Rochon & Johnston, 1991) and cDNA clones were constructed as described above using RT–PCR.

Four cDNA clones from CNV-Lc small RNAs and five cDNA clones from the de novo generated small RNAs (four from one plant and one from the other plant) were subjected to dideoxynucleotide sequencing (Sanger et al., 1977) in both directions using plasmid dsDNA templates (Sambrook et al., 1989), oligonucleotide primers and Sequenase (U.S. BioWhittaker). The sequencing results are shown in Fig. 1(a) and Table 1. The DI RNAs were sequenced using primers complementary to the first nucleotides of the three ORFs (Fig. 1(b)).

Fig. 1. (a) Comparison of cloned DI RNA sequences with the CNV genomic sequence. Portions of the CNV genomic sequence not represented in any DI RNAs are indicated within the CNV sequence in parentheses. DI RNA sequences in order of largest to smallest are indicated below the CNV sequence. Dashes indicate sequence identity with respect to CNV; nucleotides are shown only when they differ from CNV. Asterisks indicate deletions relative to either CNV or another DI RNA. Underlining indicates the AUG codon that initiates synthesis of p3 and p92. Total lengths of CNV genomic RNA and each DI RNA are indicated in brackets at the end of each sequence. Numbers at the beginning of each line correspond to the first nucleotide in each line. The 5'-terminal G and 3'-terminal C for each DI RNA were added during the cloning procedure. (b) Structure of cloned DI RNAs relative to the CNV genome. Blocks along the CNV genome represent the five ORFs of CNV, hatched blocks immediately beneath the CNV genome represent the largest limits of the three distinct domains derived from CNV by the various DI RNAs. DI RNAs with distinct overall organizations are indicated in the bottom half with broken lines between the hatched boxes indicating deletions within each domain.
Biochemicals). The complete DNA sequences of seven clones with unique sequences are given in Fig. 1(a). A simplified version of the overall organization of the DI RNAs relative to the CNV genome is given in Fig. 1(b).

Sequence alignments clearly demonstrated that both the small RNAs from CNV-Lc virion RNA and those generated de novo by high m.o.i. passaging were linear deletion mutants of CNV genomic RNA. Each DI RNA sequence examined contained nucleotide sequences derived from the CNV 5'-untranslated and 3'-terminal regions (regions I and III, respectively) and a small portion of the coding region for the putative polymerase (region II; Fig. 1b). This pattern of retained CNV genomic sequences has been observed in CNV DI RNAs recently sequenced in another laboratory (Y.-C. Chang, T. J. Morris & A. Jackson, personal communication) and those generated from the 20K mutant of CNV (Rochon, 1991) as well as DI RNAs from the related tombusviruses TBSV-Ch (Hillman et al., 1987; Knorr et al., 1991) and CyRSV (Burgyan et al., 1989, 1991; Rubino et al., 1990). At their 5' ends, all clones maintained sequences up to and immediately upstream of the CNV 33K start codon and seven of the nine clones retained the actual start codon. The largest ORF beginning with the retained CNV 33K start codon (which would remain in the original translational context) encoded 23 amino acids (DI15). At the 3' end, there was some variability in the sequences retained from the CNV translated and untranslated portions. Only one clone (DI9) contained some sequence from the 3' portion of the 20K coding region; all clones contained some sequence from the 3' portion of the 21K coding region. One clone retained nearly all of the 3' untranslated region (DI15) whereas the remaining clones contained an internal deletion in the 3' untranslated region ranging in size from 310 nucleotides (DI48) to 120 nucleotides (DI12). DI48 may be an artefact of the RT–PCR procedure since this clone lacked most of region III yet retained the last 15 nucleotides exactly complementary to the 3' oligonucleotide used for RT–PCR. There were very few nucleotide substitutions in DI RNA sequences relative to CNV genomic RNA (Fig. 1a) and none of the DI RNAs sequenced contained duplications of nucleotide sequences as has been observed with TBSV-Ch DI RNAs (Hillman et al., 1987) and CNV DI RNAs sequenced in another laboratory (Y.-C. Chang, T. J. Morris & A. Jackson, personal communication).

Each of the DI RNAs with unique sequences was tested on N. clevelandii hosts for their ability to accumulate and/or attenuate symptoms. Plants were inoculated with equal amounts (0.5 μg) of T7 RNA polymerase-derived transcripts of each DI cDNA clone alone or co-inoculated with (5 μg) T7 RNA polymerase-derived transcripts of wt CNV clones as described by Rochon (1991). Plants were monitored for symptom attenuation for up to 3 weeks, by which time controls inoculated with wt transcript alone had become desiccated and died. Six days post-inoculation total leaf RNA was extracted from one originally inoculated leaf and subjected to Northern blot analysis using 32P-labelled nick-translation CNV probes (Rochon & Johnston, 1991). Probes corresponded to the 5'-terminal 40 nucleotides (5' probe) and the 3'-terminal 540 nucleotides (3' probe) of CNV genomic RNA.

All of the transcripts generated from the DI cDNA clones replicated and were able to attenuate symptoms when co-inoculated with wt transcripts (data not shown) with the exception of clone DI48. Plants co-inoculated with transcripts from this clone displayed severe necrotic symptoms similar to those generated in wt transcript-inoculated plants. All plants that were inoculated only with transcripts generated from DI cDNA clones appeared as mock-inoculated controls. Northern blot analysis was used to determine whether DI RNAs were capable of accumulating in plants. Fig. 2 shows that in each co-inoculation experiment, it was possible to detect with both 5' and 3' probes an RNA species corresponding to the \( M_r \) of the DI RNA transcript used for co-inoculation. As expected, the synthetic DI RNA transcripts did not accumulate in plants when inoculated alone (Fig. 2). Higher \( M_r \) RNA species that were not detected in wt-infected plants also hybridized to the 5' and 3' CNV probes used and, in some cases, were the main RNA species detected. These higher \( M_r \) species were approximately twice the size of the DI RNA transcript used in the co-inoculations so it is tempting to speculate that these may be dimers of DI RNA. The existence of DI RNA dimers has been recently suggested for CyRSV (Burgyan et al., 1992). These larger dimer-sized RNA species were not observed in blots where synthetic transcripts alone were electrophoresed, showing that they are not migration artefacts due to insufficient denaturation (data not shown). In addition, they do not represent double-stranded forms of the DI RNA since control experiments demonstrated that the electrophoresis conditions were sufficient to denature CNV dsRNA (data not shown). It is possible that the dimer-sized RNA species seen in our experiments are head-to-tail dimers of DI RNA such as those known to be generated during replication of certain satellite RNAs (Roossinck et al., 1992).

This study confirms that our CNV laboratory culture carries DI RNAs. In addition, it demonstrates that DI RNAs can be generated by wt CNV inoculum de novo. The de novo generated DI RNAs have fewer deletions relative to the laboratory culture DI RNAs in region III suggesting that the latter arose from the former. The evolution of smaller DI RNAs from larger precursors
has been suggested in the case of CyRSV (Burgyan et al., 1991). In addition, comparison of DI RNAs generated de novo from wt clones and those generated from CNV 20K mutant infections (Rochon, 1991) show that these DI RNAs are highly similar in sequence and overall structure (data not shown). This suggests that the rapid appearance of DI RNAs in 20K mutant infections is not due to gross peculiarities in the DI RNA species generated which render them better able to accumulate. One clone, DI48, that was recovered was not able to be replicated by helper virus and did not attenuate symptom formation. Although examination of the sequence of this clone suggests that it may have been derived as a cloning artefact, it still shows that there are sequences in region III corresponding to between nucleotides 4380 and 4400 and/or 4587 and 4688 in the CNV sequence which are absolutely required for CNV DI RNA to be efficiently replicated by helper virus. Finally, our data indicate that alternative forms of the DI RNA can be generated in planta during a co-infection. Elucidation of the nature of these forms should provide insights into how CNV DI RNAs replicate and/or evolve.

The authors gratefully acknowledge Dr H. Sanfaçon for critically reading this manuscript. R. L. F. was supported by a scholarship from the MRC of Canada. This work was also partially supported by an operating grant from the Natural Sciences and Engineering Research Council of Canada.

**References**


Short communication


(Received 13 January 1992; Accepted 22 March 1993)