JOURNAL OF VIROLOGY, Dec. 1997, p. 9800–9802 Vol. 71, No. 12
0022-538X/97/$04.00+0


# Involvement of a Subgenomic mRNA in the Generation of a Variable Population of Defective Citrus Tristeza Virus Molecules

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Received 3 February 1997/Accepted 8 August 1997

**The fusion sites between the termini of naturally occurring defective RNAs (D-RNAs) from three citrus tristeza virus (CTV) isolates were sequenced. Seven of eight clones showed a common 3′ terminus of 940 nucleotides (nt) fused to 5′ termini with different sizes. An extra cytosine nucleotide was found at the junction site of the majority of the common 3′ D-RNAs. Molecular analysis of the plus and minus strands of the 0.9-kbp double-stranded RNA, corresponding to the CTV open reading frame 11 subgenomic RNA (sgRNA), showed that they were identical in length and sequence to the common 3′ sequence of the D-RNAs. These results imply that viral sgRNA messengers also function as building components for genomic rearrangement and exchange of complete viral genes.**

Citrus tristeza virus (CTV) is a destructive pathogen causing some of the most important diseases of citrus. The CTV virions contain a large (ca. 20 kb) single positive-stranded genomic RNA (gRNA) (2), encapsidated by two coat proteins of 25 and 27 kDa (8). The sequence of the complete CTV genome was determined for the Florida isolate T36 (10) and for the VT isolate, from Israel (17). The genome of CTV (Fig. 1A) encodes 12 open reading frames (ORFs) potentially coding for at least 17 protein products which include replication-associated proteins, a homolog of the HSP70 proteins, the two coat proteins, and several other products with unknown functions (10). Infected plants contain the large replicative-form (RF) RNA molecule and a nested set of at least nine smaller species, 3′-coterminal subgenomic RNAs (sgRNAs) corresponding to the 3′-terminal ORFs 2 to 11. Each sgRNA is present as a single-stranded RNA (ssRNA) molecule and as a corresponding double-stranded RNA (dsRNA) species (9, 15). Recombination of CTV molecules, resulting in different sizes of defective RNA molecules (D-RNAs) and different junction sites, has been demonstrated to occur in both the VT isolate of CTV (CTV-VT) and other CTV isolates (16, 18). Considerable variation was found in both the presence and the relative abundance of the CTV-VT D-RNAs (18). This paper reports the sequence analyses of several cDNA molecules obtained by reverse transcription (RT)-PCR of D-RNAs from three different CTV isolates. A large proportion of the D-RNAs showed a common 3′ terminus of 940 nt fused to 5′ termini of different sizes. The common 3′ termini were found to be identical in both size and sequence to the 3′-terminal sgRNA of ORF11.

The CTV isolates, VT (17), MorT (3), and GalT (obtained from Y. Oren), were propagated in Alemow (*Citrus macrophylla*) seedlings. Total RNAs were extracted from 100 mg of leaves with Tri-Reagent (Molecular Research Center, Inc.) and used for RT-PCR with primers P1 (5′-CTTCAGTGCTA GCTGTGTTG-3′) (CTV-VT positions 18377 to 18397) and P2 (5′-GCTACGTTCGT-CACGTATAC-3′) (CTV-VT positions 1301 to 1320) for first- and second-strand cDNA synthesis, respectively. The PCR was performed by using the UNO-Thermoblock (Biometra) with the following profile: (i) 94°C for 3 min; (ii) 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min; and (iii) 5 min at 72°C. The amplified DNA was isolated from agarose by using the QIAEX extraction kit (QIAGEN) and cloned into plasmid pUC57/T (MBI-Fermentas). The cloned cDNA fragments were sequenced from both sides by using the M13 forward and reverse primers and the Sequenase version 2.0 kit (U.S. Biochemical Corp.).

Sequence analyses (5) of RT-PCR-cloned CTV-VT D-cDNAs revealed that fusion occurred at a common 3′ site (position 18287) in seven of the eight clones. The fusion sites of the 5′ termini varied between positions 881 and 1925 (Fig. 1B). In three clones, each originating from a different CTV isolate, the 5′-terminus fusion sites were located at position 1824. Two other clones, one each from VT and GalT isolates, showed identical 5′ fusion at position 1925. An additional interesting observation was the presence of an extra C, in comparison with the gRNA sequence, at the junction sites of five of the clones. These included the three clones with identical 5′ termini of 1,824 nt and two clones with 5′ fusion sites at positions 881 and 1751 (Fig. 1C).

The considerable homogeneity of the 3′ termini and close resemblance to the ORF11 subgenomic RNA of 0.9 kb suggested the identity of these molecules. In order to test this possibility, we have isolated the 0.9-kbp dsRNA fraction from total dsRNA extracted from CTV-VT-infected bark tissue (6, 15). The dsRNA was denatured with methylmercury and the [$\gamma$-$^{32}$P]ATP-labeled P2 primer was used for cDNA extension, according to the method described in reference 14. The reaction mix was separated on a 6% polyacrylamide electrophoresis gel and run in parallel with a size marker consisting of the sequencing product of a CTV-VT clone, H72, spanning 1,070 bases from the 3′ end, obtained with primer P2. Figure 2A shows that the 5′ end of the terminal sgRNA from CTV-VT was positioned at nt 18287 of the CTV-VT genome (17), thus coinciding in its length of 940 nt and sequence with the common 3′ termini of the D-RNAs. The primer extension reaction was interrupted by several stops (Fig. 2A, lane 1), which was consistent with the putative presence of a stem-loop structure suggested by computer analysis of the secondary structure for the intergenic region between ORFs 10 and 11 (data not shown). Recent analyses of CTV-T36 have also revealed a 940-nt-long ORF11 sgRNA starting at position 18356 (11).

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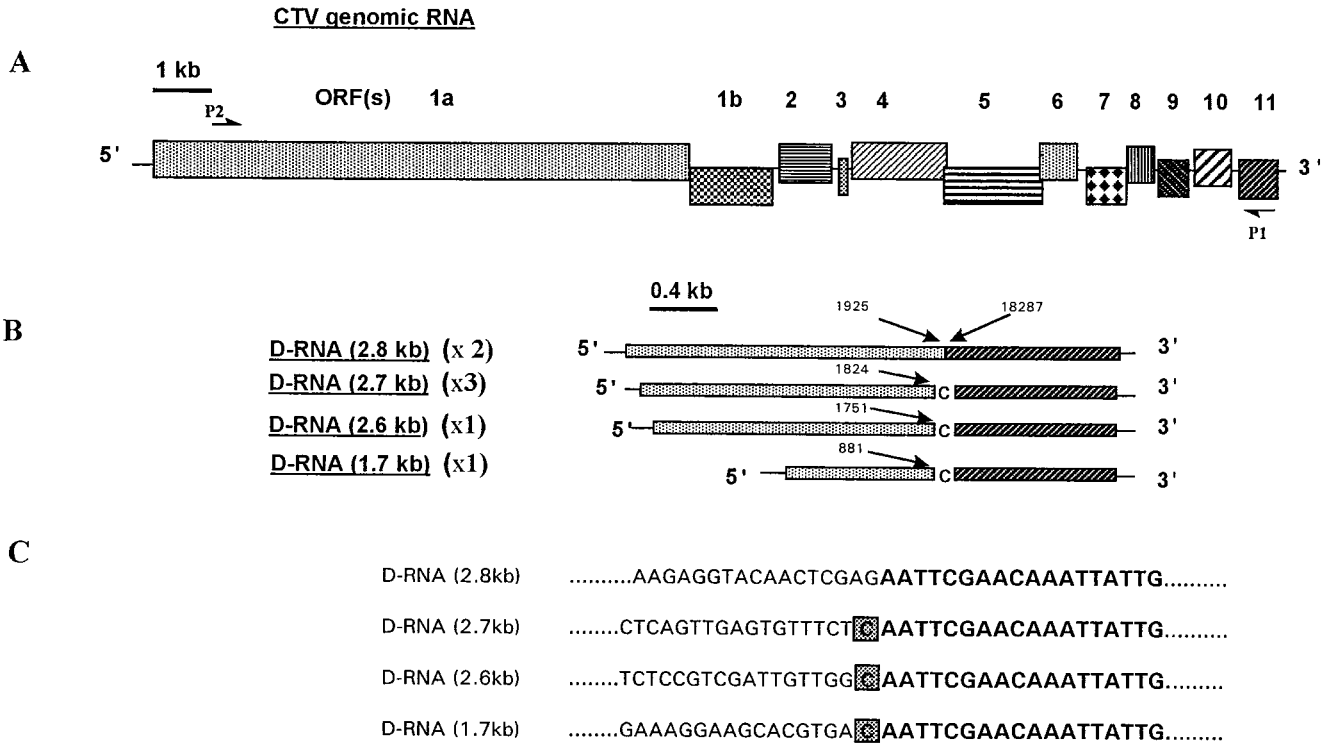


FIG. 1. (A) Schematic map of the 12 ORFs from CTV gRNA. (B) Structures of four types of D-RNA molecules derived from three CTV isolates that showed the common 3′ termini of 940 nt. The number of clones of each type is indicated in parentheses, and coordinates of the junctions are shown according to the sequence of CTV-VT (17). (C) Nucleotide sequences of junctions of four different CTV D-RNAs. The junction region includes a common 3′ terminus starting at the 5′ untranslated region of ORF11 (in bold) fused to different-sized 5′ termini of ORF1a. An extra C nucleotide at the junction site is boxed.

The presence of D-RNAs with common 3′ termini among the dsRNA population of CTV-VT-infected plants was also revealed by Northern hybridization with a $\gamma$-$^{32}$P-labeled 16-mer oligonucleotide probe, P3 (5′-TCGAATTGagaaacac-3′), where the lower- and uppercase letters indicate the complementary sequences, flanking the junction site (underlined) of the 2.7-kb common-terminus D-RNA from the 5′ and 3′ ends, respectively. Figure 2B, lane 2, shows that the probe hybridized only with the molecule of the expected size, which was present in dsRNA preparations from the plant CTV-VT5, harboring a 2.7-kbp D-RNA band, and not with CTV-VT12, which showed D-RNAs of other sizes. The 5′-end CTV-VT probe hybridized to the RF and to 2.7- and 4.5-kb D-RNAs (Fig. 2B, lanes 3 and 4).

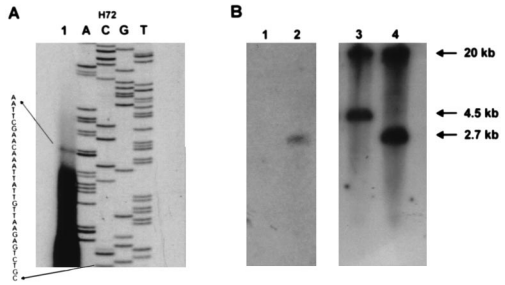


FIG. 2. (A) Primer extension analysis of the 5′ terminus of CTV (VT strain) ORF11 sgRNA, carried out in a 6% polyacrylamide sequencing gel. Lane 1, Runoff reverse transcription of the ca. 0.9-kbp ORF11 sgdsRNA template, using the $\gamma$-$^{32}$P-labeled primer. The sequencing ladder obtained for CTV-VT clone H72 with primer P2 is indicated in the adjacent lanes. The 5′-terminal sequence of the ORF11 dsRNA plus strand is presented on the left; the 5′-to-3′ direction is from top to bottom. (B) Northern blot analysis of dsRNAs from CTV-VT. The dsRNA preparations were separated by electrophoresis on a formamide-formaldehyde denaturing 1.1% agarose gel, transferred to a nylon membrane (Hybond N+; Amersham), and hybridized with a $\gamma$-$^{32}$P-labeled oligonucleotide and cDNA probes. Hybridization was carried out with a $\gamma$-$^{32}$P-labeled 16-mer oligonucleotide probe complementary to the junction site of the common-end D-RNA (lanes 1 and 2) and with an $\alpha$-$^{32}$P-labeled 5′ cDNA probe (from positions 1 to 777 of the CTV genome) (lanes 3 and 4). The dsRNAs were extracted from two different CTV-VT subisolates, VT12 (lanes 1 and 3) and VT5 (lanes 2 and 4). The 20-kb band corresponds to the CTV RF molecule.

Our finding of the extra C at the junction site was suggestive of a recombination event involving the minus strand of ORF11 sgRNA, which has an extra G at the 3′ terminus. The RT-PCR analyses of the polyadenylated minus strands of CTV-VT ORF11 dsRNA molecules from recently and from chronically infected CTV-VT plants did not, however, reveal the presence of an extra G at the 3′ end (Table 1). Similar analyses of the minus-strand molecules from the RF dsRNA revealed an extra G in two of the six clones (Table 1). The 3′ terminus of the minus strand in the RFs of the type strain of CTV (10), as in several other positive-strand RNA viruses, including Semliki Forest virus (21), cucumber mosaic virus and its CARNA-5 satellite (4), and barley stripe mosaic virus equivalents of RNAs 1, 2, and 3 (7), was found to possess an unpaired guanosine (extra G). The absolute dependence of synthesis of the positive strand of CARNA-5 on the presence of the extra G in the negative-strand RNA template was demonstrated by Wu and Kaper (22). The sgRNAs of cucumber mosaic virus (4) and barley stripe mosaic virus (7) did not show an extra G. Contrary to these findings, Karasev et al. (11) recently reported the presence of an extra G in the minus strand of the sgRNA of ORF10 of the T3 strain and its absence from T36. The absence of the extra G at the 3′ termini from CTV-VT ORF11 sgRNA may indicate a temporal presence during cer-

TABLE 1. The 3′-terminal sequences of the minus-strand RNAs from ORF11 dsRNA and from the RF molecules of the VT strain of CTV

| dsRNA | Primer[a] | Terminal sequence | No. of clones |
|---|---|---|---|
| sgORF11 | dT14V | 3′TTAAGCTTGTTT.....5′ | 4 |
| | dT14V | 3′CTAAGCTTGTTT.....5′ | 2 |
| | dT14V | 3′GTAAGCTTGTTT.....5′ | 1 |
| | dT14V | 3′CTTGTTT.....5′ | 1 |
| | P-dT | 3′TTAAGCTTGTTT.....5′ | 6 |
| RF | dT14V | 3′TTAAAGAGTTTA.....5′ | 1 |
| | dT14V | 3′CTAAAGAGTTTA.....5′ | 2 |
| | dT14V | 3′GTTAAAGAGTTTA.....5′ | 1 |
| | dT14V | 3′GAAAAAGAGTTTA.....5′ | 1 |
| | dT14V | 3′TAAAGTTAAAGAGTTTA.....5′ | 1 |

[a] Primers dT14V, where V represents either A, C, or G, and P-dT were used for first-strand cDNA synthesis from the polyadenylated templates as described elsewhere (15).

tain stages of sgRNA replication. Two common-3′-terminus molecules of 2.8-kb D-RNA did not show the extra nucleotide at the junction site (Fig. 1C). This indicates that the ORF11 sgRNA recombination in common-3′-end D-RNAs was not dependent on the presence of the extra nucleotide.

The finding of the extra C residue at the junction site of a substantial number of common-3′-end D-RNAs supports the minus-strand jumping model (19) as the possible mechanism of discontinuous transcription and recombination. Accordingly, the replication process that led to the generation of common-3′-end CTV D-RNA molecules included (i) synthesis of full-length minus-strand RNA on the genomic template; (ii) synthesis of the ORF11 plus-strand sgRNA starting 38 nt upstream from the AUG on the minus-strand template; (iii) synthesis of the minus strand sgRNA, terminated by a nontemplate nucleotide (extra G), followed by template switching toward the 5′ ends of gRNA molecules; and (iiii) synthesis of plus-strand D-RNAs. This model allows the viral sgRNAs to serve both as mRNAs and as building blocks for recombination of defective or genomic viral RNA. It also allows the shuffling of both viral and host sequences, such as is known to occur in closterovirus evolution (1). Genetic recombination and rearrangement of RNA viruses are considered essential for genome repair, to increase genetic variability, and to facilitate evolution (12, 13, 20). Genomic rearrangement in RNA viruses is facilitated by the multipartite nature of a large number of RNA plant viruses. Single-component viruses, except the coronaviruses, which use a unique mechanism for mRNA synthesis (13, 19), were expected to be less likely to undergo rearrangements that involve complete virus genes. To the best of our knowledge, this is the first report to demonstrate the involvement of full-length sgRNA molecules in a recombination process leading to the generation of D-RNA molecules. These results imply that viral sgRNA messengers also function as building components for genomic rearrangement and exchange of complete viral genes.

We thank Y. Ben-Shalom for excellent technical help.

This work was supported in part by grants from the U.S.-Israel Binational Agricultural Research and Development Fund (BARD), the Israeli Academy of Science, and the Israeli Citrus Marketing Board.

## REFERENCES

1. **Agranovsky, A. A.** 1996. Principles of molecular organization, expression and evolution of closteroviruses: over the barriers. Adv. Virus Res. **47:**119–158.
2. **Bar-Joseph, M., and R. F. Lee.** 1990. Citrus tristeza virus, revised description. CMI/AAB description of plant viruses, no. 353. Association of Applied Biologists, Warwick United Kingdom.
3. **Ben-Ze'ev, I. S., M. Bar-Joseph, Y. Nitzan, and R. Marcus.** 1989. A severe citrus tristeza virus isolate causing the collapse of trees on sour orange before virus is detectable throughout the canopy. Ann. Appl. Biol. **114:**293–300.
4. **Colmer, C. W., and J. M. Kaper.** 1985. Double-stranded RNAs of cucumber and its satellite contain an unpaired terminal guanosine. Implications for replication. Virology **145:**249–259.
5. **Devereaux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
6. **Dodds, J. A., and M. Bar-Joseph.** 1983. Double-stranded RNA from plants infected with closteroviruses. Phytopathology **73:**419–423.
7. **Dolja, V. V., and J. G. Atabekov.** 1987. The structure of barley stripe mosaic virus double stranded RNAs. FEBS Lett. **214:**313–316.
8. **Febres, V. J., L. Ashoulin, M. Mawassi, A. Frank, M. Bar-Joseph, K. L. Manjunath, R. F. Lee, and C. L. Niblett.** 1996. The p27 protein is present at one end of citrus tristeza virus particles. Phytopathology **86:**1331–1335.
9. **Hilf, M. E., A. V. Karasev, H. R. Pappu, D. J. Gumpf, C. L. Niblett, and S. M. Garnsey.** 1995. Characterization of citrus tristeza virus subgenomic RNAs in infected tissue. Virology **208:**576–582.
10. **Karasev, A. V., V. P. Boyko, S. Gowda, O. V. Nikolaeva, M. E. Hilf, E. V. Koonin, C. L. Niblett, K. C. Cline, D. J. Gumpf, R. F. Lee, S. M. Garnsey, and W. O. Dawson.** 1994. Complete sequence of the citrus tristeza virus RNA genome. Virology **208:**511–520.
11. **Karasev, A. V., M. E. Hilf, S. M. Garnsey, and W. O. Dawson.** 1997. Transcriptional strategy of closteroviruses: mapping the 5′ termini of the citrus tristeza virus subgenomic RNAs. J. Virol. **71:**6233–6236.
12. **Kirkwood, T. B. L., and C. R. M. Bangham.** 1994. Cycles, chaos, and evolution in virus cultures: a model of defective interfering particles. Proc. Natl. Acad. Sci. USA **91:**8685–8689.
13. **Lai, M. M. C.** 1992. RNA recombination in animal and plant viruses. Microbiol. Rev. **56:**61–79.
14. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, New York, N.Y.
15. **Mawassi, M., R. Gafny, D. Gagliardi, and M. Bar-Joseph.** 1995. Populations of citrus tristeza virus contain smaller-than-full-length particles which encapsidate sub-genomic RNA molecules. J. Gen. Virol. **76:**651–659.
16. **Mawassi, M., A. V. Karasev, E. Mietkiewska, R. Gafny, R. F. Lee, W. O. Dawson, and M. Bar-Joseph.** 1995. Defective RNA molecules associated with the citrus tristeza virus. Virology **208:**383–387.
17. **Mawassi, M., E. Mietkiewska, R. Gofman, G. Yang, and M. Bar-Joseph.** 1996. Unusual sequence relationships between two isolates of citrus tristeza virus. J. Gen. Virol. **77:**2359–2364.
18. **Mawassi, M., E. Mietkiewska, M. E. Hilf, L. Ashoulin, A. V. Karasev, R. Gafny, R. F. Lee, S. M. Garnsey, W. O. Dawson, and M. Bar-Joseph.** 1995. Multiple species of defective RNAs in plants infected with citrus tristeza virus. Virology **214:**264–268.
19. **Sawicki, S. G., and D. L. Sawicki.** 1990. Coronavirus transcription: subgenomic mouse hepatitis virus replicative intermediates function in RNA synthesis. Proc. Natl. Acad. Sci. USA **64:**1050–1056.
20. **Simon, A. E., and J. J. Bujarski.** 1994. RNA-RNA recombination and evolution in virus-infected plants. Annu. Rev. Phytopathol. **32:**337–362.
21. **Wengler, G., and H. J. Gross.** 1979. Replicative form of Semliki Forest virus RNA contains an unpaired guanosine. Nature (London) **282:**754–756.
22. **Wu, G., and J. M. Kaper.** 1994. Requirement of 3′-terminal guanosine in (−) stranded RNA for *in vitro* replication of cucumber mosaic virus satellite RNA by viral RNA-dependent RNA polymerase. J. Mol. Biol. **238:**655–657.