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SHORT COMMUNICATION

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Alemow (*Citrus macrophylla*) and sweet orange (*C. sinensis*) plants infected, respectively, with several Israeli and Florida isolates of the citrus tristeza virus (CTV) were found to contain multiple species of RNA molecules with features similar to defective-interfering RNAs. Northern blot hybridizations of dsRNAs extracted from serial passages of the Israeli VT isolate (CTV-VT) and from different plants infected with a single source of inoculum showed considerable variation both in the presence and in the relative abundance of the defective RNA (D-RNA) bands. The D-RNA molecules were found to be encapsidated in the CTV particles. Sequence analysis of two VT D-RNA molecules of 2.7 and 4.5 kb revealed that they were composed of two regions corresponding to 1818 and 4036 nucleotides from the 5′ and 938 and 442 nucleotides from the 3′ termini of the CTV-VT genomic RNA, respectively. A short (ca. 0.8 kb) nonencapsidated single-stranded positive-sense RNA species was also found in infected plants. This ssRNA, which copurified with dsRNAs, was shown by hybridization to encompass the 5′-terminal part of the CTV genome and might have an extensive secondary structure.

Citrus tristeza virus (CTV) is a destructive pathogen of citrus, causing either quick decline or stem-pitting in susceptible varieties and having a major impact on the citrus industry worldwide (*1*). A number of "strains" or "isolates" have been characterized, differing in symptoms and in biological as well as serological properties. CTV is a phloem-associated, aphid-transmissible virus classified as a closterovirus (*1, 2*). Flexible filamentous particles of CTV contain a single positive-stranded genomic RNA (gRNA) of 19,296 nt (*3*). The CTV genome encodes 12 ORFs potentially coding for at least 17 protein products which include replication-associated proteins, a homolog of the HSP70 proteins, a duplicate of the coat protein, and several other protein products with unknown functions (*3–5*). The 3′-proximal ORF 11 was suggested to encode a 23.5-kDa protein with RNA-binding properties (*6*).

Plants infected with CTV contain a nested set of 3′-coterminal single-stranded (ss) and double-stranded (ds) RNAs: a large RNA molecule corresponding to the CTV replicative form (RF) and at least nine smaller subgenomic RNA (sgRNA) species corresponding to 3′-terminal ORFs 2 to 11 (*7, 8*). Three of the sgRNAs of 3.2, 1.6, and 0.9 kb were identified by *in vitro* translation as templates encoding protein products of ORFs 7 (CP), 10, and 11 (*7, 9*), respectively. Extensive studies of dsRNAs isolated from citrus plants infected with different CTV isolates (*10–13*) demonstrated the presence of numerous RNA fragments which could not be assigned to any of the CTV sgRNAs (*8*). Recently, we have reported that RNA preparations from CTV particles and those of ss- and dsRNAs from CTV-infected plants contain an abundant 2.4-kb RNA species with features suggesting its designation as a defective RNA (D-RNA) molecule (*14*).

In this paper we demonstrate that the presence of multiple D-RNA species in virus-infected plants is a widespread, intrinsic characteristic of CTV. These D-RNAs differ in size and relative abundance within and between different isolates. Sequence analysis of two additional D-RNAs from the Israeli isolate CTV-VT has provided some clues in delineating the CTV genome regions involved in replication and encapsidation. In addition, in CTV-infected plants we found an unusual single-stranded positive-sense 0.8-kb RNA (LMT 5′-RNA) corresponding to the 5′-terminal part of the CTV genome.

The VT strain of CTV (*15*), originally isolated in 1970, was serially passaged at an average of once per year. Passages were done by graft transmission to 6- to 12-month-old Alemow (*Citrus macrophylla*) seedlings grown in a greenhouse with temperatures ranging between 25 and 30°. Double-stranded RNAs were isolated from bark

Sequence data from this article have been deposited with the GenBank Data Library under Accession Nos. U35119 for the 4.5-kb D-RNA molecule and U35120 for the 2.7-kb D-RNA.

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tissue of Alemow citrus by two cycles of CF-11 column chromatography (*10*) followed by an additional separation step using CC41 columns (*16*). The dsRNA preparations were denatured by treatment with methylmercury hydroxide (*17*) and separated by electrophoresis in formamide/formaldehyde denaturing agarose gels prepared in MOPS buffer followed by treatment with 50 m*M* NaOH to enable the efficient transfer of gRNA. The denatured RNAs were transferred to nylon membrane (Hybond N, Amersham) and hybridized to cDNA probes corresponding to the 5′ and 3′ termini of CTV-VT according to Maniatis *et al*. (*18*). The 5′ cDNA probe was labeled with [^{32}P]dCTP by 30 cycles of PCR using the oligonucleotide primers 27196 (5′-CAAATTCACCCGTACCCTCCG-GAAATC-3′) and 18243 (5′-AGCGAAGGATATCATCCA-3′) corresponding to nucleotides 1 to 27 and 686 to 703, respectively, of the previously described 2.4-kb D-RNA molecule (*14*). The 3′ cDNA probe was prepared using the primers 18168 (5′-TGGCGCATATGTTAATGC-3′) and 26225 (5′-ATGGACCTATGTTGGCCCCCCATAG-3′) representing the nucleotides 1811 to 1828 and 2400 to 2424, respectively, of the 2.4-kb D-RNA molecule. Northern blot hybridizations of dsRNAs extracted from single CTV-VT-infected plants from different passages using cDNA probes corresponding to the 3′ and to the 5′ regions of the CTV-VT genome are shown in Fig. 1A. The 3′-terminal probe hybridized with several dsRNA molecules ranging in size from 0.9 to 20 kb (RF) (Fig. 1A, lanes 1–8). The 5′-terminal probe hybridized consistently with the genomic (RF) band (20 kb) and with a ca. 0.8-kb ssRNA molecule, but showed considerable variation in both pattern and intensity with the smaller dsRNA molecules of 2.4 to 4.5 kb (Fig. 1A, lanes 9–16). Among the smaller RNAs, those of 2.4 to 4.5 kb also hybridized with the 3′ probe, suggesting that they share features with the previously described VT-D 2.4-kb RNA molecule (*14*). Both the 5′ and the 3′ cDNA probes failed to react with total ssRNA extracted from uninfected plants (not shown).

To check the stability of these D-RNA molecules and their ability to spread systematically following grafting, the dsRNA preparations from two groups of plants, which were graft-inoculated with a single source of inoculum, were hybridized with the 5′-terminal probe (Fig. 1B). A remarkable variability in the hybridization pattern of the dsRNA molecules of single plants from the same passage was observed.

The existence of multiple species of D-RNA molecules among several populations of CTV strains was also investigated. It was found that molecules similar in size to those in Fig. 1A could be identified among several collections of Israeli CTV strains (not shown). Figure 1C shows the hybridization pattern of the 3′ (lanes 1–4)- and the 5′ (lanes 5–8)-terminal probes with dsRNA preparations from CTV isolates from Florida. Extensive variations were also observed in both the size and the relative abundance of the D-dsRNA molecules from the tested CTV strains. The results indicated that the D-RNA phenomenon is conserved across different CTV strains obtained from different geographic areas.

The presence of the D-RNA molecule among the CTV-VT virions was investigated using ssRNA extracted from purified virus particles (*19*). Northern blot hybridization of the viral ssRNAs showed the presence of several D-RNA molecules ranging in size from 2.4 to 4.5 kb (Fig. 2), indicating that these D-RNA molecules appear among the encapsidated CTV-VT particles.

Using the 5′-terminal region of the CTV genome as a probe for Northern blots we regularly observed a distinct RNA band with a size of ca. 0.8 kb associated both with CTV-VT and with several Florida isolates (Fig. 1). This RNA molecule was detectable in total ssRNA (not shown) and dsRNA (Fig. 1A, lanes 9–16; Fig. 1B; Fig. 1C, lanes 5–8; and Fig. 2, lane 1) extracts prepared from CTV-infected plants, but not in RNA extracted from purified CTV particles (Fig. 2, lane 2). It should be noted that an 0.8-kb RNA molecule was also detected in our previous work (*14*); however, its significance was not recognized. To isolate the 0.8-kb RNA molecules, hereby designated as the low-molecular-weight tristeza 5′ RNA (LMT 5′-RNA), we separated total CTV-VT dsRNA preparation by PAGE, the gel was sliced to several fractions, and the electroeluted RNA was analyzed for the presence of LMT 5′-RNA using the 5′-terminal probe. The LMT 5′-RNA molecules were found to migrate in nondenaturing PAGE gels as a diffuse band with a mobility slightly faster than the 0.9-kb dsRNA segment. Northern blots showed that the LMT 5′-RNA molecules were precipitated by 2 *M* LiCl and hybridized to the antisense and not to the sense probe corresponding to the 5′-terminal 702 nt of the CTV-VT genome (not shown). In addition, the LMT 5′-RNA did not hybridize with cDNA probes covering nt 1056–3970 from the 5′ of the CTV-VT gRNA and with cDNA probes corresponding to 3200 nt of the 3′ end of the CTV-VT genome (data not shown). These results support the notion that the LMT 5′-RNA are positive-sense ssRNA molecules of about 0.8 kb representing the 5′ terminus of the CTV genome. The LMT 5′-RNA molecules were found consistently in plants infected by different CTV isolates (Fig. 1). The origin of the LMT 5′-RNA remains unknown; both generation, via cleavage of gRNA as in the case of the Leishmania RNA virus 1 (*20*) and through a discontinuous replication process, are possible.

For further characterization of the D-RNAs, the 2.7- and 4.5-kb dsRNA segments were isolated by electroelution using Bio-Trap-Membranes (Schleicher and Schuell), treated with DNase and denatured with methylmercury as previously described (*17*). The cDNA synthesis was carried out using a CTV-VT deoxyoligonucleotide primer, 26225. The PCR amplification was performed by using the 26225 and 27196 primers. The PCR reactions included 25 cycles of 1 min denaturation at 94°, 30 sec of annealing at 65°, and 10 min synthesis at 72°. The re-

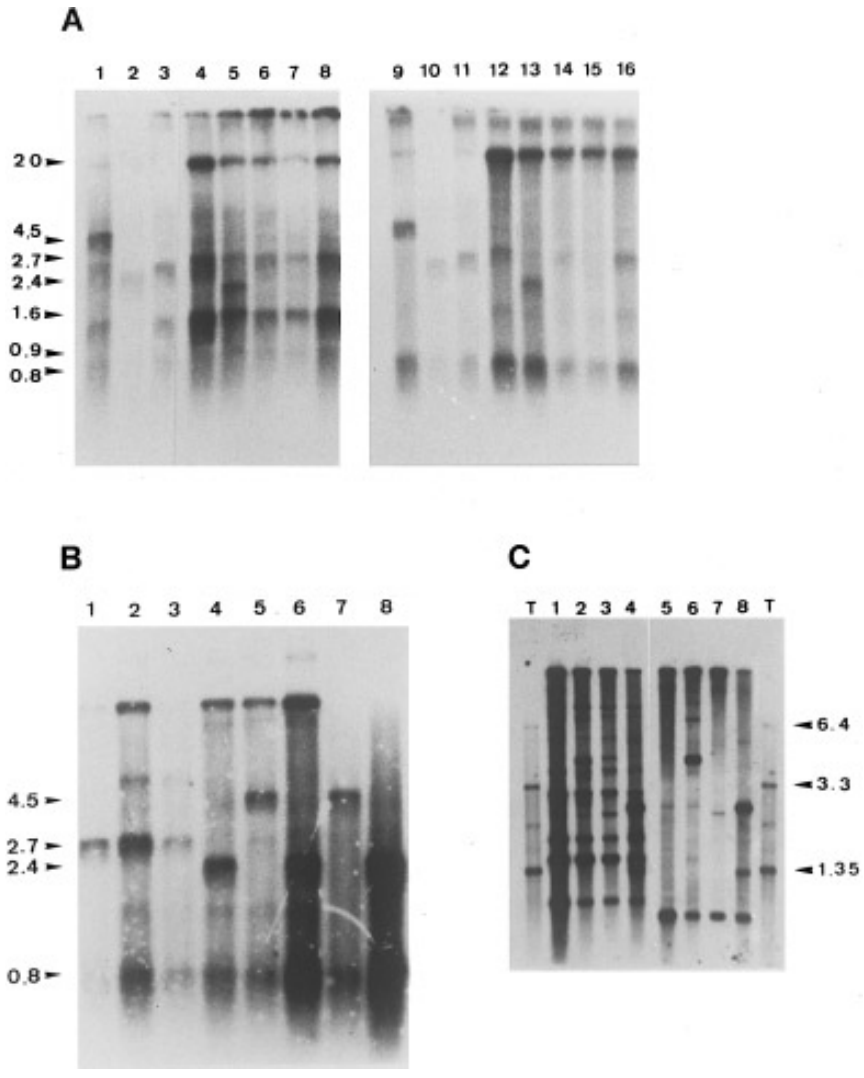

FIG. 1. Northern blot analysis of dsRNAs from the VT strain of citrus tristeza virus (CTV-VT). The dsRNA preparations were separated by electrophoresis on 1.1% formamide/formaldehyde denaturing agarose gel, transferred to nylon membrane (Hybond N, Amersham), and hybridized with ^{32}P-labeled cDNA probes. (A) The hybridizations were carried out with ^{32}P-labeled 3′ cDNA probe (between primers 18168 and 26225) (lanes 1–8) and 5′ cDNA probe (between primers 27196 and 18243) (lanes 9–16). The dsRNAs extracted from single CTV-VT-infected plants from different passages were 12/92 (lanes 1, 9), 7/93 (lanes 2, 10), 4/94 (lanes 3, 11), 10/91 (lanes 4, 12), 9/91 (lanes 5, 13), 9/90 (lanes 6, 14), 2/92 (lanes 7, 15), and 2/91 (lanes 8, 16). (B) Northern blot analysis of CTV-VT dsRNA extractions of eight single plants from the passage of 7/91 (lanes 1–4) and from the passage of 9/90 (lanes 5–8). The hybridization was performed using ^{32}P-labeled 5′ cDNA probe. (C) Northern blot analysis of dsRNAs extracted from four CTV isolates from Florida: T36 (lanes 1, 5); B181, originating from Costa Rica (lanes 2, 6); T64-1 (lanes 3, 7); and T66-1 (lanes 4, 8). The dsRNAs were denatured with DMSO and glyoxal, separated on a 1% agarose gel, blotted to nylon membrane, and hybridized with cDNA probes corresponding to p23.5 coding region (lanes 1–4) and with h15 cDNA clone (*3, 8*) representing the 5′-terminal sequence of CTV-T36 (lanes 5–8). Lanes (T) are TMV RNAs used as size markers. Numbers to the sides are in kb.

sulting DNA fragments were cloned into pUC57/T plasmid (Fermentas) according to the manufacturer's instructions. The cloned cDNA fragments were digested with the restriction enzymes *Hae*III and *Alu*I, subcloned into the *Eco*RV site of pBluescript (KS) vector, and sequenced on both strands, using the T3 and the T7 primers and the Sequenase Version 2.0 kit (USB). In addition, cDNA clones in pUC57/T vector were sequenced using CTV-VT primers. Sequence analyses were carried out using the UWGCG program (*21*). The sequence of the cDNA clone obtained from the 4.5-kb D-RNA molecule of the CTV-VT strain was determined and compared with that of the CTV-VT gRNA (Mawassi *et al.,* unpublished data). The larger part of D-VT 4.5 kb is composed of the 4036 nt corresponding to the 5′ terminus of the CTV-VT gRNA and the remainder represent 442 nt from the 3′ end (Fig. 3). The sequence identities between the 5′ end and the 3′ regions from the VT-D 4.5-kb RNA molecule and the corresponding region of CTV-VT gRNA were 99.6 and 98.6%, respectively. Sequence analysis of the RT–PCR product obtained from the 2.7-kb D-RNA molecule of the CTV-VT strain revealed a second molecule which also

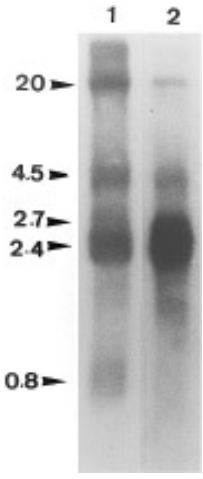

FIG. 2. Northern blot analysis of CTV-VT-specific RNAs. Both the dsRNA (lane 1) and the ssRNA from purified CTV-VT particles (lane 2) were prepared from the same plant tissue and separated by electrophoresis on 1.1% formamide/formaldehyde denaturing agarose gel followed by blotting to nylon membrane (Hybond N, Amersham) and hybridization with ^{32}P-labeled 5′ cDNA probe (between primers 27196 and 18243). Numbers to the sides are in kb. Note that the exposure times for the samples in lanes 1 and 2 were 5 and 15 hr, respectively.

originates from the 3′ and 5′ termini of CTV-VT gRNA. The larger part of 1818 nt is from the 5′ portion and the remaining fragment of 938 nt represents the 3′ terminus (Fig. 3). The 5′-UTR region and the remaining 1720 nt of ORF1 and the 3′-UTR and 662 nt from ORF11 showed 96, 99.6, 100, and 99% identity, respectively, with the corresponding regions in the CTV-VT gRNA.

Sequence analyses of both 2.7- and 4.5-kb D-RNA molecules from two separate graft passages of CTV-VT showed that both were composed of CTV-VT RNA sequences representing the two termini of the helper virus; however, the position of the recombination site was different (Fig. 3). Hybridizations and RT–PCR analyses (not shown) using CTV-VT primers located near the junction sites indicated (I) that both the 2.7- and the 4.5-kb RNA molecules were present in the preparations of ssRNA (not shown) and dsRNA and also among the populations of RNAs extracted from purified CTV particles, (II) that the CTV coat protein assembly site was preserved on the 2.7- and 4.5-kb RNA molecules, and (III) that 442 nt at the 3′ (VT-D 4.5-kb RNA) and 1159 nt at the 5′ (VT-D 2.4-kb RNA) (*14*) were sufficient for replication of D-RNA molecules. Unlike the previously characterized 2.4-kb VT D-RNA molecule which was found to contain a short (probably nonviral) segment of 14 nt at the junction site (*14*), the two newly characterized VT D-RNA molecules were composed solely from the CTV termini. The sites of the junctions were not conserved and the lengths of the 5′ and of the 3′ termini differed considerably among the three VT D-RNA molecules (Fig. 3). The sequences of the three D-VT molecules (4.5, 2.7, and 2.4 kb) showed considerable homology both among themselves and with the cDNA fragment from the corresponding VT-gRNA regions. The high levels of homology between the D-RNAs and their helper virus either indicate a strong selection pressure for sequence conservation or their recent generation from a common CTV-VT RNA template.

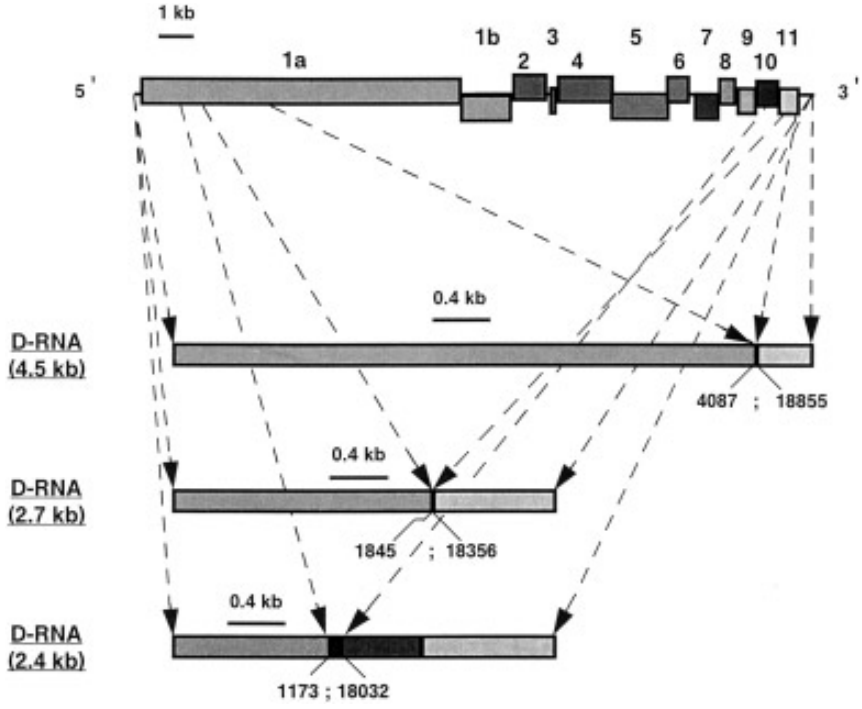

FIG. 3. Diagram of the structure of the D-RNA molecules of 4.5, 2.7, and 2.4 kb from the CTV-VT strain compared with the structure of the CTV-T36 genomic RNA (*3*). The coordinates of the junctions are indicated according to the sequence of CTV-T36 (*3, 5*).

The dsRNA patterns from different passages (Fig. 1A) and from individual plants infected by a given passage (Fig. 1B) varied considerably. The hybridization results also showed the inconsistent presence of certain D-dsRNA among single plants from different or even from the same passage. Inconsistent DI appearance in other systems was found to result either from spontaneous generation due to errors of the replicase action or from the generation of DI molecules by a cyclic synthesis process (*22*).

DI-RNA molecules were associated with a large number of animal viruses (*23*) and several plant virus groups (*24*). The DI-RNAs were usually found to compete with the helper virus. Interference with viral replication results in a reduced level of virus accumulation and a marked attenuation of viral symptoms in infected plants (*24*). This is, however, not a general rule, as in the case of turnip crinkle virus the DI molecules were found to intensify the viral symptoms (*25*). Although a marked reduction in CTV-VT gRNA was observed in some of the chronically infected plants which contained the 2.4 D-RNA (*14*), the role of D-RNAs in CTV infection remains unclear. Dodds *et al.* (*11, 12*) and Moreno *et al.* (*13*) have correlated variations in dsRNA patterns with biologically distinct CTV isolates and with passages of individual isolates through different hosts. However, none of the dsRNA patterns or the currently described VT D-RNA molecules were correlated with a unique symptom in the Alemow host plants. The assignment of biological functions to a particular CTV D-RNA molecule is a difficult process mainly because of the lack of infectious clones, difficulties with mechanical transmission, and long incubation period. The recent development of CTV infection in protoplast systems (J. Navas-Castillo, personal communication) is expected to be a useful tool for elucidating D-RNA functions at a cellular level.

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