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SHORT COMMUNICATION

Defective RNA Molecules Associated with Citrus Tristeza Virus

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Preparations of single-stranded (ss) RNA extracted from particles of the Israeli VT strain of citrus tristeza virus (CTV-VT), and ss- and double-stranded (ds) RNA preparations extracted from infected Alemow (*Citrus macrophylla*) plants, contained a population of molecules with features that suggest that they are defective RNAs. The prototype of 2424 nt was cloned and sequenced and was found to be composed of two genomic regions corresponding to the 5′ (1151 nt) and the 3′ (1259 nt) termini of the genomic CTV-RNA, with two perfect direct repeats of eight nucleotides of unknown origin at the junction site. Northern hybridization analysis demonstrated that this 2.4-kb defective RNA is an abundant species among the other CTV-specific ss- and ds-RNAs in infected plants. The 2.4-kb RNA was found encapsidated by the CTV coat protein indicating that the CTV origin of assembly is located close to the 5′ or 3′ terminus. This is the first defective RNA to be reported for a member of the closterovirus group.

The particles of citrus tristeza virus (CTV), a member of the closterovirus group, are 2,000 nm long and 10–12 nm wide (*1*). The CTV virion contains a large single positive-stranded genomic RNA (gRNA) with an estimated size of ca. 20 kb (*2*) and a single coat protein (CP) of 25 kDa (*1, 3*). Plants infected with CTV contain a large dsRNA molecule with an estimated size of 19.5 kbp, corresponding to the replicative form (RF) and several smaller dsRNAs (*4*). Recently, the 19,296-nt sequence of the complete CTV genome was determined for the Florida isolate T36 (*5*). The CTV genome encodes 12 open reading frames (ORFs) potentially coding for at least 17 protein products (*5–7*). Sequence analysis of 2,540 nt downstream of the CP gene of the VT strain from Israel observed the presence of four ORFs which showed variable but usually close levels of homology with the corresponding ORFs of CTV-T36 from Florida (*8*). During routine studies of sequence variations among different CTV isolates we revealed an unexpected deviation from the T36-like sequence in some CTV-VT-specific clones. Northern-blot analysis of subcloned fragments showed differential hybridization with the population of genomic and subgenomic RNAs from CTV-VT particles and infected plants (*8*).

In this paper we report that ssRNA preparations from CTV particles and from CTV-infected plants and preparations of CTV-VT-dsRNAs contain an abundant 2,424-nt RNA species. This RNA molecule is composed of two fragments derived from the 5′ and the 3′ terminal regions of the CTV genome. These features suggest its designation as a defective RNA.

The aphid-transmitted seedling yellows CTV strain VT (*9*), was propagated by graft infection of 6- to 12-months-old Alemow (*Citrus macrophylla*) seedlings. The CTV dsRNAs were isolated from bark tissue by two cycles of CF-11 cellulose column chromatography (*10*), followed by an additional separation step on CC41 columns (*11*), and analyzed by polyacrylamide gel electrophoresis and ethidium bromide staining (*4*). The dsRNA pattern from the CTV-VT-infected plants showed a slow migrating, major dsRNA segment of ca. 20 kbp, which is presumably the RF of the CTV genome ssRNA, and other major dsRNA segments of ca. 2.4, 1.6, and 0.9 kbp (Fig. 1). The latter two molecules were recently analyzed by *in vitro* translation and sequencing and identified as a nested set of subgenomic RNAs coding for p20 (ORF 10) and p23.5 (ORF 11), respectively (*8*). The origin of the 2.4-kbp dsRNA (Fig. 1), which was found to be an inefficient template for *in vitro* translation, was previously unknown.

To investigate the nature of the 2.4-kbp dsRNA segment, it was isolated by electroelution using Bio-Trap-Membrane, denatured with methylmercury hydroxide, and 3′-polyadenylated according to previously described procedure (*12*). The cDNA synthesis was carried out using a chimeric deoxyoligonucleotide primer consisting of a polylinker and of an oligo-dT (designated P-dT) (Table

Sequence data from this article have been deposited with the GenBank Data Library under Accession No. U20531.

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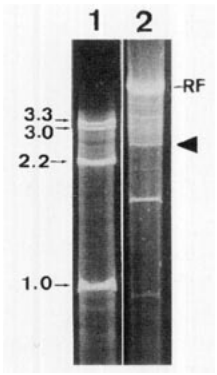

FIG. 1. Ethidium bromide-stained acrylamide gel of total dsRNAs extracted from Alemow (*Citrus macrophylla*) plants infected with the VT strain of citrus tristeza virus (CTV-VT) (Lane 2). Lane 1 shows cucumber mosaic virus dsRNAs from *Pachistachis coccinae* which were used as molecular size markers; arrow on the right points to the location of the 2.4-kbp defective RNA (D-RNA) molecule.

1). After the RNA hydrolysis with 0.1 *M* NaOH, the cDNA molecules were polyadenylated at their 3′ ends, using deoxyadenosine terminal transferase, and the PCR amplification was continued according to (*12*). The PCR fragments were digested with *Bam*HI (this site was included in the P-dT primer) and cloned into *Bam*HI-digested pBluescript KS (Stratagene) (*13*). Two different clones were obtained and found to hybridize with the CTV gRNA and with a 2.4-kbp dsRNA fragment (not shown). Based on sequence information derived from these clones, other specific primers were synthesized and used for priming, reverse transcription, and PCR (RT-PCR) of CTV-VT dsRNA templates to generate several other clones covering the entire 2.4-kbp dsRNA. To determine the 5′ sequence of CTV-VT 2.4-kb RNA, the respective dsRNA was isolated, polyadenylated, and used for cDNA synthesis using P-dT primer. PCR amplification with P-dT and a specific primer 5′ 18243 (nt 686 to 703, Table 1) enabled us to amplify, clone, and sequence the exact 5′ terminal 703-nt sequence of the 2.4-kbp dsRNA molecule. The same approach was used to amplify the exact 5′ terminus of the genome-size CTV-VT dsRNA; both genome-size and 2.4-kbp dsRNA were found to have identical 5′ termini (data not shown). We sequenced both strands of each CTV-specific clone at least twice.

The sequence of the 2.4-kb RNA is presented in Fig. 2. Surprisingly, after comparison of this 2.4-kb RNA with the complete sequence of the CTV genome, now available for Florida T36 isolate (5), it was found to be composed of two fragments, 5′ 1151 nt and 3′ 1259 nt corresponding to 5′ and 3′ termini of the CTV genome (Figs. 2 and 3). The sequence of the 2.4-kb RNA derived from the corresponding dsRNA molecule apparently has an extra C at the 5′ and an extra U at the 3′ termini comparing to the Florida T36 genome (*5;* Fig. 2). We suggest that this 5′ terminal extra C is a complement of a nontemplate extra G at the 3′ terminus of the minus strand in the 2.4-kbp dsRNA. The same nontemplate extra nucleotides were revealed in the full-length genomic dsRNA of the CTV T36 (*5*). The 5′- and 3′-derived portions of the 2.4-kb RNA are separated by a perfect 8-nt direct repeat of unknown, perhaps nontemplate origin (Fig. 2), as sequencing of the gRNA of the VT isolate in the regions adjacent to the 2.4-kbp RNA junction site did not reveal such repeats (not shown). The sequence of this CTV-VT-specific 2.4-kb RNA apparently deviates from the sequence of CTV T36, which seems to be expected, provided two isolates were maintained in geographically widely separated regions. Notably, both "halves" of the 2.4-kb RNA have remarkably different degrees of sequence conservation compared to the T36 CTV isolate, 91% identity for the 3′ fragment and 72% identity for the 5′ fragment, respectively. Previous analysis of the sequence located downstream of the coat protein gene of VT and T36 CTV isolates showed a far less dramatic variation in sequence similarities ranging from 97% for the 3′ noncoding region to 87% for ORF 11 (p23.5) (*8*).

In order to examine whether the 2.4-kb RNA was indeed an abundant species among CTV-VT-specific RNAs we performed a Northern blot analysis of total RNA isolated from Alemow plants (*16*) and from ssRNA extracted from partially purified CTV particles (*17*) and from CTV dsRNA preparations (*4*). The RNAs were denatured with methylmercury hydroxide (*12*) and separated by electrophoresis in formamide/formaldehyde denaturating agarose gels prepared in MOPS buffer. The gels were treated with 50 m*M* NaOH to enable the efficient transfer of gRNA, blotted to nylon membrane (Hybond N, Amersham), and hybridized according to Maniatis *et al.* (*13*). The ^{32}P-labeled cDNA probes specific to the 5′ and the 3′ sequences of the genomic CTV were prepared using 30 cycles of PCR on the respective plasmid templates.

The 3′ cDNA probe hybridized with several dsRNA molecules ranging in size between 0.9 to 20 kbp (RF), including the 2.4-kbp dsRNA segment (Fig. 4, lanes 1 and 2). The 5′ cDNA probe hybridized with bands corresponding to the RF (20 kbp) and the 2.4-kbp dsRNA segment (Fig. 4, lanes 3 and 4). This 5′ cDNA probe showed a strong hybridization signal corresponding to a 2.4-kb ssRNA extracted from CTV-VT-infected plants (Fig. 4, Lane 5) and to a similarly sized molecule from partially purified CTV-VT particles (Fig. 4, Lane 7). The hybridization signals with the gRNA (20 kb) in both preparations were weaker or nondetectable in some experiments which might reflect a lower amount of gRNA in these plants. The Northern blot analyses of different ss- and ds-RNA preparations isolated from CTV-VT virions and from the infected plants provided evidence that this unusual RNA species is probably a recombinant molecule, which was generated naturally and was not derived during the reverse transcriptase or polymerase chain reactions. The nucleotide sequence alignments indicate that the 2.4-kb RNA is a chimeric molecule that contains se-

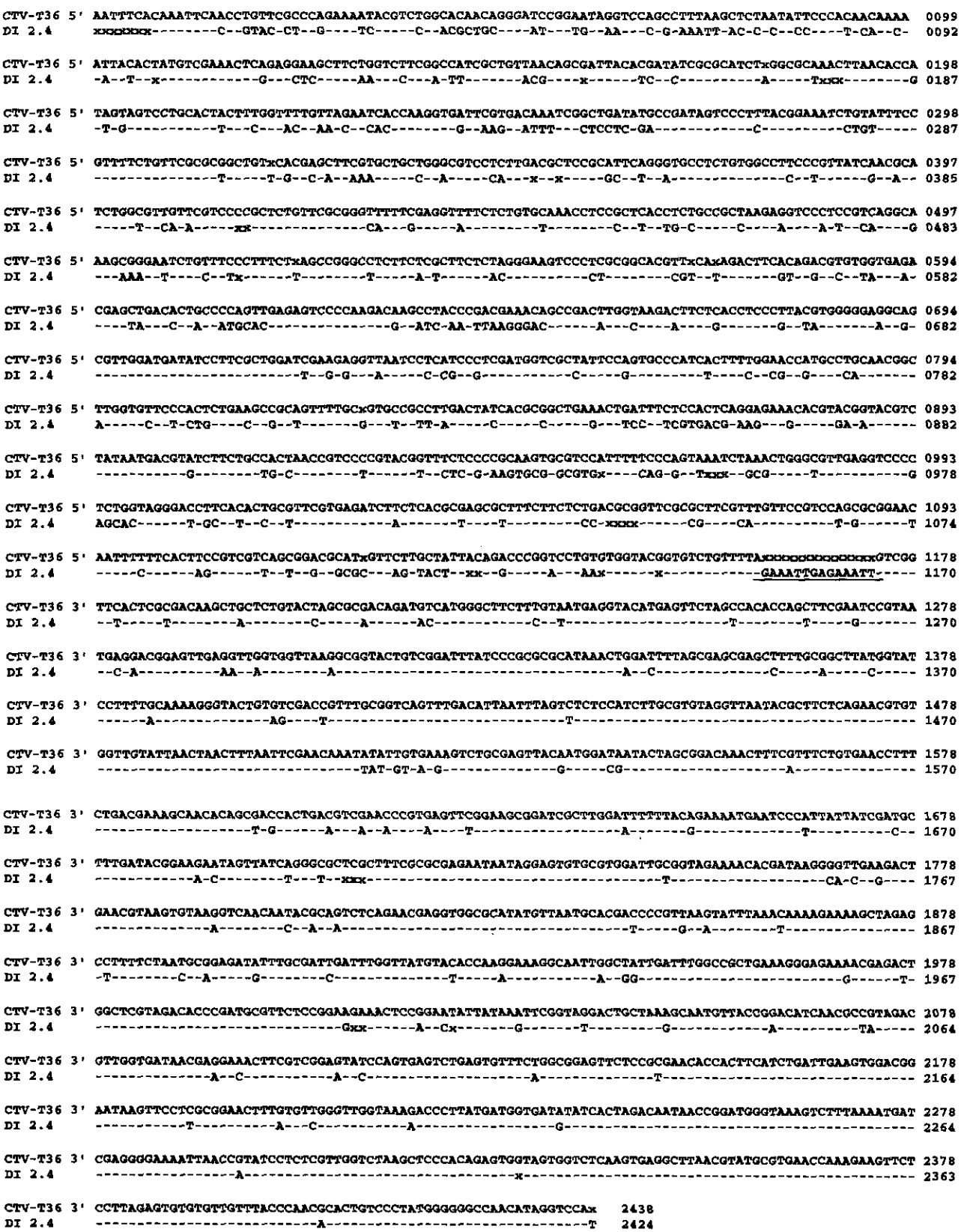

FIG. 2. Nucleotide sequence alignment, presented as a DNA, of the 2.4-kb D-RNA and the respective regions of the CTV-T36 genome in the vicinity of the junction between the 5′ and the 3′ portions of the 2.4-kb D-RNA. Residues in the D-RNA sequence that are identical to the T36 are indicated by dashes (−); deletions are indicated by (X). Two direct repeats of eight nucleotides are underlined. Sequence analysis was carried out with the Wisconsin Reference (UWGCG) program (*14*). Nucleotide sequence alignment was generated using the MACAW program (*15*).

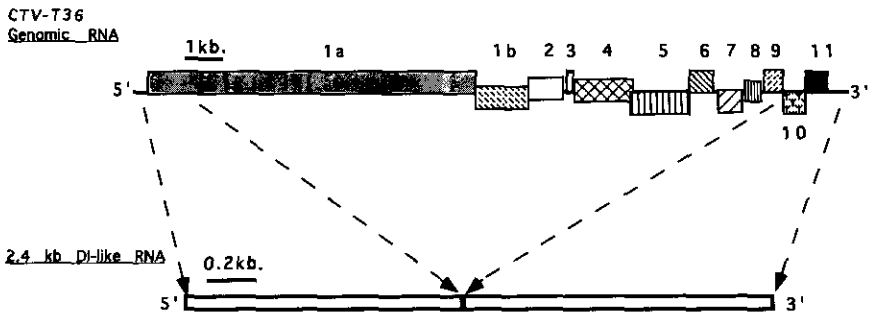

FIG. 3. Diagram of the structure of the 2.4-kb D-RNA, from CTV-VT compared with the structure of the CTV-T36 genomic RNA, the numbers along the CTV-T36 gRNA indicate the ORFs as described in (*5*).

quences derived from the 3′ and the 5′ termini of the gRNA (Figs. 2 and 3).

The abundance of the 2.4-kb RNA among the RNAs extracted from purified CTV particles (Fig. 4) indicates that the CTV coat protein assembly site was preserved on the 2.4-kb RNA molecule and is probably located at the 3′ of the CTV gRNA, as suggested recently (*8*). A distinguishing feature of this CTV-RNA is that it is clearly defective as it lacks almost 17 kb (ca. 87%) of the CTV genome. Consequently, most of the replicase-associated genes, and 8 of the 10 3′-proximal genes which are expressed via formation of subgenomic RNAs (*18*), are deleted. The only ORF that seems to be completely intact is the ORF 11 encoding a 23.5-kDa putative RNA-binding protein (*5, 19;* see Fig. 3). It would be interesting to know whether the 2.4-kb defective RNA molecule still retains the respective subgenomic promoter.

This 2.4-kb defective molecule probably had lost most of the essential coding sequences required for independent replication. These features of the 2.4 kb suggest that it is similar to defective-interfering (DI)-RNAs and that it is the first such RNA to be reported for a member of the closterovirus group. The sequence analysis of the 2.4-kb D-RNA molecule indicates that a recombination event was probably responsible for the generation of this chimeric molecule. The 14 nt of an unknown origin, located in the junction between 5′ and 3′ fragments (Fig. 2), could have been remnants of a hypothetical stem-and-loop structure which facilitated the recombination event(s). It would be interesting to investigate the molecular basis for the recombination leading to the insertion of the two direct repeats at the CTV–D-RNA junction site and to the generation of this unusual molecule.

DI RNAs have been found associated with a wide variety of animal viruses (*20*) and with different groups of plant positive-strand RNA viruses, i.e., tombusviruses (*21, 22*), carmoviruses (*23*), potexviruses (*24*), and some others (see *25* for a recent review). The DI-RNAs are usually found to compete with the nondefective virus genome for the components of replicase. This interference with viral replication results in a reduced level of virus accumulation and a marked attenuation of viral symptoms in infected plants (*25*). This, however, is not a general rule as in the case of turnip crinkle virus where the DI molecules were found to intensify the viral symptoms (*23*). Studies are in progress in order to establish if the 2.4-kb D-RNA molecules interfere with CTV-gRNA replication and to determine their possible involvement in the symptomatology of CTV-VT-infected plants.

TABLE 1

The DNA Sequences of the Synthetic Oligonucleotides Which Were Used for RT-PCR and Their Binding Sites in the 2.4-kb DI-like RNA Molecule

Primer code	Sequence (5′ to 3′)	Polarity	Binding site
P-dT	GCCGCGGATCCAAGC$(T)_{15}$	/	/
18168	TGGCGCATATGTTAATGC	+	1811–1828
18243	AGCGAAGGATATCATCCA	–	686–703
26225	ATGGACCTATGTTGGCCCCCCATAG	–	2400–2424
27196	CAAATTCACCCGTACCCTCCGGAAATC	+	1–27

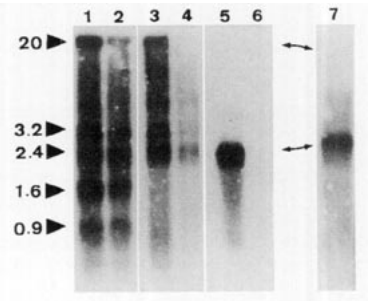

FIG. 4. Northern blot analysis of CTV-VT-specific RNAs. The dsRNAs and the ssRNA preparations were separated by electrophoresis on 1.1% formamide/formaldehyde denaturating agarose gel, transferred to nylon membranes (Hybond N, Amersham), and hybridized with ^{32}P-labeled cDNA probes complementary to the 3′-terminal region (between primers 26225 and 18168, nucleotides 1811 to 2424; see legend to Fig. 2 and Table 1) (lanes 1 and 2) and to the 5′ terminal region (between primers 18243 and 27196; nucleotides 1 to 703) (lanes 3 to 7). Lanes 1 and 2, and corresponding lanes 3 and 4, respectively, represent two different extractions of dsRNAs prepared from CTV-VT-infected plants. Lanes 5 and 6 are total ssRNAs prepared from CTV-VT-infected and healthy citrus plants, respectively. Lane 7 is the RNA extracted from purified CTV-VT particles. The numbers on the left indicate the sizes in kb.

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