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A cDNA clone from a defective RNA of citrus tristeza virus is infective in the presence of the helper virus

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A naturally occurring defective RNA of 2379 nt (D2.3) from the VT strain of citrus tristeza closterovirus (CTV) was cloned and sequenced. The D2.3 RNA is a fusion of two regions of 1521 and 858 nt from the 5′ and 3′ ends of the CTV genome, respectively. A cDNA clone of D2.3 RNA was tagged by the insertion of a 0·47 kb chimeric DNA fragment and the recombinant cDNA was inserted downstream of the cauliflower mosaic virus 35S promoter. The resulting construct was bombarded into CTV-infected tissue, which was then grafted onto virus-free plants. The presence of recombinant RNA in systemically infected leaves was demonstrated by RT–PCR. Sequencing the RT–PCR products synthesized from double-stranded RNA confirmed the presence of the chimeric segment used for tagging. This is the first report of an infectious cDNA molecule derived from CTV D-RNA.

Citrus tristeza virus (CTV), a member of the closterovirus group (Agranovsky, 1996; Bar-Joseph *et al.*, 1995; Dolja *et al.*, 1994; Coffin & Coutts, 1993), is one of the most destructive pathogens of citrus, causing a range of diseases such as quick decline and stem-pitting in susceptible cultivars. A large number of CTV strains or isolates has been characterized differing in symptomatology and serological properties (Bar-Joseph *et al.*, 1989). The CTV virions are heterodimers and, similarly to beet yellows virus (Agranovsky *et al.*, 1995), consist of two segments; the larger, ca. 2000 nm, is encapsidated by the p25 coat protein (CP) and the shorter, ca. 75–85 nm, is encapsidated by p27, a diverged coat protein (CPd) (Febres *et al.*, 1996). The CTV particles contain a single component positive-stranded genomic RNA, of 19296 nt for the T-36 strain from Florida (Karasev *et al.*, 1995) and of 19226 nt for the Israeli strain VT (Mawassi *et al.*, 1996). The genomes of these strains showed considerable sequence deviation within the 5′ half, but were found to have similar organization and to encompass 12 ORFs, which potentially code for at least 17 protein products, including the replication associated proteins, a homologue of the HSP70 proteins and the two coat proteins (Karasev *et al.*, 1995; Mawassi *et al.*, 1996). In addition to the genomic and subgenomic RNAs (Hilf *et al.*, 1995; Mawassi *et al.*, 1995*a*) plants infected with CTV contain multiple species of defective RNAs (D-RNAs). These D-RNAs consist of the 5′- and 3′-terminal segments of the genomic RNA, with extensive internal deletions. Hybridization analysis showed that the D-RNAs are abundant in infected plants and occur in most CTV isolates (Mawassi *et al.*, 1995*b*, *c*). Two of the characterized CTV VT D-RNAs of 2·7 and 4·5 kb were found to represent different fusions of the genomic termini, whereas the 2·4 kb D-RNA molecule contained a short nonviral segment of 14 nt at the junction site (Mawassi *et al.*, 1995*b*, *c*). The 2·4 kb dsRNA band, previously shown to contain the 2424 nt D-RNA (Mawassi *et al.*, 1995*b*), was later found to contain a second molecule, designated D2.3-RNA, of 2379 nt, structurally representing a fusion of 1521 nt and 858 nt from the 5′ and 3′ ends, respectively, of CTV-VT. The present paper reports the cloning and sequence characterization of a new D-2.3 RNA and its use for the construction of an infectious cDNA clone.

CTV strains VT (Mawassi *et al.*, 1996) and GalT (obtained from Mr Yair Oren, Department of Citriculture, Ministry of Agriculture, Israel), were propagated in Alemow (*Citrus macrophylla*) seedlings. Total single-stranded RNA (ssRNA) was extracted from the leaves and young bark of infected plants using Tri-Reagent (Molecular Research Center Inc.). Double-stranded (ds) RNA was isolated from bark tissue according to Dodds & Bar-Joseph (1983). The dsRNA molecules were separated by PAGE. The 2·3 kb dsRNA segments were isolated by electroelution using Bio-Trap membranes (Schleicher and Schuell), or by elution into 0·5 × TBE buffer (Maniatis *et al.*, 1982) followed by phenol and chloroform extraction and ethanol precipitation. Fig. 1(A) shows the strategy for obtaining the full-length cDNA clone of D-2.3 RNA. The 5′ end fragment of 709 nt was obtained by cDNA synthesis using primers P6 and P1 (to incorporate an *Eco*RV site) and digested with *Eco*RV. The central 1718 nt fragment of D-2.3 RNA was amplified by RT–PCR with

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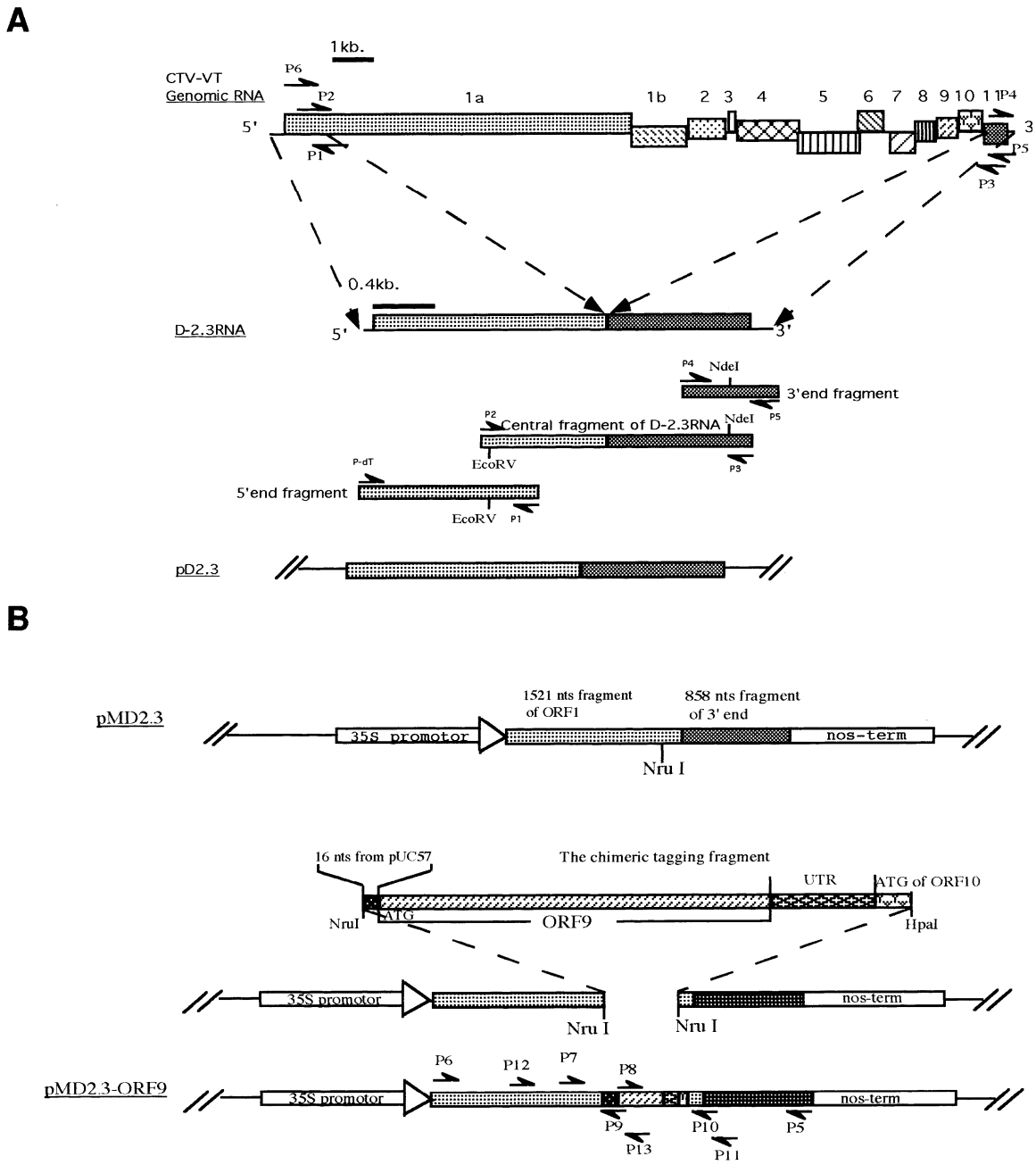

Fig. 1. (A) Cloning strategy for the full-length cDNA of the D2.3 RNA of the VT strain of CTV. Primers for RT–PCR synthesis of three cDNA fragments representing the two termini and the central part of the D2.3 RNA are shown in Table 1. (B) Schematic representation of the strategy for constructing pMD2.3-ORF9. The recombinant cDNA was inserted between the cauliflower mosaic virus 35S promoter and Nos terminator. Arrows marked on the upper side and lower side of the construct diagrams indicate primers complementary to the minus-strand and plus-strand respectively.

primers P3 and P2 (Table 1) and digested with *Eco*RV and *Nde*I. The 3′ end was polyadenylated and amplified by PCR with primers P1 and P-dT (to incorporate an *Nde*I site) (Table 1) and digested with *Nde*I. The three PCR products were ligated to form clone pD2.3 (Fig. 1A).

Clone pD2.3 was sequenced on both strands and the data were deposited with the GenBank database (accession no. U489765). Sequence comparisons with CTV-VT genomic RNA (Mawassi *et al.*, 1996) showed that the D2.3 RNA consists of 2379 nt with 1521 nt from the 5′ end and 858 nt from the 3′ end of the CTV-VT genome (Fig. 1A). The sequence identities between the 5′ and 3′ end regions of CTV-VT and D2.3 RNA, determined with the BESTFIT program (GCG), were 99·4% and 99·3% respectively.

The entire 2·3 kb pD2.3 cDNA was amplified with PCR Vent polymerase (New England Biolabs) using primers P5 and

Table 1. DNA sequences of the synthetic oligonucleotide primers used for RT–PCR

Primer code	Sequence (5′ to 3′)	Polarity	Position in the complete CTV-VT sequence (Mawassi *et al*., 1996)
P-dT	GCCGCGGATCCAAGC(T)$_{15}$		$(A)_n$ tail
P1	AGCGAAGGATATCATCCA	−	686–703
P2*	TTACCCGGGCTTCTTCACTCTTCTCACGGGAAGTCC	+	512–547
P3	TTTCCCCTCGATCATTTTAAAGACTTTACCCATCC	−	19043–19077
P4	TGGCGCATATGTTAATGC	+	18611–18628
P5	ATGGACCTATGTTGGCCCCCCATAG	−	19203–19227
P6	AATTTCTCAAATTCACCCGTACCC TCC	+	1–27
P7	GCTACGTTCGTCACGTATAC	+	1301–1320
P8*	ATGCATATGAGCATTCGACGTGT	+	17260–17276
P9	CATAATCTAGATGCATTCGCGAAG	−	pUC polylinker
P10	CTTCAGTGCTAGCTGTGTTG	−	18378–18397
P11	CAGATAACTAGTTTTCCGTA	−	18473–18492
P12	TTCGGCGCCGTGCCCACGAC	+	766–785
P13	GCGTTGCAACCTTCGCTA	−	17334–17351

* The underlined bases were added or changed, to introduce restriction sites.

P6 (Table 1). The resulting fragment was ligated between the cauliflower mosaic virus 35S promoter and Nos terminator to yield the clone pMD2.3 (Fig. 1B). The strategy for tagging pMD2.3 is shown in Fig. 1(B). A 1360 nt cDNA from the CTV-VT genome (positions 17260–18620) was cloned into pUC57/T (MBI Fermentas) (not shown). The clone was cleaved with blunt-end restriction endonucleases *Nru*I and *Hpa*I to yield a 470 bp fragment (consisting of a 16 nt segment from the vector, ORF9, the region between ORF9 and ORF10 and 21 bp from the 5′ end of ORF10) which was inserted into the *Nru*I site of pMD2.3 to generate pMD2.3-ORF9 (Fig. 1B). Aliquots of 1 μg DNA of pMD2.3-ORF9 were applied by hand-gun bombardment (Gal-On *et al*., 1997) to the cambial faces of CTV-infected bark patches, which were then graft-inoculated on virus-free plants. Four to five weeks post-inoculation, typical vein clearing symptoms of CTV were observed on the new leaves.

The recombinant D-RNA molecules were detected in systemically infected leaves by RT–PCR analyses of total ssRNA or dsRNA templates using two sets of primers: (i) P7 (Table 1) upstream to the 5′ junction site of the tagging fragment, and P10 downstream from the 3′ junction site; (ii) P8 downstream of the 5′ junction site of the tagging insert of the ORF9 and P11 downstream from the 3′ junction site in ORF11. The PCR reactions were carried out using a UNO-Thermoblock (Biometra) with the following profile: (1) 94 °C for 3 min; (2) 30 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min; (3) 72 °C for 5 min. The presence of CTV particles encapsidating D2.3-ORF9 RNA was assayed by immuno-capture/RT–PCR of systemically infected leaves, using the protocol described by Nolasco *et al*. (1993). The cDNA fragments were cloned and sequenced from both strands and analysed with the UWGCG program (Devereux *et al*., 1984). The DNAs obtained by RT–PCR amplification of virions trapped by immuno-capture, using the primers P9 (complementary to the pUC57 residue) and P12 (from the ORF1 residue) (Table 1) were transferred to Hybond N+ membranes (Amersham) and hybridized with a 353 nt cDNA probe from ORF9.

RT-PCR analyses showed the presence of recombinant ds D-RNA molecules in systemically leaves collected 2·5 to 7 months after infection. The sequence of recombinant D2.3-ORF9 cDNA was identical to that of the cloned pMD2.3-ORF9 (Fig. 2A, B). Additional evidence for the replication of D2.3-ORF9 RNA in co-infected plant tissue was obtained by RT–PCR of dsRNAs with primers P9 and P12 (Table 1) (not shown). Furthermore, the same result was obtained in the immuno-capture/RT–PCR of RNA of CTV particles from secondary co-infected plants using the same primers (P9 and P12). Southern hybridization of the amplification product from the trapped virions gave a clear signal with a cDNA probe from ORF9 (not shown). Attempts to locate replicating recombinant ds D2.3-ORF9 RNA, 1 to 7 months post-inoculation, by Northern hybridization using the tagging fragment as a probe, did not give conclusive results.

Fusions of the genomic termini were also observed for D-RNAs of 2·7 and 4·5 kb (Mawassi *et al*., 1995*c*), and the 2·4 kb D-RNA molecule (Mawassi *et al*., 1995*b*). The lengths of the 5′ and 3′ termini, and as a consequence the sites of the junctions between the termini, varied considerably among the VT D-RNAs. Interestingly, the D-2.3 RNA was the first CTV-VT D-RNA to show a putative single contiguous ORF. Con-

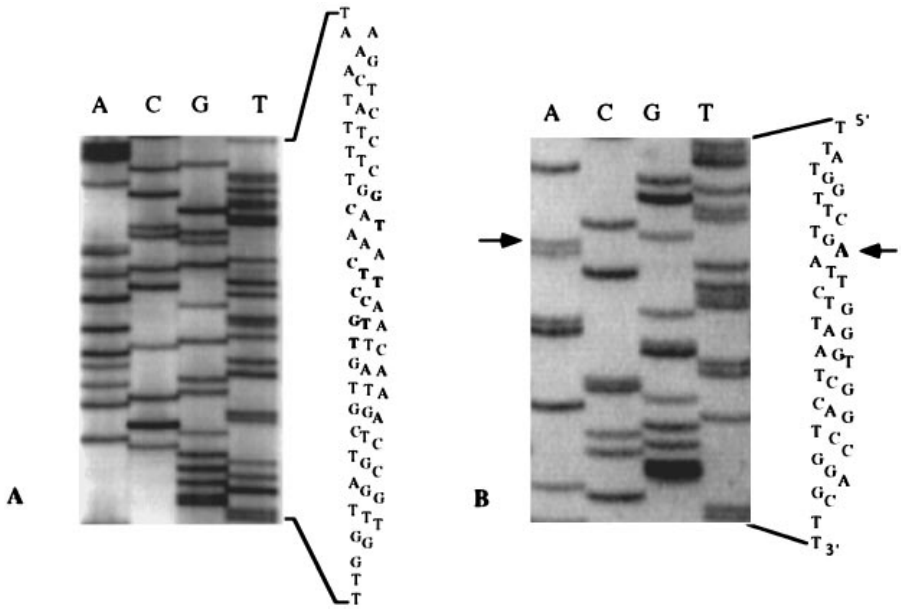

Fig. 2. Nucleotide sequences at the junction sites of recombinant D2.3-ORF9 dsRNA molecules from a plant co-infected with CTV and pMD2.3-ORF9. (A) The 5′ end junction between a part of ORF1, the 16 nt from pUC57 (in bold) and part of ORF9. (B) The 3′ end junction between part of ORF1 and a short sequence from ORF10 which was included in the tagging fragment. The arrows indicate the junction site.

tiguous ORFs have been previously reported in D-RNAs from other elongated plant viruses but their significance remains unknown (White *et al*., 1992).

The finding of two different D-RNAs within the 2·4 kb dsRNA band indicates the variable structure of the naturally occurring D-RNAs in CTV-infected plants. Indeed, sequencing RT-PCR products from a single plant infected with CTV-GalT, using primers corresponding to ORF11 and to ORF1 sequences, showed the presence of at least four different D-RNAs (Guang Yang and others, in preparation).

The instability of CTV-VT D-RNA molecules in different plants, including those graft-inoculated from a single budwood source plant (Mawassi *et al*., 1995*c*), necessitated the tagging of the cDNA molecules used for infection. In preliminary assays we could not detect a foreign gene, *bar* (White *et al*., 1989), which we inserted into the D2.3 construct by using *bar*-specific primers or by hybridization with a *bar*-specific probe (Guang Yang and others, unpublished). Confirmation of the presence of recombinant D2.3-ORF9 molecules in co-infected plants was obtained by using RT–PCR. The tagged RNA molecules were detected 2·5 months post-inoculation and onward for at least 7 months, which shows the infectious nature of the recombinant D-cDNA driven by the 35S promoter. These results demonstrate that (1) the construct used for co-infection possessed the CTV termini necessary for replication and that (2) the recombinant D-RNA was encapsidated in CTV particles. The failure to detect recombinant D-RNA by Northern hybridization was not surprising since the tagged construct does not possess any selective advantage over the multiple D-RNAs of various sizes naturally present in CTV-VT infected plants (Mawassi *et al*., 1995*c*: Guang Yang and others, unpublished).

Studies on the effects of CTV D-RNAs on the outcome of virus infections are hampered by the lack of D-RNA-free virus preparations and irregularity of D-RNA accumulation in different plants infected by a single source of inoculum (Mawassi *et al*., 1995*c*). Infection of plants with CTV-D-RNA is expected to provide the means for analysing the accumulation of these molecules in chronically infected plants and also for studying the functions of CTV genes (e.g. by transferring marker mutations between a D-RNA molecule and a helper virus, and for the expression of foreign genes), as in the case of the even larger coronavirus genomes (Liao, 1995). However, considerably more research on the factors affecting the expression of selectable markers and foreign genes along with different safety aspects will have to be studied before considering the practical use of infectious CTV-D molecules.

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