cDNA Clones of CTV That Discriminate Severe and Mild Strains

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ABSTRACT. CTV dsRNA purified from a Valencia Frost orange from Concordia, Argentina, was reverse transcribed to cDNA, ligated to plasmid vector pcDNAII and cloned in *E. coli* INV αF' competent cells. Ampicillin resistant colonies were screened with ^{32}P labelled short copies of cDNA of a highly purified CTV dsRNA. Fifty clones were isolated with inserts of 0.4 to 2.3 kbp which showed different electrophoresis patterns after digestion with several restriction enzymes. These results suggest that the clones are different from each other and cover most of the CTV genome. To perform Southern analysis, all clones were cut with *Bam*HI, *Xho*I and *Hin*fI or *Bam*HI, *Xho*I and *Dde*I, electrophoresed in agarose gels, blotted and hybridized with short copy cDNA of the dsRNA from the mild T312 or severe T387 strains of CTV. Several fragments of the clones hybridized with one probe but not with the other, providing useful tools for the discrimination of these strains of CTV.

Citrus tristeza virus (CTV) is a member of the closterovirus group (1). The extent to which it causes severe disease in citrus plantings is dependent on the *Citrus* spp. grown, the scion/rootstock combination, the efficiency of local vectors and the virus strains present in a particular area (2). Severe strains of CTV cause considerable economic losses due to decline of trees grafted on sour orange rootstock. On the other hand, mild CTV strains produce very weak to no noticeable symptoms on susceptible plants. The technical capacity of detecting mild isolates is of great practical significance because of the ability of some of these CTV strains to protect the infected plants from more severe ones (10).

Currently, the severity of a given isolate can be determined only by the time consuming procedure of inoculating different indicator plants (6). The application of strain-specific monoclonal antibodies (MAbs), such as CTV MCA13, can be highly useful to develop even more specific probes (7). Peptide map analysis has been also assayed to show differences among CTV strains (12), but as with MAbs only differences in the coat protein become apparent in this procedure. This gene represents only about 3.4% of the viral genome.

Analysis of dsRNAs purified from infected plant tissues allowed to gain some insight on the diversity of CTV strains present in field trees, but it is not adaptable for rapid large-scale screening assays (4, 11).

There is a need for a rapid and reliable diagnostic method to identify specific severe isolates of CTV. In a previous work, Rosner *et al.* reported the cloning of unrelated CTV cDNA sequences that hybridized differentially with the RNA of a severe Florida isolate (13). The analysis of other cDNA sequences derived from different parts of the virus genome and/or from other virus isolates will be required to extend nucleic acids hybridization as a more general technique for the differentiation of CTV strains. Working in this direction we set out to develop cDNA clones for different genomic regions and to use them to screen for restriction fragments that could discriminate CTV strains. Here we describe the synthesis and testing of such cDNA probes.

MATERIALS AND METHODS

Isolation of double-stranded RNAs (dsRNAs). CTV dsRNAs were purified from the bark of shoots of a naturally infected Valencia Frost orange tree from Concordia, Argentina. Tissue samples were frozen at –135 C, ground to powder and dsRNAs purified according to the method described by Dodds *et al.* (4). After the phenol-detergent extraction, the aqueous phase

containing nucleic acids was adjusted to 16% ethanol and passed through a Whatman CF-11 cellulose column. The dsRNA eluted from the column was ethanol-precipitated, redissolved in sterile water, and treated with RNase-free DNAse in 0.3 M NaCl plus 10 mM $MgCl_2$. This preparation was reprecipitated with ethanol and analyzed by agarose gel electrophoresis in 1X TBE (0.9 M Tris-borate, 0.002 M EDTA) (14). The dsRNA corresponding to the largest band was recovered from the gel, phenol extracted and ethanol precipitated.

Molecular cloning of cDNAs. Complementary DNA (cDNA) to viral RNA was prepared by the procedure of Gubler and Hoffman (9) using the Librarian II cloning kit (Invitrogen Corp., USA) and dsRNA as the template. The cDNA was blunt ended with T4 DNA polymerase and ligated to *Bst*XI adapters. The resulting cDNA was size fractionated on a 1% PurElute™ agarose gel (Invitrogene, USA) and selected fractions (3.0 kbp-0.3 kbp) purified by adsorption to glass powder (GeneClean, BIO 101, USA). The cDNA was then ligated into *Bst*XI digested pcDNAII plasmid and cloned by transformation of competent *E. coli* INV αF′ cells according to standard procedures (14).

Selection of clones containing inserts complementary to CTV RNA. Transformed bacteria were plated out on LB agar containing 100 μg/ml ampicillin, IPTG and X-gal (14). White colonies were picked and transferred onto duplicate LB agar plates. Replicas were transferred to Whatman 541 filters and the recombinant plasmids containing CTV sequences were detected by hybridization with ^{32}P labelled short copy cDNA using viral RNA as template (8). Subsequently, the same filters were hybridized with probes derived from healthy plant nucleic acids prepared by the same procedure as the infected ones so as to discard plant clones.

Analysis of cloned cDNA sequences. Plasmid DNA was isolated from the clones by the alkaline lysis procedure (14). The cDNA inserts were released by digestion of the recombinant plasmids with the restriction endonucleases *Bam*HI plus *Xho*I. The size of each insert was determined by electrophoresis on 0.8% agarose gel using *lambda* DNA digested with *Hind*III or with *Bst*EII as size markers.

To perform Southern analyses, all recombinant clones were cut with the following combinations of enzymes: *Bam*HI, *Xho*I and *Hin*fI or *Bam*HI, *Xho*I and *Dde*I, electrophoresed on 2% agarose gels and transferred by capillary blotting onto nylon membranes (Genescreen, NEN, Dupont de Nemours, USA). Blots were hybridized first with a short copy cDNA probe reverse-transcribed from the dsRNA isolated from the mild CTV strain T-312 and then rehybridized with the severe CTV strain T-387 provided by Dr. J. Guerri (IVIA, Moncada, Valencia, Spain).

RESULTS AND DISCUSSION

Identification of cDNA clones containing CTV sequences. A library of cloned cDNA sequences complementary to CTV dsRNAs was prepared. Fifty clones were obtained that hybridized positively with a short copy cDNA of CTV dsRNA. None of these clones gave a positive reaction with labelled cDNA synthesized from a nucleic acid preparation obtained from a healthy plant. It is important to mention that since it is a field isolate, the clones can be derived from both mild and severe strains that could be infecting the same citrus plant.

Sizing of cDNA inserts. In order to determine the size of the inserted viral cDNA sequences, purified plasmid DNA was digested with restriction endonucleases *Bam*HI plus *Xho*I and subjected to electrophoresis on 0.8% agarose gels (Fig. 1). The insert size was estimated to range between 2.3 and 0.4 kbp (Table 1). According to the number of clones obtained, their sizes and the fact that random primers were used to generate the cDNA, we could estimate that more than 85% of the CTV genome is represented in this library.

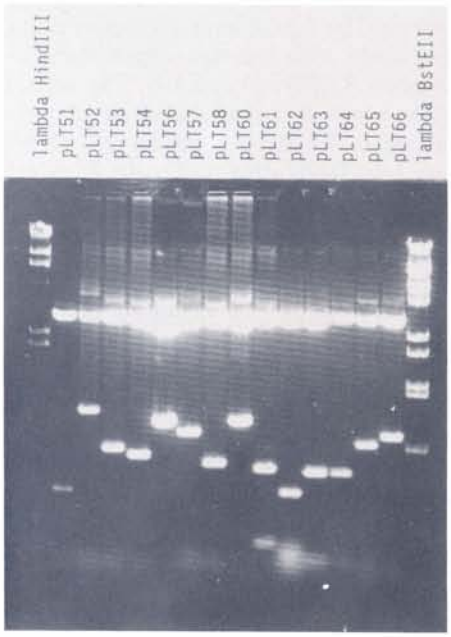

Fig. 1. Size of the CTV cDNA inserts in the recombinant plasmids. The plasmid pcDNAII harboring CTV cDNA sequences was digested with *Bam*HI + *Xho*I endonucleases and electrophoresed in a 0.8% agarose gel in 1 X TBE. *Lambda* DNA *Hind*III or *Bst*EII digested were used as size markers.

Restriction-hybridization analysis of CTV clones. To screen for putative strain-specific regions in the different cDNA inserts, all recombinant plasmids were cleaved with the following enzyme combinations: *Bam*HI + *Xho*I + *Dde*I or *Bam*HI + *Xho*I + *Hin*fI. The DNA digests were analyzed by Southern blot, and subsequent hybridization with two short copy cDNA probes generated from dsRNA from a mild (T-312) and a severe (T-387) CTV isolate, respectively (Fig. 2 and 3).

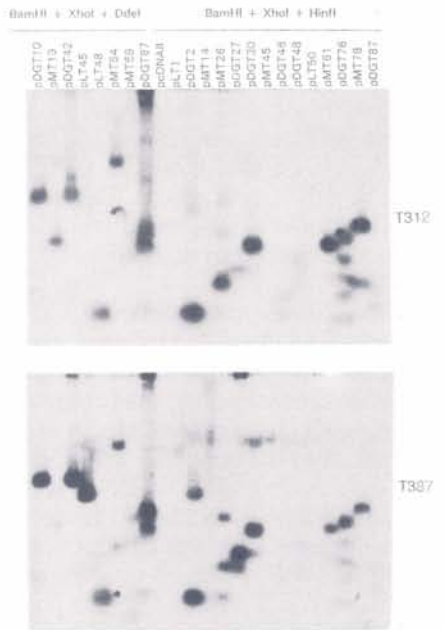

Fig. 2. Southern-blot hybridization of cloned CTV cDNA sequences. Recombinant plasmids were digested with *Bam*HI + *Xho*I + *Dde*I or with *Bam*HI + *Xho*I + *Hin*fI endonucleases, electrophoresed in a 2% agarose gel, blotted and hybridized with a short copy cDNA reverse transcribed from dsRNA of mild CTV strain T-312 or the severe CTV strain T-387. Clone pLT45 (lane 4) shows a 200-bp fragment that hybridizes only with T-387 probe and clone pDGT2 (lane 11) shows a 195-bp fragment that hybridizes strongly with T-387 probe, and mildly with T-312 probe.

TABLE 1
SIZE OF cDNA INSERTS IN CTV CLONES

Clones	Size (kb)	Clones	Size (kb)	Clones	Size (kb)	Clones	Size (kb)
pLT1	0.45	pLT49	1.1	pDGT2	0.6	pMT14	0.4
pLT2	0.5	pLT50	0.7 + 1.6	pDGT10	0.8	pMT19	0.4
pLT3	0.5	pLT51	0.4	pDGT27	0.8	pMT26	0.7
pLT5	0.5	pLT52	1.0	pDGT30	0.6	pMT45	0.4
pLT6	0.5	pLT53	0.7	pDGT42	0.9	pMT54	2.0
pLT7	0.8	pLT54	0.64	pDGT46	0.6	pMT59	1.1
pLT39	0.7	pLT56	0.94	pDGT48	0.8	pMT61	0.4
pLT40	0.7 + 0.6	pLT57	0.84	pDGT76	1.3 + 1.0	pMT78	0.7
pLT41	0.6	pLT58	0.6	pDGT86	0.7		
pLT42	0.8	pLT60	0.94	pDGT87	1.3		
pLT43	0.8	pLT61	0.53 + 0.16				
pLT44	0.8	pLT62	0.4				
pLT45	1.0	pLT63	0.5				
pLT46	0.6	pLT64	0.5				
pLT47	0.7	pLT65	0.74				
pLT48	0.9	pLT66	0.78				

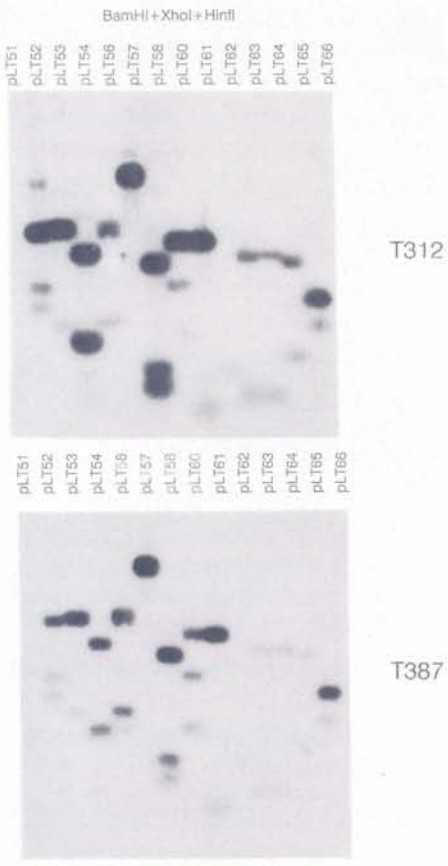

Fig. 3. Southern-blot hybridization of cloned CTV cDNA sequences. Recombinant plasmids were digested with *Bam*HI + *Xho*I + *Hin*fI endonucleases, electrophoresed in a 2% agarose gel, blotted and hybridized with a short copy cDNA reverse transcribed from dsRNA of mild CTV strain T-312 or the severe CTV strain T-387. Clone pLT54 (lane 4) shows a 165-bp fragment that strongly hybridizes with T-312 probe.

Several fragments hybridized with one probe but not with the other, or hybridized strongly with one probe and weakly with the other. Examples of these selective hybridizations were clones pLT45 (200 bp fragment, Fig. 2), pDGT2 (195 bp fragment, Fig. 2) and pLT54 (165 pb fragment, Fig. 3).

Numerous CTV strains have been characterized exhibiting considerable diversity in their biological and serological characteristics and in dsRNA pattern (3, 5, 6, 7). Such variations should be a reflection of the differences in nucleotide sequence of the CTV genome. In this paper we have presented evidence of the existence of several strain specific regions of the CTV genome (restriction fragments of cloned cDNA in the size range of 100 to 200 bp). Our results demonstrate that strain-specific probes can be developed, providing a useful diagnostic tool. The cDNA sequencing of the differential CTV clones obtained in this study and the gene assignment that could be derived therefrom will certainly contribute to our understanding of the diversity of this serious plant pathogen. The biological significance of the changes found at the genome level will need an extensive multidisciplinary analysis.

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