Molecular Characterization of the Coat Protein Gene of Citrus Tristeza Closterovirus Isolates from Cuba

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ABSTRACT. Citrus tristeza virus (CTV) is present in all citriculture areas of Cuba. Samples of CTV isolates Cu2 and Cu22 from Cuba were collected and analyzed by DASI-ELISA using a panel of monoclonal antibodies in order to determine possible serological relationships. Reverse transcription PCR was used to amplify the coat protein gene (CPG) of CTV, and the amplified products were cloned into a *SmaI/XbaI*-cut pUC 19 vector. Recombinant clones were selected by restriction enzyme reactions, and their nucleotide and amino acid compositions determined. Nucleotide and amino acid sequences were compared with the reported CPG sequences of CTV. A phylogenetic analysis showed the highest homology of these isolates with isolates classified as mild, in concordance with the absence of symptoms of the original source plants in the field.

Citrus tristeza virus (CTV) occurs in most citrus producing areas of the world and is one of the most economically important viral diseases of citrus (1). The filamentous, flexous virions are composed of a single-stranded positive-sense RNA strand encapsidated with a coat protein (CP) of M*r* 25 kDa with a CP homolog product of the p27 open reading frame (ORF) encapsidating about 5% of one end (13, 16). Recently, the complete genome sequences of two CTV isolates were fully sequenced. The 20 kb CTV genome encodes 12 ORFs which potentially codes for at least 17 protein products (7, 11).

CTV is transmitted by several species of aphids and also by grafting. There are many CTV strains, usually occurring as mixtures, which cause various symptoms depending on the citrus scion and/or rootstock combinations. In terms of severity of symptoms, CTV can be divided into two general groups: mild, not causing noticeable damage in common citrus hosts nor causing decline on sour orange; and severe strains which may cause decline on sour orange rootstock, and/or seedling yellows and/or stem pitting on grapefruit and/or sweet orange (5).

The ability to detect and differentiate CTV strains is critical for management of tristeza. Several procedures have been developed including biological, serological and nucleic acid based-methods (5, 12, 14), however, the best option will be according with the particular purpose. Sequencing of viral genes will be always be an useful and powerful tool, not only to accurately characterization the isolates, but also to generate specific probes for diagnostics and to develop CTV resistance by transgenic methods.

CTV has been detected in Cuba in different citrus areas and many efforts have been made for its diagnosis and control. Molecular characterization of the CTV strains in Cuba will provide useful information about the characteristics of the CTV isolates present and help develop better control methods.

MATERIALS AND METHODS

Virus sources. The CTV isolates Cu2 and Cu22 were collected from a Cuban citrus field and were maintained under glasshouse conditions in the collection at the IICF (Citrus Research Institute). The serological characteristics of these

isolates were determined by ELISA-DASI using a panel of 15 monoclonal antibodies (4, 9). The original source trees of the virus isolated remained asymptomatic in the field throughout this study.

Nucleic acid extraction. Total nucleic acids were extracted from fresh bark tissue samples collected from healthy and CTV infected plants. Approximately 300 mg were pulverized under liquid nitrogen, then gently mixed with 500 µl of extraction buffer (0.1 M Tris, 2mM EDTA, 2% SDS) and 500 µl of a phenol-chloroform mix (v:v). Incubation at 70°C for 5 min was followed by centrifugation at room temperature for 10 min. Then 200 µl of supernantant was removed and an equal volume of cold isopropanol added and then incubated for 10 min at -20°C. The pellet was collected after centrifuging 8,000 g for 10 min, then washed with 70% ethanol. The pellet was resuspended in 30 µl of water immediately before using as a template for the reverse transcription (RT) PCR reactions.

Reverse transcription PCR reactions. One tube RT-PCR assays were used. Primers 4112 (5'-CATGGCCATGGACGACGAAA-CAAAGAAATTG-3') and 4113 (5'-GCTCTAGAGAATTCTCAACGTGT-GTTAAATTTC-3') were designed according to the reported CTV CPG sequences for CTV isolates around the world (13). RT-PCR mixtures containing 1 µl of total nucleic acid preparation (diluted 1:50), 0 25 mM of each dNTP (dATP, dCTP, dGTP and dTTP) (Promega), 10 mM DTT, 25 pmoles of each primer, 50 mM KCl, 10 mM Tris-HC1 pH 8.8, 2.5 mM $MgC1_2$, 10 units of RNAsin (Promega), 5 units of AMV Reverse Transcriptase (Promega) and 10 unit of Taq Polymerase (Promega) were prepared in 100 µl final volume. The template was heated at 70°C for 5 min before adding to the reaction mixture. Tubes were subject to successive cycles of amplification in a thermocycler as follow: 42°C for 45 min (RT), denaturation for 1 min at 95°C, annealing for 1 min at 45°C and extension for 1 min at 72°C for 40 cycles. In the last step, samples were maintained for 10 min at 72°C. PCR products were analyzed by electrophoresis in a 0.8% agarose gel, stained with ethidium bromide (0.5 µg/ml) and visualized by UV light.

Cloning and sequencing of PCR products. Amplified products were purified after electrophoresis using the Sephaglas BandPrep Kit (Pharmacia Biotech, Sweden). Purified DNA fragments were digested with the enzyme Xba I (New England Biolabs) and ligated into a *SmaI/XbaI*-cut pUC 19 vector. Competent cells of *Escherichia coli,* strain XL 1-Blue, were transformed on culture medium containing 80µg/µl X-Gal and 10 mM IPTG and 100 µg/µl ampicillin. Plasmid DNA preparations from white colonies were subjected to the double digestion using Hind III/Sac 1 and Pst I/Eco R1, and the products were analyzed by agarose gel electrophoresis (15).

Nucleotide sequencing was performed in the automatic sequencer Vistra DNA 725 (Amersham) using the Thermosequenase pre-mixed cycle sequencing kit. Nucleotide sequences were analyzed by using the computer program CLUSTAL W (17) and the phylogenetic relationships were generated using the UPGA algorithm, Easy Tree 1.31 Program (6).

RESULTS AND DISCUSSION

CTV isolates Cu2 and Cu22, from Cuba, showed different reaction patterns when tested against a panel of 15 monoclonal antibodies. Distinct serological behavior against the antibodies ECTV 176, 4E5A1 and 6D8, indicated that should be some differences in the amino acid composition to account for such differences in monoclonal epitopes (Table 1). In

TABLE 1
REACTIVITY OF 15 MONOCLONAL ANTIBODIES TO TWO CITRUS TRISTEZA VIRUS ISOLATES FROM CUBA BY DOUBLE ANTIBODY SANDWICH-ELISA

	Citrus tristeza virus isolate[z]	
Monoclonal antibody	Cu2	Cu22
3DF1	+	+
3CA5	+	+
3C1F10	+	+
IIIAD5	+	+
4E5-A1	-	+
4B1-3	+	+
ECTV-176	-	+
3E10-3	+	+
4G12	+	+
7F24	+	+
MCA13	-	-
MCA14	-	-
ECTV-178	-	-
4F3A1	-	-
6D8	+	-

[z]+ = positive reaction; - = negative reaction.

general, serological studies such as DASI-ELISA, immunoprinting and other antibody-based techniques, have been applied to several CTV isolates to confirm the epitope diversity among CTV isolates (2, 3, 8, 9).

The CPGs of Cu2 and Cu22 were cloned from total nucleic acid preparations using the same CPG-specific primer pair. A PCR-amplified DNA fragment of approximately 700 pb was observed following RT-PCR from sample from infected plants but not from healthy plants. The yield of amplified fragment was estimated to be 0.65 µg per 100 µl reaction. Similar length products have been reported following RT-PCR for the CPG of several CTV isolates (10, 13). More than 25 recombinant clones carrying the CTV CpG were obtained for each isolate.

Sequences analysis of the cloned products corresponding to the isolates Cu2 and Cu22 revealed a DNA fragment of 672 nucleotides, similar to the CPGs previously reported for CTV (16). Deduced amino acid sequence included 223 amino acid residues for each of the two isolates. There was over 98% similarity at the nucleotide level and more than 91% similarity at the amino acid level between the Cuban isolates, Cu2 and Cu22. No coincident nucleotides were located in the positions 27, 43, 141, 161, 166, 168, 289, 326, 515, 613 and 631; whereas there were eight distinct amino acid residues in the positions 15, 47, 54, 97, 109, 172, 205 and 211. The majority of these divergent amino acid residues corresponded to functionally conserved substitutions (Fig. 1).

There were more than 80% homology at both the nucleotide and amino acid levels among the 11 CTV isolates analyzed by Pappu et al. (13) and more than 90% homology among four CTV isolates from India (10).

The phylogenetic tree generated using the deduced amino acid sequences from the Cuban isolates and CTV isolates from around the world showed two main clusters (Fig. 2). The first cluster included the isolates T397, S9, S10, B274, B272 and B32, which are isolates considered to be mild plus the two Cuban isolates which have not yet be biologically characterized. The

Cu2 MDDETKKLKNKNKE**S**KEGDNVVAAESSFGSVDHIDPTLITMNDVR**H**LST

Cu22 MDDETKKLKNKNKE**T**KEGDNVVAAESSFGSVDLHIDPTLITMNDR**Q**LST
**************.******************************.***

Cu2 QQN**A**ALNRDLFLALKGKYPNLPDKDKDFHIAMMLYRLAVKSSSLQS**N**DDT

Cu22 QQN**V**ALNRDLFLALKGKYPNLPDKDKDFHIAMMLYRLAVKSSSLQS**D**DDT
.**.

Cu2 TGITYTRE**G**VEVDLSDKLWTDIVYNSKGIGNRTNALRVWGRTNDALYLAF

Cu22 TGITYTRE**D**VEVDLSDKLWTDIVYNSKGIGNRTNALRVWGRTNDALYLAF
********.***

Cu2 CRQNRNLSYGGRPLDAGIPAG**S**HYLCADFLTGAGLTDLECAVYIQAKEQL

Cu22 CRQNRNLSYGGRPLDAGIPAG**Y**HYLCADFLTGAGLTDLECAVYIQAKEQL
*********************.****************************

Cu2 LKKR**G**ADEVV**L**TNVRQLGKFNTR

Cu22 LKKR**R**ADEVV**V**TNVRQLGKFNTR
****.*****.************

Fig. 1. Alignment of deduced amino acid sequences of coat protein genes from the Cuban isolates Cu2 and Cu22. Bold letters indicate the divergent amino acid. Asterisks indicate identical amino acid residues and dots indicate conserved substitutions.

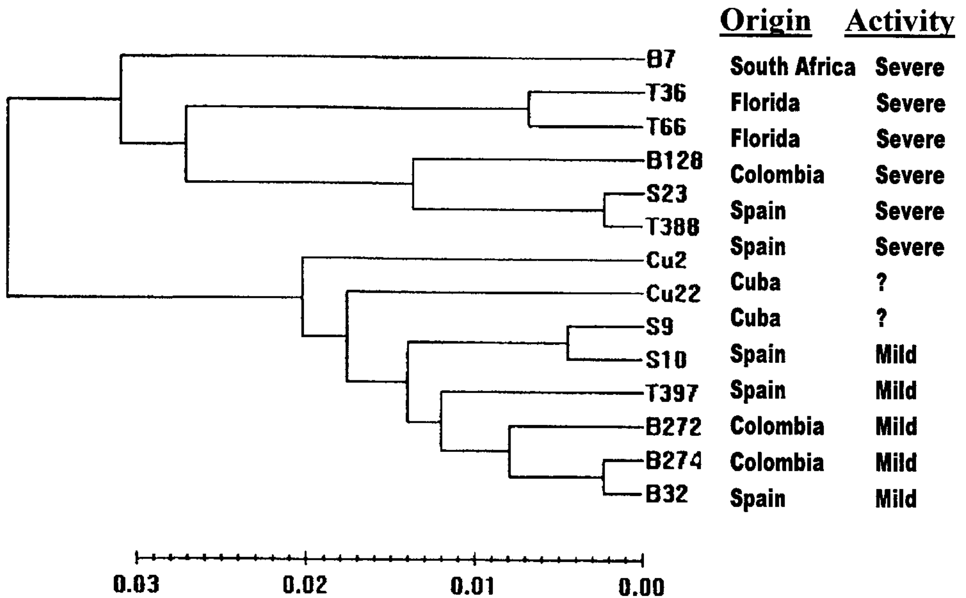

Fig. 2. Phylogenetic trees from alignment of the amino acid sequence of coat protein from several citrus tristeza virus isolates.

TABLE 2
COMPARISON OF THE SIMILARITY (%) OF THE DEDUCED AMINO ACID SEQUENCE OF THE COAT PROTEIN OF THE CUBAN CITRUS TRISTEZA VIRUS ISOLATES CU2 AND CU22 WITH OTHER CTV ISOLATES

Cuban isolate	CTV isolates compared											
	T36	B128	T66	S9	S23	S10	B274	B272	397	388	B32	B7
Cu2	93	91	93	95	93	95	97	95	95	93	96	91
Cu22	94	92	94	96	93	96	98	96	95	94	97	91

second cluster included T66, B128, S23, B7, T36 and T88, isolates which are considered severe.

The grouping of CTV isolates into clusters according their biological properties is an interesting and frequent event when the phylogenetic analysis is based on CTV CPG sequence. If an exhaustive analysis of the nucleotide sequence of several isolates of CTV is made, about 15 representative nucleotides are associated with biologically behavior, e.g., severe or mild.

Although the biological characterization of the isolates Cu2 and Cu22 is still in progress, the original source trees of these isolates remain asymptomatic in the field. While these two isolates were collected from trees in the same citrus field, they contained two different CTV isolates based on the results of the nucleotide and amino acid sequence information. The serological tests using the panel of 15 monoclonal antibodies provided the initial evidence that they were distinct CTV isolates, however, we do not eliminate the possibility of mixed infections. Additional work is in progress to study and characterize the Cuban CTV strains.

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