

## Sequence of Coat Protein Gene of the Severe Citrus Tristeza Virus Complex Capão Bonito

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**ABSTRACT.** The coat protein gene (CPG) of the citrus tristeza virus (CTV) complex known as Capão Bonito from Pera sweet orange was cloned and sequenced. Results indicated that there are at least two CTV strains in the source plant. These isolates were designated CB3-22 and CB3-104 and they differed in 59 nucleotides which changed 11 amino acids in the coat protein. A dendrogram was generated which grouped isolate CB3-22 with mild isolates and CB3-104 with severe isolates from other countries. In addition, hybridization results also indicated that CB3-104 titer is higher than of CB3-22 in the plant. A discussion of the ramification of these results is discussed.

*Index words.* Capão Bonito CTV complex, 'Pera' sweet orange, coat protein gene.

Capão Bonito and neighboring counties in the South of São Paulo State (Brazil) are at risk for sweet orange production due to the occurrence of the Capão Bonito complex of citrus tristeza virus (CTV). This strain is highly virulent and affects plantings on tolerant rootstocks including Rangpur lime which is tolerant to common CTV isolates in Brazil. The occurrence of decline in sweet orange grafted on tolerant rootstocks was first reported by Müller et al. (3) in 1968. At that time, the damage caused by this CTV complex was not economically significant, because this area was not a citrus-growing area. Since then, however, production of sweet orange varieties in the region has grown. Preimmunization of several sweet orange healthy clones with protective isolates selected from other areas was carried out but the results showed inconsistent protection. Some preimmunized plants showed severe CTV symptoms that could be attributed to a breakdown of cross protection. This was particularly true for the Pera which is the main sweet orange variety growing in São Paulo State. In spite of the preimmunization, Pera always shows stem pitting in the trunk (4, 5).

In the last decade, there has been a huge increase of citrus acreage in the Capão Bonito area and many

plants are now affected. Hence, there is a great interest to select locally mild CTV isolates that can cross protect citrus against the Capão Bonito CTV complex.

Molecular characterization of CTV was first based mainly on the coat protein gene (CPG) and the CPG from a number of biologically and geographically different isolates have been cloned and sequenced. The analysis of the nucleotide sequence identified an open reading frame (ORF) of 669 nucleotides coding a protein of 223 amino acids (2, 7, 8, 10). Some association between the nucleotide sequence and the biological activity of the isolates was observed with some isolates. Therefore, it is possible to infer from CPG sequences if an unknown CTV isolate is likely to be mild, quick decline or stem pitting, by comparison with the CPG sequence of isolates that have been biologically indexed (6). The CPG of three Brazilian isolates have already been cloned and sequenced (11). They are: i) the mild isolate used in cross-protection of Pera sweet orange in São Paulo State; ii) Pera Ipigua isolate; and iii) Galego lime isolate.

The present work was carried out to characterize the Capão Bonito complex which may help to identify mild isolates to be used in cross protection.

## MATERIAL AND METHODS

**Source of virus.** CTV dsRNA was isolated from five samples collected in the Capão Bonito E. S., Capão Bonito - SP - Brazil. The sample from a 19-yr-old Pera sweet orange tree grafted on Rangpur lime called CB3 was selected for CPG cloning and sequencing. This tree, from hereon will be called the source plant, was inoculated with the Capão Bonito complex at the nursery stage by grafting and its symptoms include: severe stunting; generalized pitting in the scion and rootstock; short axillary sprouts, deficiency-like patterns in the leaves; and small and dry fruits.

**Double-stranded RNA isolation.** CTV double-stranded (ds) RNA was isolated according to the procedure described by Valverde et al. (12). One gram of infected freeze dried bark tissue was ground to a powder in liquid nitrogen, transferred to 50 ml centrifuge tubes and 7 ml of 2× STE [1× STE is 0.1 M NaCl; 0.05 M Tris (hydroxymethyl) amino-methane]; 1 mM EDTA (ethylene diamine tetraacetic acid); pH 6.8], 14 ml of phenol: chloroform: isoamyl alcohol (24:24:1) and 1.6 ml of 20% SDS (sodium dodecyl sulfate) were added. The homogenate was shaken vigorously for 30 min and centrifuged at 8,000 g for 10 min at 4°C. After centrifugation, the aqueous phase was transferred to a clean tube, adjusted to 16% ethanol and applied to a 10 ml column of Whatman CF-11 cellulose powder prepared in 1× STE containing 16% ethanol (2 g of CF-11 dry powder, 50 ml of STE containing ethanol). The column was washed with 80 ml of STE containing 16% ethanol and with 5 ml of STE. DsRNA was eluted with 20 ml of STE and ethanol was added to the eluate to a final concentration of 16%. Sample was applied to a second column containing half the volume of the first one, as described above. DsRNA was

eluted in 6 ml of STE and precipitated with 0.1 volumes of 3M sodium acetate pH 5.2 and 3 volumes of ethanol, overnight at -20°C. The dsRNA was collected by centrifugation at 8,000 g for 25 min at 4°C, washed with 70% ethanol, resuspended in 50 µl of sterile water and analyzed by electrophoresis in 1% non denaturing agarose gel containing ethidium bromide.

**First strand cDNA synthesis, coat protein gene amplification and sequencing.** Ds RNA was denatured at 75°C for 10 min and used as a template for 1st strand cDNA synthesis by MuMLV reverse transcriptase and random hexamers primers (9). The CPG was amplified by PCR using specific primers containing EcoRI and BglII sites. The obtained fragments were cloned into pBluescript KS+ (9). DNA was sequenced using the Sequenase version 2.0 kit (USB). The sequences were analyzed using the software Seqaid II, 3.60.

**DsRNA dot blot and hybridization.** CTV dsRNA was isolated from the source plant and blotted on Hybond N membrane, according to the specifications of Bio-Rad for the "Bio-Dot SF Microfiltration Apparatus". RNA isolated from healthy tissue was used as a negative control. The cloned CPG from Pera CB-3 was labeled by PCR using the "DIG DNA Labeling and Detection Kit Non Radioactive" (Boheringer Mannheim Biochemica). pBluescript KS+ DNA was used as a negative control. The hybridization was done according to the specifications of Boheringer Mannheim Biochemica for the kit above mentioned. After the hybridization, the membrane was washed twice 10 min at room temperature with 2× SSC, 0.1% SDS; once 15 min at 65°C with 1× SSC, 0.1% SDS and once 15 min at 65°C with 0.5x SSC, 0.1% SDS. The membrane was exposed to Hyperfilm M-AP (Amersham) for 10 min and the film was developed.

## RESULTS AND DISCUSSION

A 669 bp fragment corresponding to the CPG was amplified by PCR in all five isolates studied (data not shown). The digestion of these fragments with *Eco*RI generated fragments of 669, 500 and 169 bp and indicated the presence of at least two different CTV strains: one with the *Eco*RI site generating fragments of 500 and 169 bp, and other without the *Eco*RI site. In the next step, two clones, CB3-22 (no *Eco*RI site) and CB3-104 (with *Eco*RI site), were selected and sequenced (Fig. 1, lanes 1 and 2, respectively). Fig. 2 shows the nucleotide sequence of the two Capão Bonito strains. The analysis of the nucleotide sequence showed 59 different bases between them corresponding to a homology

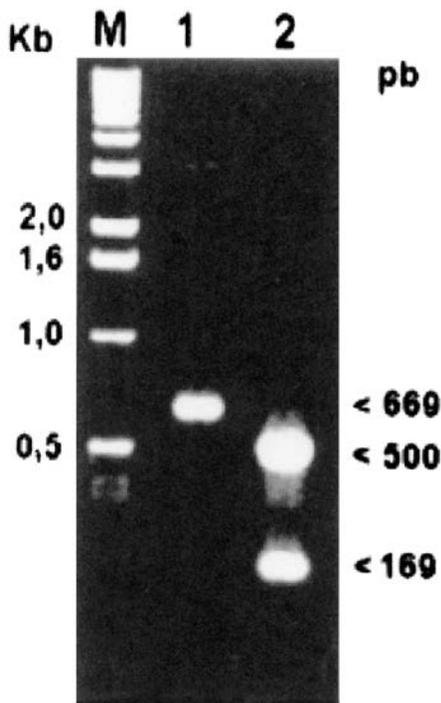


Fig. 1. Electrophoretic analysis in 1% non denaturing agarose gel of coat protein gene, isolate Pera, cloned into *Eco*RI and *Bgl*II digested pBluescript KS+. 1. Clone CB3-22; 2. Clone CB3-104. Lane M = 1 Kb DNA Ladder.

of 91.2%. The differences changed 11 amino acids in the coat protein (Fig. 3). Considering that strain CB3-22 and CB3-104 are present in the same plant, the differences are significant. The CPG from CTV isolates which are biologically and geographically different showed 90 to 100% homology (7). Our dendrogram (Fig. 4) indicated that CB3-22 strain has high similarity with the T-30 mild isolate from Florida (USA); whereas CB3-104 was very similar to the severe stem pitting isolates from Colombia (B-129) and India (B-227). The other three Brazilian isolates formed another separate cluster, differing from other mild or severe isolates in the dendrogram (11).

To confirm the occurrence of two CTV strains in the same source plant and to verify the concentration of each, hybridization was carried out using dsRNA isolated from the source plant and digoxigenin-labeled CPG from the two strains. Fig. 5 A (lanes 2 and 3) shows the products of the amplification and labeling reaction of Pera CB3-22 and CB3-104. Fragments of approximately 670 bp were obtained from both samples. DNA from pBlue-script KS+ was the negative control (Fig. 5 A, lane 1). Figure 5 B shows that both probes hybridized to CTV dsRNA isolated from the source plant, which confirm that they were present in the same plant. The intensity of the hybridization was stronger when DNA from CB 3-104 was used as probe, indicating that this CTV strain is present in higher titer. RNA isolated from healthy tissue was used as a negative control and no hybridization was detected (data not shown).

According to Lee et al. (1), one of the characteristics of mild cross protective CTV isolates is the high viral concentration in the plant tissue coupled with mild symptoms in all hosts. We demonstrated that the CB3-104 strain grouped with severe

CTV-CB3-22 ATGGACGACGAAACAAAGAAATTGAAGAACAACAAAGGAAACGAAAGA 50  
 CTV-CB3-104 ATGGACGACGAAACAAAGAAATTGAAGAACAACAAAGGAAACGAAAGA

CTV-CB3-22 AGGCGACGATGTTGTTGCTGCTGAGTCTTCTTTGGTTCCGTAAACTTAC 100  
 CTV-CB3-104 AGGCGACGATGTTGTTGCTGCTGAGTCTTCTTTGGTTCCGTAAACTTAC

CTV-CB3-22 ACATCGATCCGACTCTGATAACGATGAACGATGTGCGTCAGTTGAGTAAC 150  
 CTV-CB3-104 ACATCGATCCGACTCTGATAGCGATGAACGATGTGCGTCAGTTGGGTACC

CTV-CB3-22 CCACAGAATGCTGCTTTGAACAGGACTTATTTCTTGCTCTGAAAGGGAA 200  
 CTV-CB3-104 CCACAGAATGCTGCTTTGAATAGAGATTTGTTCTTACCTTGAAAGGGAA

CTV-CB3-22 GTATCCTAACTTGCCTGACAAAGATAAGGACTTTCACATAGCTATGATGT 250  
 CTV-CB3-104 GTATCCCAACTTACCTGACAAAGATAAGGACTTTCACATAGCTATGATGT

CTV-CB3-22 TATACCGTTTAGCGGTTAAGAGTTCATCATTGCAAAGTGATGATGACACC 300  
 CTV-CB3-104 TATATCGTTTAGCAGTTAAGAGTTCATCATTACAAAGCGACGGTGATACC

CTV-CB3-22 ACGGGCATAACGTACACTCGGGAAGGTGTTGAAGTAGATTGTCTGACAA 350  
 CTV-CB3-104 ACGGGTGTGACGTACACTCGGGAAGGTGTTGAAGTAGAGTTGTCTGACAA

CTV-CB3-22 ACTTTGGACCGACATCGTGTATAATTCTAAGGGTGTGGTAACCGAACTA 400  
 CTV-CB3-104 ACTTTGGACAGACGTCTGTGTTAACTCTAAGGGTATTGGTAACCGTACTA

CTV-CB3-22 ACGCCCTTCGAGTCTGGGGTAGAACTAAAGATGCTCTTTACCTAGCCTTT 450  
 CTV-CB3-104 ACGCCCTTCGAGTCTGGGGTAGAACTAAAGATGCTCTTTACCTAGCCTTT

CTV-CB3-22 TGTAGACAGAACCGTAATTTGAGTTATGGCGGACGTCCGCTAGATGCAGG 500  
 CTV-CB3-104 TGTAGACAGAACCGCAACTTGAGTTATGGTGGACGTCCGTTAGATGCAGG

CTV-CB3-22 GATTCGCGCCGGGTATCATTATTTGTGTGCAGATTTCTTGACCGGAGCTG 550  
 CTV-CB3-104 AATTCAGCCGGGTATCACTACTGTGTGCAGATTTCTTGACCGGAGCTG

CTV-CB3-22 GCTTGACTGATTTAGAATGTGCTGTGACATACAAGCTAAAGAACAAATTA 600  
 CTV-CB3-104 GCTTGACTGATTTAGAATGTGCAAGTGTACTTACAAGCTAAAGAGCAATTA

CTV-CB3-22 TTGAAAAGCGAGGGGCTGATGAGGTTGTAGTTACGAATGTCAGGCAGCT 650  
 CTV-CB3-104 TTAAGAAGCGAGGGGCTGATGAGTCTGTAGTTACCAATGTCAGGCAGCT

CTV-CB3-22 TGGGAAATTTAACACACGTTGA 672  
 CTV-CB3-104 TGGGAAATTTAACACACGTTGA

Fig. 2. Coat protein gene sequence of CTV-CB3-22 and CTV-CB3-104 Pera Capão Bonito strains.

CTV-CB3-22 MDETKKLKKNKKEKKEGDDVVAESSFSGSVNLHI DPTLITMNDVRQLST 50  
 CTV-CB3-104 MDETKKLKKNKKEKKEGDDVVAESSFGSLNLHI DPTLIAMNDVRQLGT

CTV-CB3-22 QQNAALNRDLFLALKGKYPNLPDKDKDFHIAMMLYRLAVKSSSLQSDDDT 100  
 CTV-CB3-104 QQNAALNRDLFLTLLKGKYPNLPDKDKDFHIAMMLYRLAVKSSSLQSDGDT

CTV-CB3-22 TGITYTREGVEVDLSDKLWTDIVYNSKGVGNRTNALRVWGRNDALYLAF 150  
 CTV-CB3-104 TGVTYTREGVEVELSDKLWTDVVFNSKGI GNRTNALRVWGRNDALYLAF

CTV-CB3-22 CRQNRNLSYGGRPLDAGIPAGYHYLCADFLTGAGLTDLECAVYIQAKEQL 200  
 CTV-CB3-104 CRQNRNLSYGGRPLDAGIPAGYHYLCADFLTGAGLTDLECAVYLQAKEQL

CTV-CB3-22 LKKRGADEVVVTNVRQLGKFNTR 223  
 CTV-CB3-104 LKKRGADEVVVTNVRQLGKFNTR

Fig. 3. Amino acid sequence deduced from the coat protein gene sequence of CTV-CB3-22 and CTV-CB3-104 Pera Capão Bonito strains.

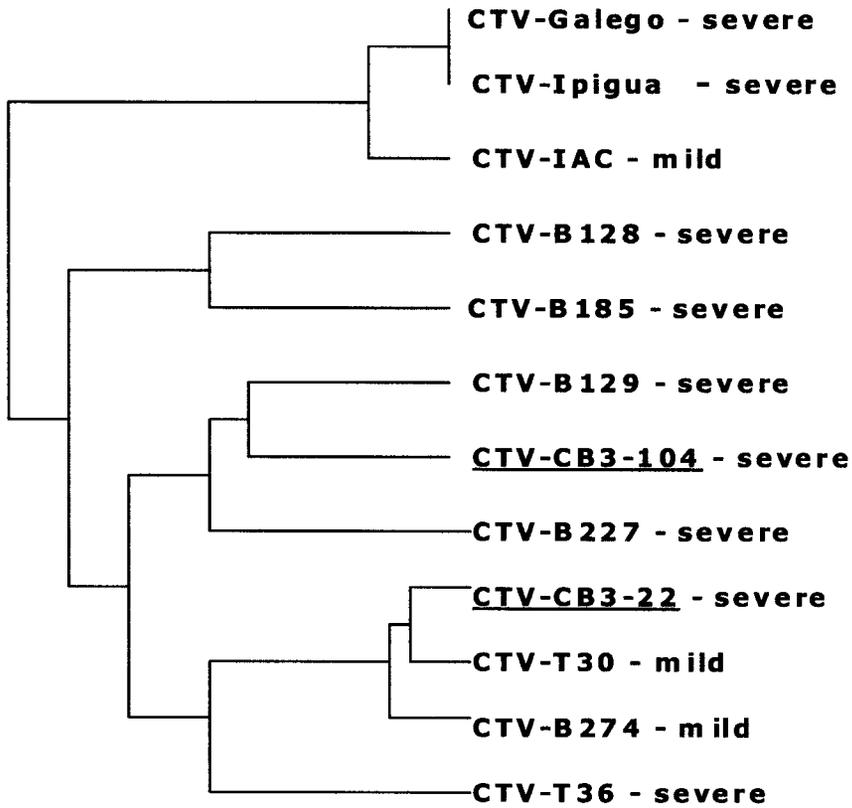


Fig. 4. Cluster dendrogram generated from the multiple alignment of the amino acid sequences of coat proteins from different CTV isolates.

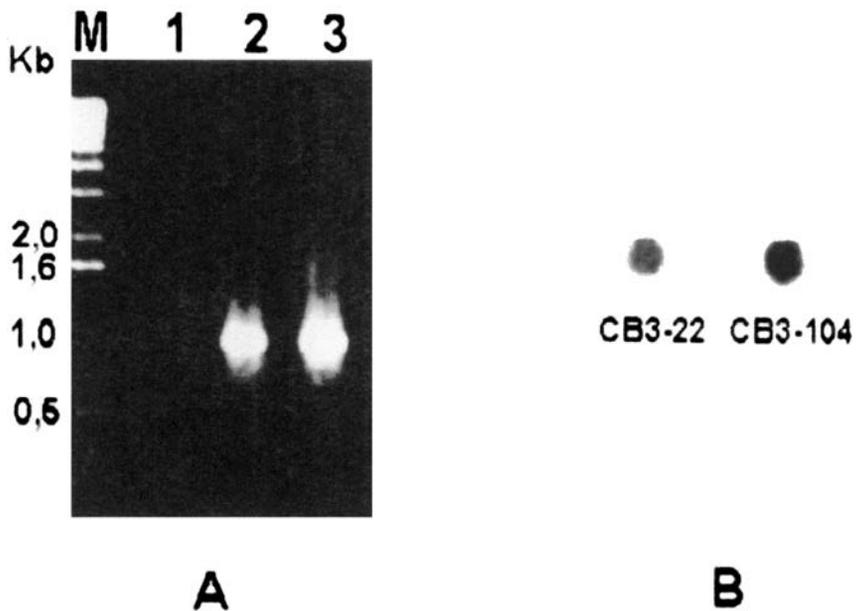


Fig. 5. Labeling of coat protein gene of CTV strains and Dot blot. A. Electrophoresis in 1% agarose gel of coat protein gene of the two Pera Capão Bonito CTV strains digoxigenin labeled. (1) pBluescript KS+ DNA; (2) CB3-22 DNA; (3) CB3-104 DNA. Lane M, 1 Kb DNA Ladder. B. Dot blot of dsRNA isolated from Pera Capão Bonito.

isolates from other countries; whereas the CB3-22 grouped with the mild isolates. It was also demonstrated that in the source plant, CB3-104 was detected in higher concentration than CB3-22. Based on these results, we conclude that the high concentration of CB3-104 probably inhibited

the replication of the CB3-22 isolate and this could be the reason for the damage observed in the source plant.

These results pointed out the complexity of the very severe Capão Bonito complex and research is now underway to characterize each CTV strain present in the complex.

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