# Application of Bi-Directional PCR to Citrus Tristeza Virus: Detection and Strain Differentiation

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ABSTRACT. The monoclonal antibody MCA13 reacts predominantly with severe strains of citrus tristeza virus (CTV) occurring in many regions of the world. Its specificity is based on a single nucleotide (A/T) difference at position 371 of the capsid protein gene (CPG). Based on this single nucleotide difference, we designed two internal primers, one specific for mild strains and the other specific for severe strains of CTV. These primers, along with two terminal primers for the ends of the CPG, were used to develop a bi-directional, reverse transcription/polymerase chain reaction (BD/PCR) to differentiate mild and severe strains of CTV. Under our standard PCR conditions, a full length CPG (~700 bp) and a 400 bp DNA fragment were produced with mild strains, and a 700 and a 300 bp DNA fragment were produced with severe strains. With a mixture of mild and severe strains, the 700, 400 and 300 bp DNA fragments were all produced. Reducing both the terminal primer concentrations and extension times eliminated the 700 bp fragment. Thus, this method can be manipulated for different purposes. Tests of BD/PCR with samples from naturally-infected trees indicated that BD/PCR were more sensitive than ELISA, because BD/PCR amplified a 300 bp fragment from some samples which were MCA13 negative by ELISA tests.

Citrus tristeza virus (CTV), the causal agent of tristeza disease of citrus, is the most destructive and economically important virus of commercial citrus worldwide (2, 10). It is a phloem limited closterovirus whose virions are thread-like filamentous particles, about 2000 × 11 nm in size (1, 3). The molecular weight of the positive sense, single-stranded RNA (ssRNA) genome of CTV was estimated to be about $6.5 \times 10^6$ Daltons (3). The recently completed sequence of strain T36 from Florida revealed the genome size to be 19,296 nucleotides (9).

There are many strains of CTV and they cause distinct symptoms on different hosts. In Florida, mild strains cause no noticeable symptoms on commercially grown varieties, whereas the severe strains cause decline on trees grafted on sour orange rootstock. Some severe strains may also induce a seedling yellow reaction and /or stem pitting on some grapefruit or sweet orange scions.

The monoclonal antibody MCA13 reacts predominantly with severe (decline inducing and/or stem pitting) strains of CTV from many regions of the world. However, some severe strains from Spain, Israel and California and most mild strains generally fail to react with MCA13. Thus, MCA13 (16) is now commonly used as a first step for differentiation of mild and severe strains of CTV in Florida, South America and the Caribbean region. The molecular basis for the MCA13 structural epitope has been described (12, 13, 14). Analysis of capsid protein (CP) amino acid sequences of a number of mild and severe strains of CTV revealed a consistent difference at amino acid position 124. The amino acid was always tyrosine (Y) in mild strains, while it was always phenylalanine (F) in severe strains. When the Y in a mild strain and the F in a severe strain were changed to F and Y, respectively, by site-directed oligonucleotide mutagenesis of the capsid protein gene (CPG), the mutant mild strain protein reacted with MCA13, whereas the mutant severe strain protein did not react with MCA13. The reactivity of MCA13 is thus controlled by a single nucleotide (A-T) change at position 371 of CTV CPG (12).

The development of polymerase chain reaction (PCR) (11) has brought a new approach to diagnostic plant pathology. PCR has been widely used as a sensitive and specific method for detection of various pathogens in their hosts (7, 15, 18). PCR also is used for differentiation of closely related viruses and different strains of the same virus (4, 19).

In this study, we have designed two internal primers based on the single nucleotide difference in the MCA13 epitope, one specific for generally mild MCA13 unreactive strains, and the other specific for generally severe MCA13 reactive strains of CTV. Using these primers with two terminal primers for the ends of the CPG, we have developed a bi-directional reverse transcription polymerase chain reaction (BD/PCR) technique which enables detection and differentiation of the mild and severe strains of CTV, as well as detection of both types of strains of CTV in mixed infections.

## MATERIALS AND METHODS

**Virus strains.** Two well-characterized Florida strains of CTV, T30 and T36, were used for standardizing BD/PCR. T30 causes mild symptoms on Mexican lime only. T36 induces vein clearing, stunting and stem pitting on Mexican lime, causes quick decline of sweet orange on sour orange rootstock and causes a mild seedling yellows reaction on sour orange and grapefruit. Tissue samples of T30 and T36 were obtained from the Exotic Citrus Pathogens Collection, Beltsville, MD where they are maintained and identified as B2 and B3, respectively. Naturally infected field samples were collected by M. Irey of U.S. Sugar Corporation, Clewston, FL. All of these samples reacted in ELISA with polyclonal antibodies (PAbs) to CTV, but not with MCA13 (Table 1).

**Oligonucleotide primers.** Four different primers were used in this study. All the primers were synthesized in the DNA Synthesis Core of the Interdisciplinary Center for Biotechnology Research at the University of Florida. The sequences and other characteristics of the primers are described in Table 2 and Fig. 1.

**Extraction of crude nucleic acids.** Samples of infected leaf or

TABLE 1
COMPARISON OF ELISA AND BI-DIRECTIONAL PCR ASSAY RESULTS OF CTV FIELD SAMPLES FROM COMMERCIAL PLANTINGS

| Isolates | ELISA | | Bi-directional PCR | |
|---|---|---|---|---|
| | PAbs[z] | MCA13[y] | 400 bp[x] | 300 bp[w] |
| USS1 | + | - | + | - |
| USS2 | + | - | + | - |
| USS3 | + | - | + | - |
| USS4 | + | - | + | + |
| USS7 | + | - | + | - |
| USS8 | + | - | + | - |
| USS9 | + | - | + | + |
| USS10 | + | - | + | + |
| USS18 | + | - | + | + |
| USS24 | + | - | + | + |
| USS25 | + | - | + | + |

[z]Polyclonal antibody that reacts with all strains of CTV.
[y]A monoclonal antibody that reacts predominantly with severe strains of CTV.
[x]The size of the DNA fragment amplified from generally mild strains of CTV.
[w]The size of the DNA fragment amplified from generally severe strains of CTV.

TABLE 2
OLIGONUCLEOTIDE PRIMERS USED FOR BD/PCR TO DETECT AND DIFFERENTIATE MILD AND SEVERE STRAINS OF CTV

| Primers | Sequence | Tm (°C)[z] | Product size |
|---|---|---|---|
| CN119 | 5' AGATCTACCATGGACGACGAAACAAAG 3' | 52 | 700 bp[y] |
| CN120 | 5' GAATTCGCGGCCGCTCAACGTGTGTTAAATTTCC 3' | 54 | 700 bp[y] |
| CN218 | 5' TTTGGACTGACGTCGTGTT 3' | 56 | 300 bp[x] |
| CN219 | 5' TTACCAATACCCTTAGAATTAT 3' | 56 | 400 bp[w] |

[z]Melting point temperatures (Tms) calculated by the equation Tm = 4 × (number of GC pairs) + 2 × (number of AT pairs).
[y]The approximate product size of the primer pair CN119 and 120.
[x]The approximate product size of the primer pair CN120 and 218.
[w]The approximate product size of the primer pair CN119 and 219.

bark tissue were prepared (see accompanying paper by Cevik et al.), flash-frozen in liquid nitrogen and stored at -80°C until used. To prepare known mixtures of T30 and T36, equal volumes of the supernatants were mixed prior to Sephadex G-50 column chromatography.

**Bi-directional reverse transcription polymerase chain reaction (BD/PCR).** A single two-step RT/PCR protocol with two internal(CN218 and CN219) and two terminal (CN119 and CN120) primers, was applied to amplify the full-length CPG (~700 bp) and its smaller (400 and 300 bp) strain specific fragments (Fig. 1). BD/PCR amplification was conducted in 100 µl of reaction mixture containing 1X PCR buffer (10X solution: 500 mM KCl, 100 mM Tris-HCl pH 9.0 at 25°C, and 1% Triton X-100), 10 mM DTT, 1.5 mM $MgCl_2$, 50 µM of each deoxyribonucleotide triphosphate (dNTP) (U. S. Biochemical), 10 units of RNAsin (Promega), 2.5 units of *Taq* DNA polymerase (Promega), 10 units of AMV reverse transcriptase (Promega), 200 ng of each internal and terminal primer, and 40-50 µl of template extract pre-heated at 70°C for 5 min. The temperature cycler, Coy Tempcycler Model 60 (Coy Laboratory Inc.), was programmed for synthesizing first strand cDNA at 50°C for 45 min. For amplification: 94°C for 1 min denaturation; 50°C for 2 min annealing of the primers; 72°C for 1 min elongation for 40 cycles, followed by one cycle of denaturation for 1 min and then extended elongation at 72°C for 10 min. To eliminate the full-length CPG and to amplify only the strain specific DNA fragments, the concentrations of terminal primers were reduced to 40 ng, and extension times were decreased to 30 sec. The other conditions remained the same. This is referred to as modified BD/PCR.

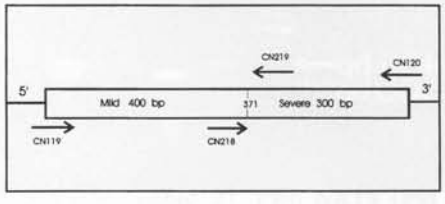


**Fig. 1. The positions of the oligonucleotide primers used for BD/PCR amplification of mild and severe strain specific fragments from the CPG of CTV.**

**Analysis of the BD/PCR amplification products.** Aliquots (10 µl) of each BD/PCR reaction and a standard (0.5 g of 100 bp DNA ladder) were separated in a 1% (w/v) agarose gel containing ethidium bromide (10 ng/ml) by electrophoresis in TBE buffer with constant power at 100 V for 1 hr. The products were visualized over a UV transilluminator and photographed using Polaroid 57 film.

**Western blot analysis.** A small amount (1 $cm^2$) of CTV-infected cit-

rus leaf tissue was chopped into fine pieces with a razor blade. The tissue was then transferred into a 1.5-ml microfuge tube containing 0.5 ml 2X western extraction buffer (0.125 M Tris-Cl pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol). The tissue was further crushed with an applicator stick in the extraction buffer, boiled for 3 min, vortexed for 15-20 sec, and centrifuged for 30 sec. A 10 l aliquot of the extract was loaded into a 12% SDS polyacrylamide gel and separated by electrophoresis (20). The separated proteins were then transferred to a nitrocellulose membrane (Stratagene Inc., La Jolla, CA.) using a Mini Transblot electrophoretic liquid transfer cell (Bio-Rad Inc., Richmond, CA) as suggested by the manufacturer. Specific antibody was applied and developed as previously described Still et al. (20).

## RESULTS

**BD/PCR amplification.** Using the normal BD/PCR, a full-length CPG (~700 bp) and a 400 bp fragment were produced with the mild T30 strain (Fig. 2A, lane 2). A 700 bp product along with a 300 bp fragment were amplified from the severe T36 strain (Fig. 2A, lane 3). From the mixed T30 and T36 template, the 700 bp, 400 bp and 300 bp DNA fragments were all produced (Fig. 2A, lane 5).

In the modified method of BD/PCR, the concentration of terminal primers and extension times were decreased. Under these conditions, the full-length CPG was no longer produced. Only the 400 bp fragment was produced with mild T30 strain (Fig. 2B, lane 2), and the 300 bp DNA fragment was amplified with the severe T36 strain (Fig. 2B, lane 3). When the mixture of both strains was used as a template, both the 400 and the 300 bp fragments were produced (Fig. 2B, lane 5). No DNA fragments were produced from the healthy tissue extract in either application of BD/PCR. (Fig. 2A and B, lanes 4).

**Testing of CTV infected field samples with BD/PCR.** The modified BD/PCR method was applied to test naturally infected field samples that were all CTV positive, but

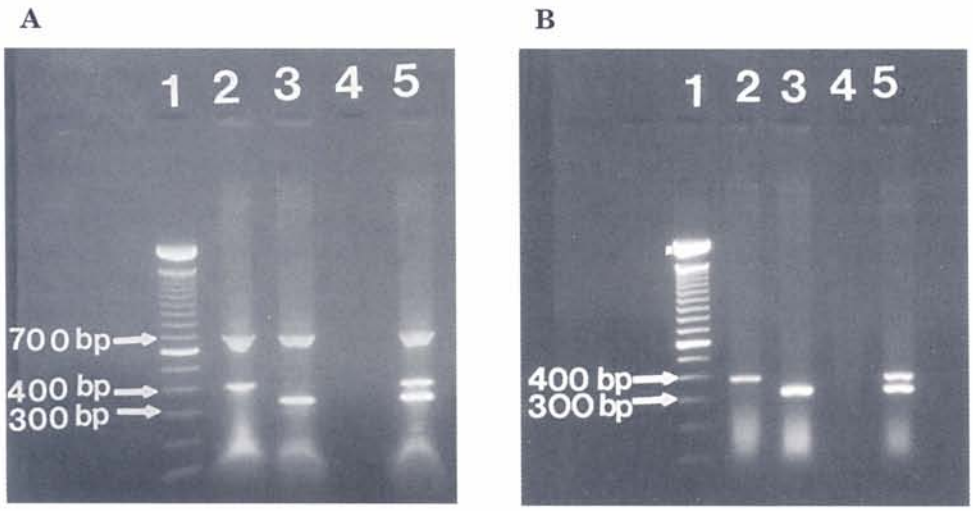


**Fig. 2. DNA fragments amplified by A) normal BD/PCR and B) modified BD/PCR, and separated on 1% agarose by electrophoresis and stained by ethidium bromide. Lane 1 is 100 bp DNA ladder marker, lane 2 is BD-PCR amplification from T30, lane 3 is from T36, lane 4 is from healthy tissue and lane 5 is from mixture of T30 and T36.**

MCA13 negative by ELISA, suggesting that they all were infected with only mild strain(s) of CTV. Mild strain T30, severe strain T36, artificial mixtures of T30 and T36, and a healthy sample collected from greenhouse grown citrus plants were used as positive and negative controls. From some of these samples, USS4, USS9, USS10, USS18, USS24 and USS25, both the 400 and 300 bp DNA fragments were amplified, indicating that some of these trees were infected with both mild and severe strains of CTV (Fig. 3, lanes i, l, m, n, o and p, respectively). From the rest of the samples only the 400 bp fragment was amplified (Fig. 3, lanes f, g, h, j and k).

**Western blot analysis of CTV field samples.** Three samples were selected for western blot analysis. These were USS1 which tested MCA13 negative in ELISA and did not produce the 300 bp strain specific fragment, and USS24 and USS25 which also tested MCA13 negative in ELISA, but did produce the 300 bp strain specific DNA fragment in BD/PCR. The mild strain T30 and uninfected tissue, the negative controls, did not react with MCA13 (Fig. 4, lanes 5 and 6, respectively), nor did samples USS1 or USS24 (Fig. 4, lanes 2 and 3, respectively). However, both the severe strain T36 (the positive control), and the sample USS25, which produced the strongest 300 bp severe strain specific DNA fragment in BD/PCR, reacted positive with MCA13 (Fig. 4, lanes 1 and 4, respectively). The intensity of the band produced by USS25 was much lower than the band produced by the T36 control (Fig. 4, lanes 4 and 1, respectively). This indicates that the titer of the MCA13 reactive strain in sample USS25 was much lower than in T36. This also may explain why the sample USS24 did not react with MCA13 in ELISA or the western blot, although it did produce 300 bp product in BD/PCR (Fig. 3, lanes n and o).

## DISCUSSION

A BD/PCR method was developed based on the single nucleotide change at the MCA13 epitope position 371 in the nucleotide sequence

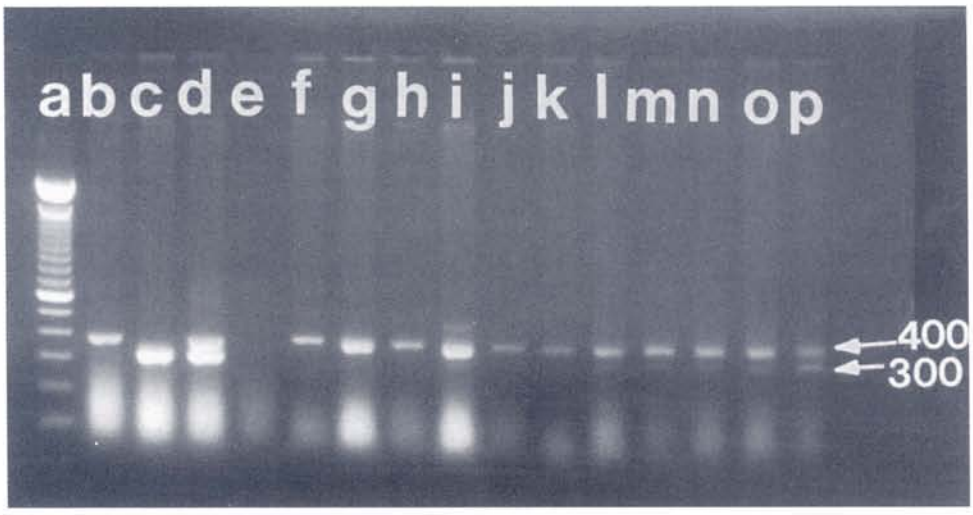


**Fig. 3. Analysis of BD/PCR amplification products of CTV field samples. Lane a is the 100 bp DNA size marker and the lanes b, c, d, and e are T30, T36, healthy and mixture of T30 and T36, respectively. The lanes f through p are product from field samples USS1 (f), USS2 (g), USS3 (h), USS4 (I), USS7 (j), USS8 (k), USS9 (l), USS10 (m), USS18 (n), USS24 (o) and USS25 (p).**

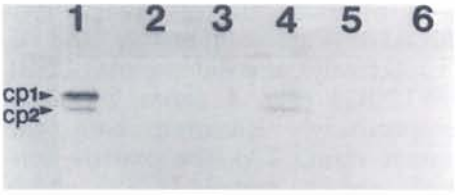


**Fig. 4. Western blot analysis of CTV infected field samples with MCA13. The lane 1 is T36 as a positive control. Lanes 2, 3, and 4 are USS1, USS24 and USS25, respectively. Lane 5 is T30, and lane 6 is healthy tissue as negative controls.**

of the CTV CPG. This method was used for detection and differentiation of MCA13 positive and MCA13 negative strains of CTV. Although this method was developed utilizing only a single nucleotide difference between CPGs of both types of strains of CTV, it has several advantages over serological methods utilizing MCA13 and some other previously reported methods for strain differentiation.

First, BD/PCR is able to determine the presence or absence of CTV in an infected tree, and if CTV is present, it also can determine simultaneously whether the tree is infected with an MCA13 positive or MCA13 negative strain or both strains of CTV in a single reaction in one tube. In addition, the strain specific DNA fragments can be amplified from crude nucleic acid extracts prepared from very small quantities of fresh or desiccated leaf tissue without laborious and expensive purification procedures. Like ELISA, the entire procedure of BD/PCR can be completed in a 5-hr period, thus, is easy and rapid enough to apply to at least a moderate scale of testing. Its drawback is that it is expensive. In comparison, the other methods used for detection and differentiation of CTV strains such as dsRNA analysis (5), cDNA hybridization (17), and peptide map analysis (6) are more laborious and complex. Some of them require more material and time to perform and they are not adaptable to rapid, large scale assays.

Second, since only a small portion of the BD/PCR amplification product is used for detection purposes, the remaining product can be used for cloning and sequencing or for other DNA-based assays such as nucleic acid hybridization.

Finally and most importantly, the BD/PCR method is more sensitive than conventional methods for detection and differentiation of CTV strains. Testing of the field samples with BD/PCR showed that some samples which did not react with MCA13 in ELISA because of low virus titer could be detected by BD/PCR since the target molecule was amplified. In other studies, it also has been reported that PCR methods are, in general, 10-100 times more sensitive than ELISA or western blot analyses (7, 8).

The BD/PCR method can be used for detection of mixed strain infection in a single tree. Thus, it might provide useful information about the kinetics of cross protection and accelerate the identification of mild strains useful for cross protection. It could be utilized to screen cross protected trees in the field, to test for the presence of purposefully inoculated mild strains, and the absence of severe strains in valuable budwood source trees prior to propagation.

One deficiency of the BD/PCR approach is its dependence on the single T residue at position 371 in the CPG to reveal the presence of severe strains. Severe strains containing a different nucleotide at that position would not be detected by either MCA13 or its epitope sequence-based oligonucleotide CN218 used in BD/PCR.

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