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A New Method for the Detection of Minor Populations of Citrus Tristeza Virus Strains Infecting Single Citrus Trees

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Abstract: Fifteen different biologically important citrus tristeza virus (CTV) isolates (B14, B15, B23, B31, B52, B57, T36, D9, D10, D11, D12, D15, D16, D20 and D32) were tested using strain-specific biotin-labeled oligonucleotide probes. Coat protein genes (CPGs) of the samples were amplified by PCR, and the products were hybridized with probes (direct PCR hybridization (DPH)) and sequenced. In the DPH, all samples having probe 0 reactions were infected with CTV. Some isolates giving more than one specific probe reaction showed that they may be infected by mixtures of different CTV strains. In order to investigate this, CPGs from individual bacterial colonies were used as PCR templates and the PCR products were tested. This method developed in this study was called colony PCR hybridization (CPH). It was found that samples such as B57, which were apparently mixtures of strains, were indeed mixtures and other samples, which apparently contained a single strain (B23, D9, D11 and D16), actually contained one or more additional strains not detectable by DPH or other methods. CPH is a sensitive method for the detection and differentiation of CTV strains infecting single citrus trees.

Key Words: CTV, Probes, PCR, hybridization, Colony PCR hybridization.

Turunçgil Ağaçlarında İnfeksiyona Neden Olan Düşük Yoğunluktaki Tristeza Virus Izolatlarının Yeni Bir Yöntem ile Tespiti

Özet: Bu çalışmada, biyolojik olarak önemli ve farklı turunçgil tristeza virüsünün (CTV) 15 izolatı (B14, B15, B23, B31, B52, B57, T36, D9, D10, D11, D12, D15, D16, D20, D32), biotinle işaretlenmiş oligonukleotid problar kullanılarak test edildi. Bu ırkların, kılıf protein genleri PCR ile çoğaltıldı, problarla hibridizasyonu (Doğrudan PCR Hibridizasyonu-DPH) ve sequence analizleri yapıldı. Bunun sonucunda, tüm örnekler Probe 0 ile reaksiyon girerek, CTV ile bulaşık oldukları tespit edildi. Bazı izolatların birden fazla probe ile reaksiyona girmesi, bu izolatların farklı CTV ırklarını birarada karışım olarak bulundurabileceğini gösterdi. Bunu araştırmak amacıyla, herbir bakteri kolonisinden elde edilen kılıf protein geni kullanılarak PCR yapıldı ve PCR ürünleri test edildi. Bu çalışmada geliştirilen bu methoda Koloni PCR Hibridizasyonu (KPH) adı verildi. Bu method ile, değişik CTV virus ırklarından oluştuğu tahmin edilen B57 izolatının durumu belirlendi ve diğer izolatlarında (B23, D9, D11 and D16), daha önce uygulanan yöntemlerle tespit edilemeyen bir yada birden fazla düşük yoğunluktaki virus ırkını içerdigi ortaya kondu. KPH , tek bir turunçgil ağacında bulunan karışık CTV ırklarının tespit edilmesinde ve bu ırkların birbirinden ayrılmasında oldukça hassas bir yöntemdir.

Anahtar Sözcükler: CTV, Probes, PCR, hibridizasyon, Koloni PCR hibridizasyon

Introduction

Citrus tristeza virus (CTV) is one of the most destructive virus diseases of citrus in the world (Bar-Joseph et al., 1989). It is a phloem-limited closterovirus and is transmitted by aphids in a semi-persistent manner (Roistacher and Bar-Joseph, 1987). CTV and the common vector, *Aphis gossipii*, Glover, have been known in Turkey for many years without causing any serious damage in citrus orchards, but it remains a serious potential threat to citrus in Turkey, because of the use of sour orange as a rootstock (Cinar et al., 1993). CTV consists of several different strains causing distinct symptoms on different hosts. The biological properties of CTV strains can be separated into 5 major groups as mild, seedling yellows, quick decline on sour orange, stem-pitting on grapefruit and stem-pitting on sweet orange (Garnsey et al., 1987). If the host is infected by more than one strain of CTV, the disease can involve any combination of these symptoms.

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Several different procedures have been developed to diagnose CTV, such as symptom development on differential indicator plant hosts (Garnsey et al., 1987), monoclonal antibodies (MCA 13) (Vela et al., 1986; Permar et al., 1990), DNA probe hybridization (Rosner et al., 1986), double-stranded RNA (dsRNA) analysis (Dodds et al., 1987), polypeptide map analysis (Guerri et al., 1990), restriction fragment length polymorphism (RFPL) analysis (Gillings et al., 1993; Akbulut, 1995), single aphid transmission (Broadbent et al., 1996), and bi-directional reverse transcription polymerase chain reaction (Cevik, 1995).

Nucleic acid hybridization has been used for the detection and/or differentiation of many plant pathogens. A number of hybridization methods utilizing both radioactively or non-radioactively labeled cDNA, and oligonucleotide probes have been reported for the differentiation of strains of specific viruses or related viruses, including CTV (Rosner et al., 1986), potato virus Y (Singh et al., 1995) and gemini viruses (Gilbertson et al., 1991).

Non-radioactive nucleic acid probes are labeled or detected with an enzyme, which releases a colorimetrically detectable substrate. They are able to hybridize to their complementary sequence of nucleic acids. This technique was developed for CTV by Cevik (1995). The probes are specific to the strain positions for which they were developed, and depend on 1-2 nucleotide differences in specific positions of CPG. Probe 0 is a "universal" probe containing a conserved sequence in the CP gene of all known CTV strains and recognizes them. Probe I hybridizes the quick decline inducing strain, T36. Probe II hybridizes strains that primarily cause severe stem-pitting on sweet orange, B53. Probes III, IV and V hybridize with different groups of stem-pitting strains, B185, T3, and B249, subsequently. Probe VI hybridizes with mild strains from Florida, T30. Probe VIII hybridizes with all mild strains of CTV (Cevik, 1995). Although these methods are all sensitive and reliable, they can only differentiate mild and severe strains of CTV. There is no method available to differentiate between mild and severe strains.

The objective of this study was to develop a sensitive technique for the detection of minor populations of CTV strains present in a single infected citrus tree using strain-specific probes. Undetected minor populations of CTV strains can be important in nurseries and in mild strain cross protection. Furthermore, such minor populations may become the dominant populations depending upon the impacts of environmental conditions, host species, and graft or aphid transmission.

Materials and Methods

CTV Isolates: Six CTV isolates (B14, B15, B23, B31, B52, and B57) were obtained from the Exotic Citrus Pathogens Collection in Beltsville, MD, USA. The biological and serological properties of these isolates are given in Table 1. Eight citrus trees of the DPI (Division of Plant Industry, FL, USA) fruit fly trap line were sampled as D9, D10, D11, D12, D15, D16, D20 and D32, collected from Delray Beach in Florida. D20 and D32 were collected from Key Lime and the others were collected from Calamondin. Isolate T36, used as a control, causes quick decline of sweet orange on sour orange rootstock and induces seedling yellows on sour orange and grapefruit, was provided by Dr. R. F. Lee, CREC, Lake Alfred, FL, USA.

ELISA: A few terminal leaves of each isolate were collected for ELISA. Midveins of terminal leaves were processed by a tissue pulverizer (Model 4200; KLECO, Visilia, CA, USA). ELISA was performed using the double-antibody sandwich indirect (DAS-I) method (Garnsey and Cambra, 1991). The test was run with monoclonal antibody 13 antiserum (MCA 13), provided by Dr. Keremane Manjunath, which detects CTV strains causing a decline or stem-pitting in Florida. ELISA optical density at 405 nm ($OD_{405\ nm}$) readings that was greater than 2 times the values for healthy citrus extracts was considered positive.

Oligonucleotide Primers: The primers were designed as complementary to 5' and 3' ends of the coat protein gene and the restriction enzyme sites for *Xba*I and *Eco*RI were added to the 5' end of the primers CN150 and CN151 to facilitate the cloning process. The sequences of the primers were: CN150 5' ATATATTTACTCTAG ATCTACCATGGACGACGAAACAAA 3' and CN151: 5' GAATCGGAACGCGAATCCTCAACGTGTGTTAAATTTCC 3'.

Nucleic Acid Extraction: Approximately 1 cm^2 leaf tissue was quick frozen in liquid nitrogen, and ground to powder in a microfuge tube (Sambrook et al., 1989). Then 300 µl of extraction buffer (0.1 M Tris-HCl pH 8.0, 0.2 mM EDTA and 2% SDS) was added, and stirred, and then 300 µl of phenol-chloroform (1/1, v/v) was added.

Table 1. Biological and serological properties of selected CTV isolates (data provided by Beltsville Agricultural Research Center, MD, USA).

Origin and Donor Identification[a]	BARC Code[b]	G-604[c]	ELISA (MCA-13)[d]	Decline[e]	SY[e]	SP[e]	Aphid Transmission[f]
Brazil							
1932/534	B14	+	+	+	+	+	+
1932/126	B15	+	+	+	+	+	-
Israel							
Mor 4-8	B23	+	+	-	-	-	+
Hawaii							
Kauai #1	B57	+	-	ND	ND	ND	ND
Japan							
HS-34	B31	+	+	-	-	+	+
Florida							
T55	B52	+	-	-	-	-	+
T36	B3	+	+	+	+	+	-

[a] Identification was by a local scientist and represents isolate characterization according to local standards.
[b] Code number assigned to isolates housed in the CTV collection under quarantine at the Beltsville Agricultural Research Center (BARC).
[c] G-604 = Reaction with the polyclonal antibody G-604 (- = no reaction, + = positive reaction).
[d] MCA13 = Reaction with the monoclonal antibody MCA13.
[e] General reactions of isolate on diagnostic indicator plants based on information from cooperator and data from assays at Beltsville. Decline in sweet orange on sour orange rootstock; seedling yellows (SY) in sour orange; stem-pitting (SP) in Duncan grapefruit; and/or cv. Madam Vinous sweet orange.
[f] Transmission test conducted with either 10 or 20 aphids (*Aphis gossypii*) per Mexican lime receptor plant.
ND = no data available

The mixture was vortexed and heated at 70° C for 5 min, and centrifuged for 5 min at room temperature. The supernatant was passed through a 1 ml Sephadex G50 column, and stored at -80 °C.

Reverse Transcription (RT) and Polymerase Chain Reaction (PCR) of Coat Protein Genes (CPGs): A single tube, two 2 RT-PCR reaction was used to amplify the CPGs (Pappu et al., 1993). The RT-PCR reaction was prepared in 50 µl of mixture containing 10 X PCR buffer, 2.5 mM of $MgCl_2$, 10 mM of DTT, 200 µM of each dNTP, 10 units of RNAsin (Promega Inc. WI), 2.5 units of Taq DNA polymerase (Promega), 15 units of reverse transcriptase (Promega), 1 µl of each primer (1 µg μl^{-1}), and 31 µl of plant extract, heated at 70 °C for 5 min before adding to the mixture. The cycler (Biometra UNO II Thermoblock) was programmed for synthesizing first cDNA at 42 °C for 1 h and then for PCR amplification as follows: 94 °C for 1 min 30 s, 45 °C for 1 min, 72 °C for 2 min 30 s and 40 cycles followed by 1 cycle of denaturation for 30 s then extended at 72 °C 10 min.

Cloning and sequencing of the CPGs: Amplified DNA products were purified using Qiagen PCR purification kits according to the manufacturer's instructions. Fifteen microliters of purified CPG products was ligated into the pGEM-T plasmid vector using 10 units of T4 DNA ligase, 2 µl of 10X ligation buffer and 1 µl of plasmid vector in a total reaction volume of 20 µl by incubating the mixture at 16 °C overnight. Competent cells of *E. coli* DH5α strain were transformed with the recombinant pGEM-T plasmid vector by heat-shocking at 42 °C for 90 s. The recombinant colonies were identified by color on media containing X-gal and tested for the presence of the CPGs as described previously (Pappu et al., 1993).

PCR Screening of the Bacterial Colonies: After recombinant colonies were identified by their white color, a small portion of each colony was transferred using a sterile toothpick to a microfuge tube containing 50 µl of 1% Triton-X100, 20 mM of Tris-HCl pH 8.5, and 2 mM of EDTA, boiled for 15 min and then cooled on ice. The

bacterial debris was removed by centrifugation at 14,000 rpm for 10 s at 4 °C and 0.5 µl of the supernatant was used for 50 µl PCR reactions. The PCR reactions were performed in 50 µl reaction mixture containing 10X PCR buffer, 2.5 mM of $MgCl_2$, 10 mM of DTT, 200 µM of dNTP, 2.5 units of Taq polymerase and 1 µl of each primer (1µg μl^{-1}). The thermocycler was programmed for PCR amplification as follows: 94 °C for 1 min 30 s, 45 °C for 1 min, 72 °C for 2 min 30 s and repeated for 40 cycles followed by one cycle of denaturation for 30 s then extended elongation at 72 °C for 10 min.

Dot Blotting and Hybridization of CTV CPGs with Oligonucleotide Probes: DNA was diluted in a final volume of 50 µl of 20 X sodium saline citrate (SSC). Samples were applied to a damped positively charged nylon membrane in the dot blot apparatus. DNA was denatured by incubating the membrane in 0.4 NaOH for 10 min at room temperature with gentle shaking. It was neutralized by incubating the membrane in 0.2M Tris-Cl pH 8.0, 0.1% SDS and 1X SSC at room temperature for 10 min, then DNA was fixed to the membrane by UV cross linking. Prehybridization of the membrane was performed for all probes at 37 °C for 1 h with gentle shaking in a hybridization bag containing 0.1 ml of the prehybridization solution. Additionally 0.2 mg of salmon sperm DNA per ml of the prehybridization solution was added to the hybridization bag. After 1 hour, 50 ng of probe was added to the prehybridization solution and hybridized overnight at 37 °C with gentle shaking. The membrane was washed twice with 6 X SSC and a stringent wash with 4 X SSC and 1% SDS was performed at 42 °C to remove the nonspecific bound probes (Cevik, 1995). A summary of the sequences and biological activity of strain specific CTV probes is given in Table 2.

Detection of Biotinylated Probes on Nylon Membrane: Streptoavidin-horseradish peroxidase conjugate was used according to the manufacturer's instructions. A amount of 0.1 ml conjugate was used per cm^2 of the membrane with gentle shaking for 45 min. The membrane was rinsed several times with TBS-T. Supersignal chemiluminescence substrate was used for the detection of horseradish peroxidase labeled streptoavidin-biotin complex. Then the membrane was exposed to X-ray film. To re-probe the membrane, it was first incubated at 0.4 NaOH at 42 °C for 30 min and then in a neutralization solution at 42 °C for 30 min. The membrane was then rinsed in 6 X SSC and prehybridized and hybridized with another probe (Cevik, 1995).

Table 2. Summary of the sequence and biological activity of strain specific CTV probes developed by Cevik (1995). Probe sequences are protected by United States Patent # 6869761.

Strain Specific Probe	Type Strain	Sequence	Symptoms
Probe 0	Universal	TTACACATCGATCC	Recognize all CTV strains
Probe I	T36	ACTTGTGTGCGGATTTC	Decline on sour orange, seedling yellows
Probe II	B53	ATGAATGACGTGCGTC	Decline on sour orange, seedling yellows, stem-pitting on grapefruit
Probe III	B185	AGGGCGTCGAAGTGG	Decline on sour orange, seedling yellows, stem-pitting on grapefruit
Probe V	B249	ACGATGATACCACGGGTGT	Decline on sour orange, seedling yellows, stem-pitting on grapefruit and sweet orange
Probe VIII	T30	ACCGACATCGTGTATAA	Recognize mild Florida strains and all known mild CTV strains

Results and Discussion

Population Diversity within CTV Isolates from Single Citrus Trees: ELISA (MCA 13) reactions and direct PCR hybridizations (DPH) of CPG products are shown in Table 3. It was demonstrated that all samples were infected with CTV by having strong probe 0 reactions and then successively with other probes. Isolates B14 and B15 hybridized with probes III and V, but no reaction was detected with any other probe. B23 hybridized only with probe III. Isolate B31 hybridized with probes II, III, and VIII. B52 hybridized with probes VI and VIII, and B57 hybridized with probes V and VIII. Except for isolate B23, all 6 isolates seemed to contain the mixtures of at least 2 strains, which occurred in sufficient concentration to be detectable by DPH. PCR products of the other isolates strongly hybridized only with probe III, however, D-11 and D-16 were later shown to contain mixtures.

Hybridization of PCR products to more than a single specific probe demonstrates population diversity within the biologically and geographically different CTV isolates. It is also indicated that the trees from which B14, B15, B31, and B57 isolates were obtained were infected with several strains of sufficiently high concentrations to be detected directly by hybridization to their PCR products.

Detecting Minor Populations of CTV in Single Citrus Trees: Seventeen individual colonies of B14 and 14 individual colonies of B15 were hybridized with biotin-labeled oligonucleotide probes (Table 3) and all reacted only with probe V and none with probe III. This indicates that the trees from which B14 and B15 were isolated were probably infected with 2 strains differing in their concentration. One clone from each isolate representing the probe V reaction, B14-6 and B15-30, were sequenced in order to see the probing sites (Figure 1). B23 is one of the more interesting isolates studied. When the PCR product of B23 was hybridized, it only reacted with probe III (Table 3). When the 21 individual colonies were screened, 3 hybridized with probe I, 10 with probe III and 8 with both probes VI and VIII (Figures 1 and 2). This indicates that a low copy number of strains reacting with probe I, III, VI and VIII were present below the level of detectability by direct PCR hybridization (DPH).

Table 3. Results of ELISA and population complexity of CTV isolates detected by direct PCR hybridization (DPH) and colony PCR hybridization (CPH).

Isolates[a]	ELISA[b] (MCA13)	DPH[c]	CPH[d]							
			Probe 0	Probe I	Probe II	Probe III	Probe IV	Probe V	Probe VI	Probe VIII
B14	+	0,III,V	17	0	0	0	0	17	0	0
B15	+	0,III,V	14	0	0	0	0	14	0	0
B23	+	0,III	21	3	0	10	0	0	8	8
B31	+	0,II,III,VIII	15	0	15	0	0	0	0	0
B52	+	0,VI,VIII	24	0	0	0	0	0	24	24
B57	+	0,V,VI, VIII	14	0	0	0	0	13	1	1
T36	+	0,I	14	14	0	0	0	0	0	0
D9	?	0,III	19	0	0	19	0	0	0	0
D10	?	0,III	9	0	0	9	0	0	0	0
D11	+	0,III	19	1	0	18	0	0	0	0
D12	?	0,III	13	0	0	13	0	0	0	0
D15	+	0,III	31	0	0	31	0	0	0	0
D16	-	0,III	33	0	0	31	0	0	2	2
D20	+	0,III	17	0	0	17	0	0	0	0
D32	+	0,III	18	0	0	18	0	0	0	0

[a] B code number isolates are from the Collection of Exotic Citrus Pathogens maintained under quarantine in Beltsville, MD, USA; D code isolates are from the field in Delray Beach in South Florida. T36 is a well known Florida quick decline strain.

[b] Enzyme-linked immunosorbent assay (ELISA), notation + = positive, - = negative, ? = questionable, borderline

[c] Direct PCR Hybridization (DPH) products from CTV infected samples with strain-specific probes.

[d] Colony PCR hybridization (CPH) of the strain-specific probes with clones. Number of clones hybridized with each probe is shown.

Cloning of B23 PCR products and then screening the CPGs that had increased in bacteria revealed that frequently there were low copy number strains of CTV present in the infected tree. Probe VI detects Florida mild strains and probe VIII detects mild strains all over the world (general mild). Only one clone was picked to represent the mild strain in B23 isolate. Clones B23-2, B23-7, and B23-15 representing probes III, I and VIII reactions were sequenced (Figure 1).

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                 Probe 0 site                    Probe II site

          90                                                   150
T36-15    GTAAACTTACACATCGATCCGACTTTGATAACGATGAACGATGTGCGTCAGTTGAGTACC
B14-6     GTAAACTTACACATGGATCCGACTCTGATAGCGATGAACGATGTGCGTCAGTTGGGTACC
B15-30    TTAAACTTACACATGGATCCGACTCTGATAGCGATGAACGATGTGCGTCAGTTGGGAACC
B23-2     GTAAACTTACACATGGATCCGACTTTGATAACGATGAACGATGTGCGTCAGTTGAGTACC
B23-7     GTAAACTTACACATGGATCCGACTTTGATAACGATGAACGATGTGCGTCAGTTGAGTACC
B23-15    GTAAACTTACACATGGATCCGACTCTGATAACGATGAACGATGTGCGTCAGTTGAGTACC
B31-12    ATAAACTTACACATGGATCCAACTCTGATAGCGATGAATGACGTGCGTCAGTTGGGTACC
B52-27    GTAAACTTACACATGGATCCGACTCTGATAACGATGAACGATGTGCGTCAGTTGAGTACT
B57-7     GTAAACTTACACATGGATCCGACTCTGATAACGATGAACGATGTGCGTCAGTTGGGTACT
B57-31    GTAAACTTACACATGGATCCGACTCTGATAGAGATGAACGATGTGCGTCAGTTGGGTACC
D9-5      GTAAACTTACACATGGATCCGACTTTGATAACGATGAACGATGTGCGTCAGTTGAGTACC
D10-10    GTAAACTTACACATGGATCCGACTTTGATAACGATGAACGATGTGCGTCAGTTGAGTACC
D11-5     GTAAACTTACACATGGATCCGACTTTGATAACGATGAACGATGTGCGTCAGTTGAGTACC
D12-12    GTAAACTTACACATGGATCCGACTTTGATAACGATGAACGATGTGCGTCAGTTGAGTACC
D15-16    GTAAACTTACACATGGATCCGACTTTGATAACGATGAACGATGTGCGTCAGTTGAGTACC
D16-3     GCAAACTTACACATGGATCCGACTTTGATAACGATGAACGATGTGCGTCAGTTGAGTACC
D16-43    GTAAACTTACACATGGATCCGACTCTGATAACGATGAACGATGTGCGTCAGTTGAGTACT
D16-44    GTAAACTTACACATGGATCCGACTCTGATAACGATGAACGATGTGCGTCAGTTGAGTACT
D20-8     GTCAACTTACACATGGATCCGACTTTGATAACGATGAACGATGTGCGTCAGTTGAGTACC
D32-5     GTAAACTTACACATGGATCCGACTTTGATAACGATGAGCGATGTGCGTCAGTTGAGTACC

                 Probe VI site                   Probe V site

          241                                                  300
T36-15    GCTATGATGTTGTATCGTTTAGCAGTTAAGAGTTCATCATTACAAAGCGATGACGACGCC
B14-6     GCTATGATGTTGTATCGTTTAGCAGTTAAGAGTTCATCGTTACAAAGCGACGATGATACC
B15-30    GCTATGATGTTATATCGTTTAGCGGTTAAGAGTTCATCATTACAAAGCGACGATGATACC
B23-2     GCTATGATGTTGTATCGTTTAGCAGTTAAGAGTTCATCATTACAAAGCGATGACGACGCC
B23-7     GCTATGATGTTGTATCGTTTAGCAGTTAAGAGTTCATCATTACAAAGCGATGACGACGCC
B23-15    GCTATGATGTTATACCGATTAGCGGTTAAGAGTTCATCATTGCAAAGTGATGATGACACC
B31-12    GCTATGATGTTGTATCGTTTAGCAGTTAAGAGTTCATCATTACAAAGCGATGACGACACT
B52-27    GCTATGATGTTATACCGATTAGCGGTTAAGAGTTCATCATTGCAAAGTGATGATGACACC
B57-7     GCTATGATGTTATACCGATTAGCGGTTAAGAGTTCATCATTGCAAAGTGATGATGACACC
B57-31    GCTATGATGTTGTATCGTTTAGCAGTTAAGAGTTCATCGTTACAAAGCGACGATGATACC
D9-5      GCTATGATGTTGTATCGTTTAGCAGTTAAGAGTTCATCATTACAAAGCGATGACGACGCC
D10-10    GCTATGATGTTGTATCGTTTAGCAGTTAAGAGTTCATCATTACAAAGCGATGACGACGCC
D11-5     GCTATGATGTTGTATCGTTTAGCAGTTAAGAGTTCATCATTACAAAGCGATGACGACGCC
D12-12    GCTATGATGTTGTATCGTTTAGCAGTTAAGAGTTCATCATTACAAAGCGATGACGACGCC
D15-16    GCTATGATGTTGTATCGTTTAGCAGTTAAGAGTTCATCATTACAAAGCGATGACGACGCC
D16-3     GCTATGATGTTGTATCGTTTAGCAGTTAAGAGTTCATCATTACAAAGCGATGACGACGCC
D16-43    GCTATGATGTTATACCGATTAGCGGTTAAGAGTTCATCATTGCAAAGTGATGATGACACC
D16-44    GCTATGATGTTATACCGATTAGCGGTTAAGAGTTCATCATTGCAAAGTGATGATGACACC
D20-8     GCTATGATGTGTTATCGTTTAGCAGTTAAGAGTTCATCATTACAAAGCGATGACGACGCC
D32-5     GCTATGATGTGTTATCGTTTAGCAGTTAAGAGTTCATCATTACAAAGCGATGACGACGCC
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Figure 1. Multiple alignment of the nucleotide sequences of the CPGs of several CTV isolates, showing probe sites. The sequence from reacting with specific probe is highlighted.

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                Probe V site          Probe III site          Probe VIII site

              301                                                          360
T36-15        ACGGGTATAACGTACACTCGGGAGGGTGTTGAAGTGGATTTGTCTGACAAACTTTGGACT
B14-6         ACGGGTGTGACGTACACTCGGGAGGGTGTTGAAGTGGATTTGTCTGACAAACTTTGGACT
B15-30        ACGGGTGTGACGTACACTCGGGAGGGTGTTGAAGTGGATTTGTCTGACAAACTTTGGACT
B23-2         ACGGGTATAACGTACACTCGGGAGGGCGTCGAAGTGGATTTGTCTGACAAACTTTGGACT
B23-7         ACGGGTATAACGTACACTCGGGAGGGTGTTGAAGTGGATTTGTCTGACAAACTTTGGACT
B23-15        ACGGGTATAACGTACACTCGGGAGGGTGTTGAAGTGGATTTGTCTGACAAACTTTGGACC
B31-12        ACGGGTATAACGTACACTCGGGAGGGTGTTGAAGTGGATTTGTCTGACAAACTTTGGACT
B52-27        ACGGGTATAACGTACACTCGGGAGGGTGTTGAAGTGGATTTGTCTGACAAACTTTGGACC
B57-7         ACGGGTATAACGTACACTCGGGAGGGTGTTGAAGTGGATTTGTCTGACAAACTTTGGACC
B57-31        ACGGGTGTGACGTACACTCGGGAGGGTGTTGAAGTGGATTTGTCTGACAAACTTTGGACT
D9-5          ACGGGTATAACGTACACTCGGGAGGGCGTCGAAGTGGATTTGTCTGACAAACTTTGGACT
D10-10        ACGGGTATAACGTACACTCGGGAGGGCGTCGAAGTGGATTTGTCTGACAAACTTTGGACT
D11-5         ACGGGTATAACGTACACTCGGGAGGGTGTTGAAGTGGATTTGTCTGACAAACTTTGGACT
D12-12        ACGGGTATAACGTACACTCGGGAGGGCGTCGAAGTGGATTTGTCTGACAAACTTTGGACT
D15-16        ACGGGTATAACGTACACTCGGGAGGGCGTCGAAGTGGATTTGTCTGACAAACTTTGGACT
D16-3         ACGGGTATAACGTACACTCGGGAGGGCGTCGAAGTGGATTTGTCTGACAAACTTTGGACT
D16-43        ACGGGTATAACGTACACTCGGGAGGGTGTTGAAGTGGATTTGTCTGACAAACTTTGGACC
D16-44        ACGGGTATAACGTACACTCGGGAGGGTGTTGAAGTGGATTTGTCTGACAAACTTTGGACC
D20-8         ACGGGTATAACGTACACTCGGGAGGGCGTCGAAGTGGATTTGTCTGACAAACTTTGGACT
D32-5         ACGGGTATAACGTACACTCGGGAGGGCGTCGAAGTGGATTTGTCTGACAAACTTTGGACT

                Probe VIII site                    Probe I site

              361            378            518                  540
T36-15        GACGTCGTCTTTAACTCT.....TTACTTGTGTGCGGATTTCTTG
B14-6         GACGTCGTCTTTAACTCT.....CTACCTGTGTGCAGATTTCTTG
B15-30        GACGTCGTCTTTAACTCT.....CTACCTGTGTGCAGATTTCTTG
B23-2         GACGTCGTCTTTAACTCT.....TTACCTGTGTGCAGATTTCTTG
B23-7         GACGTCGTCTTTAACTCT.....TTACTTGTGTGCGGATTTCTTG
B23-15        GACATCGTGTATAATTCT.....TTACCTGTGTGCAGATTTCTTG
B31-12        GACGTCGTCTTTAACTCT.....TTACCTGTGTGCAGATTTCTTG
B52-27        GACATCGTGTATAATTCT.....TTACCTGTGTGCAGATTTCTTG
B57-7         GACATCGTGTATAACTCT.....TTACCTGTGTGCAGATTTCTTG
B57-31        GACGTCGTCTTTAACTCT.....TTACCTGTGTGCAGATTTCTTG
D9-5          GACGTCGTCTTTAACTCT.....TTACCTGTGTGCAGATTTCTTG
D10-10        GACGTCGTCTTTAACTCT.....TTACCTGTGTGCAGATTTCTTG
D11-5         GACGTCGTCTTTAACTCT.....TTACTTGTGTGCGGATTTCTTG
D12-12        GACGTCGTCTTTAACTCT.....TTACCTGTGTGCAGATTTCTTG
D15-16        GACGTCGTCTTTAACTCT.....TTACCTGTGTGCAGATTTCTTG
D16-3         GACGTCGTCTTTAACTCT.....TTACCTGTGTGCAGATTTCTTG
D16-43        GACATCGTGTATAATTCT.....TTACCTGTGTGCAGATTTCTTG
D16-44        GACATCGTGTATAATTCT.....TTACCTGTGTGCAGATTTCTTG
D20-8         GACGTCGTCTTTAACTCT.....TTACCTGTGTGCAGATTTCTTG
D32-5         GACGTCGTCTTTAACTCT.....TTACCTGTGTGCAGATTTCTTG
```

Figure 1. continued.

The direct hybridization from the PCR product of B31 indicated a mixture of up to 3 strains hybridized with probes II, III, and VIII (Table 3). However, 15 individual colony PCR products of B31 hybridized only with probe II (Table 3). This suggests that the concentration of the probe II strain is much higher than that of the other strains, and this is suggested by the lower level of probe III and VIII reactions, and clone B31-12 representing the probe II was sequenced (Figure 1).

PCR products of isolate B52 hybridized only with probes VI and VIII (Table 3) representing mild isolates. Twenty-four individual colony PCR products were

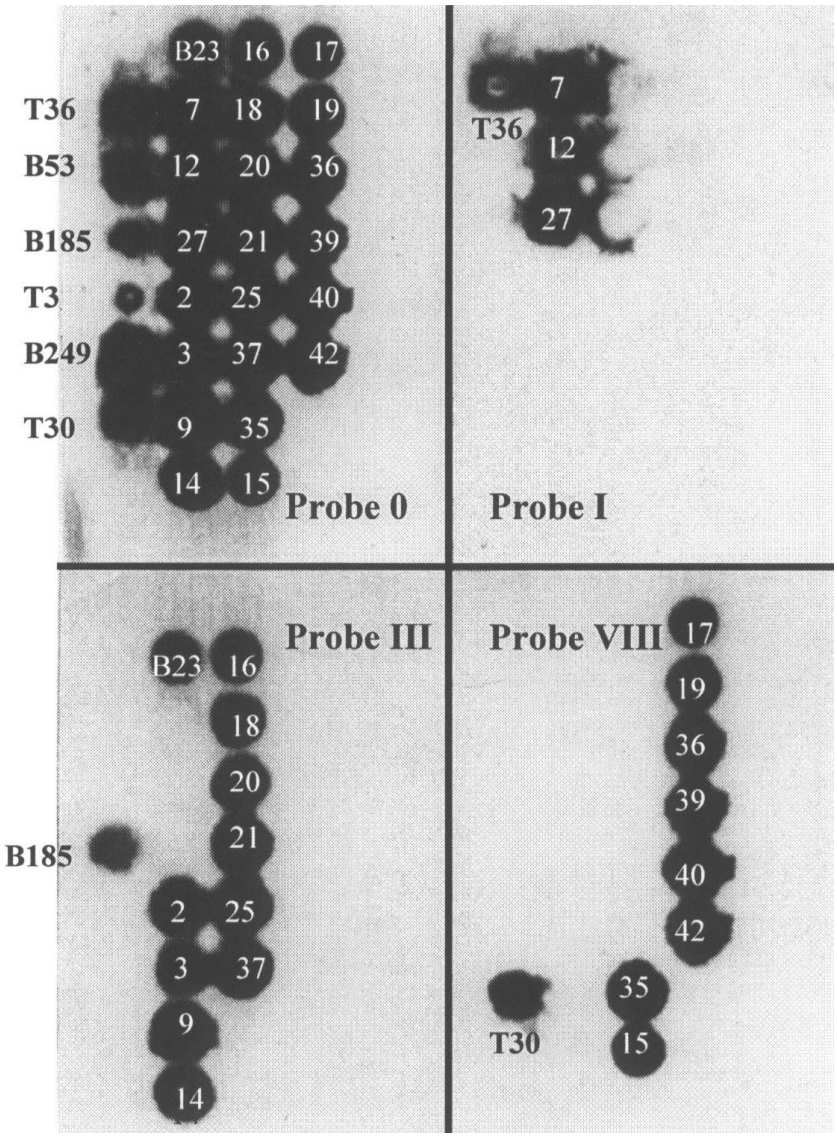

Figure 2. Hybridization of clones obtained from isolate B23. Bold numbers on the left refer to controls and white numbers refer to RT-PCR product of B23 and total 21 clones.
Probe 0: RT-PCR product of B23 and the clones reacted with probe 0.
Probe I: Clones of 7, 12, and 27, reacted with probe I.
Probe III: Clones 2, 3, 9, 14, 16, 18, 20, 21, 25, and 37, reacted with probe III.
Probe VIII: Clones 35, 15, 17, 19, 36, 39, 40, and 42, reacted with probe VIII.

screened, and all hybridized only with probes VI and VIII (Table 3). This suggests that isolate B52 is a mild isolate having a reaction with the Florida mild (probe VI) and general mild (probe VIII). Clone B52-27 representing the probe VI and VIII reactions was sequenced (Figure 1).

B57 is a mixture of at least 2 strains that hybridized with probes V, VI and VIII as the PCR product. Fourteen individual colony PCR products of B57 were screened by dot blot hybridization. Thirteen of them hybridized with probe V and I, B 57-7, hybridized with probes VI and VIII (Table 3). Clones B57-31 and B57-7 representing the

probe V and probe VI and VIII reactions, respectively, were sequenced (Figure 1).

The PCR product and the 14 individual colony PCR products of T36 hybridized only with probe I, representing the expected quick decline isolate. Clone T36-15 was sequenced (Figure 1). PCR products of certain D code isolates hybridized only with probe III (Table 3). Nineteen individual colony PCR products of isolate D9, 9 colonies of isolate D10, 13 colonies of isolate D12, 31 colonies of isolate D15, 17 colonies of isolate D20, and 18 colonies of isolate D32 were screened by dot blot hybridization and they hybridized only with probe III. Clones D9-5, D10-10, D12-12, D15-16, and D20-8, all representing probe III reactions, were sequenced (Figure 1). Nineteen individual colony PCR products of isolate D11 were screened. Only one colony hybridized with probe I, and all others hybridized with probe III. Clone D-11-5 was sequenced (Figure 1).

Thirty-three individual colony PCR products of isolate D16 were screened. Clones D16-42 and D16-43 hybridized with probes VI and VIII and the other 31 colonies hybridized only with probe III. Clone D16-3 was sequenced, representing the probe III reaction. In addition, clones D16-42 and D16-43 were sequenced, representing probes VI and VIII (Figure 1). This indicated that isolates D11 and 16 are both mixtures, and that low copy numbers of strains could be detected by hybridization of the PCR products amplified from the individual colonies, thus representing single clones of those sequences.

Analysis of the Nucleotide Sequences of the CPGs of the CTV Isolates: The nucleotide sequences of the clones were examined for the presence of the appropriate probe sites. The clones of isolates D16-43, D16-44, B23-15, B52-27, and B57-7 contain sites for probes VI and VIII at positions 251-265 and 357-374 in the nucleotide sequences of their CPGs (Figure 1). Similarly, all clones from other isolates contain the expected probe sites representing probes I, II, III, and V.

Mild and decline strains of CTV have been previously reported in Florida, (Brown, 1997; Garnsey, 1995); however, the incidence of stem-pitting strains has not been documented before. Molecular analyses of selected Delray Beach samples (D9, D10, D11, D12, D15, D16, D20 and D32) with the strain-specific probes indicate the presence of severe stem-pitting strains of CTV in Florida. Additionally, the relationship between the probes' reactivity and the biological activity has been demonstrated before by probe reactions with strains of known biological properties and also with field strains whose biological activity was determined in standard citrus indicator plants (Garnsey et al., 1991). A reaction to probe III has been relatively rare in Florida isolates (Cevik, 1995; Ochoa et al., 2000). This suggests that there has been an increase in the diversity of strains and severity of CTV infection in Florida. The occurrence of previously uncommon CTV strains may be due to the introduction of the brown citrus aphid (Susan et al., 1995). It was also demonstrated that a known stem-pitting isolate B23 (originally from Israel) is a mixture of 4 different strains, reacting with probes I, III, VI and VIII. The dot blot hybridization result of isolate B23 is shown in Figure 2 and the result was confirmed by sequencing clones of each individual strain.

Cevik (1995) developed biotin-labeled oligonucleotide probes to differentiate CTV isolates. This method was further improved in this study to detect minor populations of occurring in infected plants as low copy number strains and present below the level detectable by direct dot blot hybridization of RT-PCR products.

Conclusion

Biologically important strains might not be detected if they occur in low concentrations. In order to study this, the specific probes were hybridized directly with CP gene and PCR products of infected plants (DPH). Some samples reacted strongly with several differential probes (Table 3, isolates B31 and B57). With other samples, there were only strong reactions with a single differential probe (isolate B23). The DNA isolated from individual bacterial colonies was then used as PCR templates and the PCR products were tested with the specific probes (CPH). The CPH method revealed that samples such as B57, which were apparently mixtures of strains, were certainly mixtures; other samples, apparently containing a single strain (B23, D9, D11 and D16), actually contained one or more additional strains not detectable by DPH. This would not cause a serious problem if the undetected strain was a mild strain of CTV, as sample D15. On the other hand, it would be extremely important if the contaminating strain induced a decline or stem-pitting, as in isolates B23 and D11.

The CPH method, which is more sensitive for the detection and differentiation of strains of CTV infecting single citrus trees, was developed in this study. This method clearly demonstrated that DPH is not adequate to determine whether a tree is infected with a single or multiple strains of CTV. Thus, the development of a CPH method using strain-specific probes will enable the rapid detection, identification and classification of newly discovered and previously undetectable CTV strains. It has the additional advantage of providing clones of the low copy number strains for their further characterization and differentiation. Such undetected minor populations may be very important in disease management, especially in nurseries and mild strains cross protection situations.

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