Detection and Isolate Differentiation of *Citrus tristeza virus* in Infected Field Trees Based on Reverse Transcription–Polymerase Chain Reaction

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ABSTRACT

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Reverse transcription–polymerase chain reaction (RT-PCR) was compared with enzyme-linked immunosorbent assay (ELISA) and direct tissue blot immunoassay (DTBIA) for detection of non-decline-inducing and decline-inducing isolates of *Citrus tristeza virus* (CTV) in 21 field sweet orange and grapefruit plants on sour orange rootstock in Fort Pierce, FL. Among these samples, seven, six, and eight were infected with decline-inducing, non-decline-inducing, and both decline-inducing and non-decline-inducing isolates of CTV, respectively. However, there was not a good correlation between field symptoms and detection of the decline-inducing isolate. The results confirmed that RT-PCR is not only able to detect and differentiate decline-inducing and non-decline-inducing isolates of CTV in Florida, but also can detect both isolate types in a single field sweet orange or grapefruit tree. For most samples, results from RT-PCR, ELISA, and DTBIA were the same. However, the 320-bp fragments produced only from decline-inducing isolates were amplified from two sweet orange and two grapefruit samples that did not react with decline-inducing CTV-specific monoclonal antibody MCA13 in ELISA or DTBIA, indicating that RT-PCR has a higher sensitivity than these immunological tests for field sweet orange or grapefruit samples. Thus, RT-PCR is a simple, rapid, and specific procedure for CTV identification applicable to both research and diagnostic needs.

Additional keywords: cross-protection, diagnosis

Citrus tristeza virus (CTV), a member of the genus *Closterovirus* of the *Closteroviridae*, is distributed worldwide and is the causal agent of one of the most economically important diseases of citrus (3,4). Virus particles are flexuous, threadlike, and 2,000 nm in length and 10 to 12 nm in diameter (4). The single-stranded RNA genome of CTV is about 20 kb and contains 12 open reading frames (ORFs) that potentially encode at least 19 proteins (16,17). The virus is genetically and biologically diverse and can cause stunting, slow decline, quick decline, stem pitting, or no symptoms depending on the virus isolate, time of infection, rootstock, citrus cultivar, and environmental conditions (10,21). Decline isolates kill citrus trees on sour orange or related rootstock, and stem pitting isolates make citrus production

uneconomical because of poor tree vigor and small fruit. During the last century, CTV has devastated entire citrus industries in several countries (2).

Measures to control losses caused by CTV include quarantine systems to avoid introduction of exotic isolates, certification programs to prevent CTV spread, and cross-protection with mild isolates (27). These measures require rapid procedures to detect CTV and to discriminate between mild and severe isolates. Isolates of CTV that induced different field symptoms were traditionally differentiated by inoculating the appropriate indicator plants and observing the symptoms. This bioassay was time-consuming, expensive, and often imprecise. In recent years, remarkable progress in the development of quick, accurate techniques for the detection and differentiation of isolates of CTV has been achieved (22). These techniques involved SDS-Immunodiffusion (3,5,9), direct tissue blot immunoassay (DTBIA) (11), restriction fragment length polymorphism (RFLP) (12), serologically specific electron microscope (SEM) (5), enzyme-linked immunosorbent assay (ELISA) (3,7,23,26), dot-immunobinding assay (28,29), radio-immunosorbent assay (RISA) (26), in situ immunofluorescence

(ISIF) (6), in situ immunoassay (ISIA) (19), Western blot assay (13,18,26), single-strand conformation polymorphism (SSCP) (30), and reverse transcription–polymerase chain reaction (RT-PCR) (8,15,20). In this study, RT-PCR has been applied for detection and differentiation of the non-decline-inducing and decline-inducing isolates of CTV in infected field trees in Florida, as well as for detection of both isolate types in mixed infections. The purpose was to test the usefulness of this technique and to probe the relationship between field symptoms and detection of decline-inducing isolates in different varieties of citrus. Moreover, this technique was also compared with immunological techniques for field diagnosis of CTV. The information will be helpful for the development of field detection systems for CTV.

MATERIALS AND METHODS

Virus isolates and plant materials.

Four isolates of CTV, maintained in the greenhouse at the Indian River Research and Education Center, Fort Pierce, FL, were used to standardize the procedure of RT-PCR. The biological characteristics of the isolates were described previously (19). Field samples were from 14 sweet orange trees on sour orange rootstock (SSO) and 7 grapefruit trees on sour orange rootstock (GF) at the Indian River Research and Education Center. All the sweet orange trees were declining, whereas the grapefruit trees were not declining. The mature shoots of citrus plants were collected from east, west, south, and north locations and mixed for the tests. Stems of shoots were used for DTBIA and the extraction of nucleic acid for RT-PCR. Petioles of the same shoots were used to prepare extractions for ELISA experiments.

ELISA. Indirect double antibody sandwich (I-DAS) ELISA (3) was performed to diagnose citrus trees infected by CTV. One polyclonal antiserum (PCA) 1212 and two monoclonal antibodies (MAbs), 17G11 and MCA13, were used in this study. Their characteristics were described previously (19). The IgG of PCA 1212 was used as coating antibody, and the two others were used as intermediate antibodies. Extractions were prepared for ELISA from 0.5 g of petioles with 5 ml of 1× PBST buffer (0.15 M NaCl, 0.015 M NaH₂PO₄, 0.05%

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Tween 20, pH 7.0). Positive reactions were defined as an OD_{415nm} 2 times higher than the healthy control.

DTBIA. Direct tissue blot immunoassay (11) was also performed on stems of healthy or infected citrus plants. A smooth fresh cut was made with a razor blade, and the cut surface was pressed gently and evenly to the nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The blotted membranes were allowed to dry for 10 to 30 min, incubated in plastic bags with MAb 17G11 or MCA13 for 2 h at 37°C, and rinsed with PBST buffer three times (3 min each time). The blots were labeled with alkaline phosphatase conjugated goat antimouse Ig (H+L)-Ap at a 1:2,000 dilution for 1 to 2 h at 37°C, rinsed with PBST-PVP (PBST with 2% polyvinylpyrrolidone) three times (3 min each time), and then incubated in a petri dish with the freshly prepared substrate mixture of nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) for 5 to 20 min to develop the reaction. After stopping the reaction by washing the blots with water, the blots were observed under a light microscope at ×10 magnification. A positive reaction was the development of a purple color in the phloem tissue. Blots where no purple color developed were considered negative.

Nucleic acid extraction from citrus tissues. Total nucleic acid extracts of samples were used as templates for RT-PCR. The extraction procedure as described by Hung et al. (15) was modified slightly and used in this study. In short, stems (approximately 250 mg) were placed in a canister and pulverized with liquid nitrogen for 40 s using a KLECO Model 4200 Pulverizer (Kinetic Laboratory Equipment Company, Visalia, CA) and then collected into a 1.5-ml sterile Eppendorf tube. Each sample was suspended in nucleic acid extraction buffer (0.1 M Tris-HCl [pH 8.0], 0.05 M EDTA, 0.5 M NaCl, 1% N-lauroylsarcosine) and incubated at 55°C for 1 h. After centrifugation (6,000 rpm) for 5 min, the supernatant (800 µl) was treated with 100 µl of 5 M NaCl and 100 µl of 10% hexadecyl-trimethyl-ammonium-bromide (CTAB) at 65°C for 10 min and then extracted with chloroform/isoamyl alcohol (24:1). The aqueous supernatant was re-extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The nucleic acid was precipitated from the aqueous layer with 0.6 volume of isopropanol

at room temperature for 15 min. The pellet was collected by centrifugation at 12,000 rpm for 10 min and washed with 70% ethanol, dried, and resuspended in 20 µl TE buffer (pH 8.0).

Primers. Two internal primers, one (CP₄) specific for non-decline-inducing (T₃₀) and the other (CP₃) specific for decline-inducing isolates (T₃₆) of CTV, and two terminal primers (HCP₁ and HCP₂) for the ends of the capsid protein gene were designed on the basis of the reported studies by Cevik et al. (8) and the published nucleotide sequence of CTV isolates T₃₆ and T₃₀ (2,17). The characterization of primers is described in Table 1.

cDNA synthesis and polymerase chain reaction amplification. The first-strand cDNA was synthesized using total nucleic acid extracted from citrus stems as templates and HCP₂, CP₄ as the primers. The total reaction volume was 20 µl, which contained 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM each of the four dNTPs, 0.75 µM of primer HCP₂, 0.75 µM of primer CP₄, 100 units of SuperScript II RNase H- Reverse Transcriptase (Invitrogen, Carlsbad, CA), 40 units RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen), and 8 µl of nucleic acid extract. The contents were mixed gently and incubated at 42°C for 50 min. The PCR amplification was performed in 50 µl of reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM each of the four dNTPs, 1.5 mM MgCl₂, 0.5 µM primer HCP₁, 0.5 µM primer HCP₂, 0.5 µM of primer CP₃, 0.5 µM of primer CP₄, 2.5 units of *Taq* DNA polymerase (Invitrogen), and 2 to 2.5 µl of RT mixture. The PCR cycling profile was one cycle at 94°C for 10 min, followed by 35 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min, with a final extension step at 72°C for 20 min. Aliquots of PCR-amplified fragments were electrophoresed in 2% agarose gels in Tris-borate (TBE) buffer (0.09 M Tris base, 0.09 M boric acid, 0.002 M EDTA, pH 8.0). After electrophoresis, the gels were stained with ethidium bromide at 0.5 µg/ml and then analyzed using a BIO imaging system (Syngene, Frederick, MD). A 100-bp DNA Ladder (Promega, Madison, WI) was used as a nucleic acid marker.

RESULTS

Detection and isolate differentiation of the known CTV isolates by RT-PCR.

Different amplified fragments of different isolate types were obtained by using HCP₁, HCP₂, CP₃, and CP₄ as primers and nucleic acids as templates (Fig. 1). For non-decline-inducing isolates (T₂₆ and T₃₀), a 672-bp full-length CP gene fragment and a 392-bp fragment were produced (Fig. 1, lanes 3 and 4). The 672- and 320-bp fragments were amplified from decline-inducing isolates (T₃ and T₃₆) (Fig. 1, lanes 5, 6, and 7). From the infection with both decline-inducing and non-decline-inducing isolates (T₃₀ + T₃₆), the 672-, 392-, and 320-bp fragments were all produced (Fig. 1, lane 8). No fragment was obtained from the healthy tissue extract (Fig. 1, lane 2).

Detection and isolate differentiation of CTV in infected field samples by RT-PCR. RT-PCR was applied to test 21 CTV-infected field samples from sweet orange and grapefruit plants on sour orange rootstock (Figs. 2 and 3). Decline-inducing isolate T₃₆, non-decline-inducing isolate T₃₀, artificial mixtures of T₃₆ and T₃₀, and a healthy sample were used as positive and negative controls. The 672-, 392-, and 320-bp fragments were amplified from eight of these samples, indicating that they were infected with both decline-inducing and non-decline-inducing isolates of CTV (Fig. 2A, lanes 6 and 11; Fig. 2B, lanes 7, 8, 9, 10, and 11; Fig. 3, lane 10). From seven of these samples, the 672- and 320-bp fragments were produced, indicating that they were infected only with decline-inducing isolates of CTV (Fig. 2A, lane 7; Fig. 2B,

Fig. 1. Analysis of reverse transcription–polymerase chain reaction (RT-PCR) products of the known isolates of *Citrus tristeza virus* (CTV). Lane 1: 100-bp DNA ladder; lane 2: extract of healthy plant; lane 3: T₂₆; lane 4: T₃₀; lane 5: T₃; lane 6: T₃₆; lane 7: T₃₆; lane 8: T₃₀ + T₃₆.

Table 1. Primers used for reverse transcription–polymerase chain reaction (RT-PCR) to detect and differentiate isolates of *Citrus tristeza virus* (CTV)

Primers	Polarity	Sequence	Position ^a	Tm(°C)	Predicted product size ^b
HCP ₁	+	5'-ATGGACGACGAAACAAAGAA-3'	16155-16174	56	672 bp
HCP ₂	–	5'-TCAACGTGTGTTGAATTTCC-3'	16826-16807	56	672 bp
CP ₃	+	5'-TTTGACTGACGTCGTGT-3'	16507-16525	56	320 bp
CP ₄	–	5'-TTACCAATACCCTTAGAATTAT-3'	16546-16525	56	392 bp

^a Nucleotide position of primers in sequence of CTV isolates T₃₆.

^b 672-bp fragment is the product of primer pair HCP₁ and HCP₂. 320-bp fragment is the product of primer pair CP₃ and HCP₂. 392-bp fragment is the product of primer pair HCP₁ and CP₄.

lanes 6 and 12; Fig. 3, lanes 6, 8, 9, and 11). From the rest of these samples, the 672- and 392-bp fragments were produced, indicating that they were infected only with non-decline-inducing isolates of CTV (Fig. 2A, lanes 5, 8, 9, and 10; Fig. 3, lanes 7 and 12).

Comparison of DTBIA, ELISA, and RT-PCR. Twenty-one CTV-infected field samples were also tested by DTBIA and ELISA with MA17G11 and MCA13. A comparison of the results of DTBIA, ELISA, and RT-PCR is shown in Table 2. RT-PCR results of most samples were consistent with those obtained with DTBIA and ELISA. However, the 320-bp

fragments were amplified from samples SSO-3, SSO-7, GF-5, and GF-6, indicating that they were infected with decline-inducing isolates of CTV, even though they were MCA13-negative in ELISA or DTBIA.

DISCUSSION

With the development of polyclonal antisera and monoclonal antibodies, serological methods have become a dependable tool for CTV detection and isolate differentiation. Among antibodies, MCA13 is especially useful because it reacts with most severe isolates. This positive reaction has been associated with tree decline in

Florida (22). Thus, MCA13 is now commonly used as a first step for differentiation of decline-inducing and non-decline-inducing isolates of CTV in Florida. It had been demonstrated by site-directed mutagenesis that the epitope for MCA13 is based on a single nucleotide difference at position 371 of the capsid protein gene, mostly being T for severe isolates and A for mild isolates (24). Based on this characteristic, two internal primers (CN218 and CN219) and two terminal primers (CN119 and CN120) were designed by Cevik et al. (8) and used for differentiating CTV isolates. In this study, the two inter-

Fig. 2. Analysis of reverse transcription-polymerase chain reaction (RT-PCR) products of *Citrus tristeza virus* (CTV)-infected field samples from sweet orange trees on sour orange rootstock. **A**, lane 1: 100-bp DNA ladder; lane 2: T₃₀; lane 3: T₃₆; lane 4: T₃₀ + T₃₆; lane 5: SSO-1; lane 6: SSO-2; lane 7: SSO-3; lane 8: SSO-4; lane 9: SSO-5; lane 10: SSO-6; lane 11: SSO-7; lane 12: extract of healthy plant. **B**, lane 1: 100-bp DNA ladder; lane 2: extract of healthy plant; lane 3: T₃₀; lane 4: T₃₆; lane 5: T₃₀ + T₃₆; lane 6: SSO-8; lane 7: SSO-9; lane 8: SSO-10; lane 9: SSO-11; lane 10: SSO-12; lane 11: SSO-13; lane 12: SSO-14.

Fig. 3. Analysis of reverse transcription-polymerase chain reaction (RT-PCR) products of *Citrus tristeza virus* (CTV)-infected field samples from grapefruit trees on sour orange rootstock. Lane 1: 100-bp DNA ladder; lane 2: T₃₀; lane 3: T₃₆; lane 4: T₃₀ + T₃₆; lane 5: extract of healthy plant; lane 6: GF-1; lane 7: GF-2; lane 8: GF-3; lane 9: GF-4; lane 10: GF-5; lane 11: GF-6; lane 12: GF-7.

Table 2. Comparison of direct tissue blot immunoassay (DTBIA), enzyme-linked immunosorbent assay (ELISA), and reverse transcription-polymerase chain reaction (RT-PCR) for detection and isolate differentiation of *Citrus tristeza virus* (CTV)

Plant	DTBIA			ELISA			RT-PCR			
	17G11 ^a	MCA13	Comment ^b	17G11	MCA13	Comment	672 bp	392 bp	320 bp	Comment
SSO-1	+	–	M	+	–	M	+	+	–	M
SSO-2	+	+	S	+	+/-	WS	+	+	+	M & S
SSO-3	+	–	M	+	–	M	+	–	+	S
SSO-4	+	–	M	+	–	M	+	+	–	M
SSO-5	+	–	M	+	–	M	+	+	–	M
SSO-6	+	–	M	+	–	M	+	+	–	M
SSO-7	+	+	S	+	–	M	+	+	+	M & S
SSO-8	+	+	S	+	+	S	+	–	+	S
SSO-9	+	+	S	+	+	S	+	+	+	M & S
SSO-10	+	+	S	+	+	S	+	+	+	M & S
SSO-11	+	+	S	+	+	S	+	+	+	M & S
SSO-12	+	+	S	+	+	S	+	+	+	M & S
SSO-13	+	+	S	+	+	S	+	+	+	M & S
SSO-14	+	+	S	+	+	S	+	–	+	S
GF-1	+	+/-	WS	+	+	S	+	–	+	S
GF-2	+	–	M	+	–	M	+	+	–	M
GF-3	+	+	S	+	+	S	+	–	+	S
GF-4	+	+	S	+	+	S	+	–	+	S
GF-5	+	–	M	+	–	M	+	+	+	M & S
GF-6	–	–	UD	–	–	UD	+	–	+	S
GF-7	+	–	M	+	–	M	+	+	–	M

^a + = positive reaction, – = negative reaction, +/- = uncertain reaction.

^b M = T₃₀-like, S = T₃₆-like, WS = unidentified, UD = undetected.

nal primers were the same as those of Cevik et al. (8), but the two terminal primers were modified. The 5'-terminus of primers HCP₁ and HCP₂ contained no enzyme digestion site, and the length of primer HCP₁ was changed so that all four primers had the same melting point temperature (Table 1). These primers were used to successfully detect decline-inducing and non-decline-inducing isolates of CTV in both sweet orange and grapefruit trees in the field. There was not a good correlation between field symptoms and detection of decline-inducing CTV by any method. Four SSO plants with declining symptoms were infected only with non-decline-inducing isolates by ELISA, DTBIA, and RT-PCR. Likewise, three GF plants with non-declining symptoms were infected with decline-inducing isolates. This can be explained by the observations that the SSO trees had significant citrus root weevil damage and that grapefruit on sour orange rootstock can be infected with decline-inducing CTV for at least 6 years without going into decline (C. A. Powell, *unpublished*).

An important distinction of perennial crops is their ability to accumulate multiple pathogens over the years of cultivation, including multiple closteroviruses. There are numerous, genetically distinct isolates of CTV in citrus. They may be present in an infected plant separately or in mixed infections, and they provide an ideal reservoir of infection (1,16). Often up to 25% of the samples were infected with more than one isolate of CTV (22). Tests of 21 infected field samples with RT-PCR showed that 8 samples contained mixed infections (Table 2). The result also indicated that this method is not only able to detect and differentiate the decline-inducing and non-decline-inducing isolates of CTV, but also to detect both isolate types in both single grapefruit and sweet orange trees. It is suggested that the method might be used for field diagnosis of CTV, to evaluate cross-protection, and to select non-decline-inducing isolates useful for cross-protection from healthy appearing trees in groves severely affected by CTV.

It has been demonstrated that sweet orange and grapefruit plants that were infected with both decline-inducing and non-decline-inducing isolates of CTV may carry the decline-inducing isolates at a concentration or distribution that eluded detection by ELISA with MCA13 (25). Other studies have also reported that PCR methods are more sensitive than ELISA for the detection of CTV and *Cucumber mosaic virus* (CMV) (8,14). We have confirmed that the RT-PCR method is more sensitive than ELISA for the field diagnosis of CTV. RT-PCR results showed that some samples that were MCA13-negative in ELISA and/or DTBIA were infected with decline-inducing or a mixture of de-

cline-inducing and non-decline-inducing isolates of CTV (Table 2, samples SSO-3, SSO-7, GF-5, and GF-6).

The total nucleic acid extract, prepared from very small quantities (about 250 mg) of stems of citrus, can be used as the template for RT-PCR. In our extraction procedure of nucleic acid, stems were placed in a canister and pulverized with liquid nitrogen using a KLECO Model 4200 Pulverizer. It is less time-consuming and labor-intensive than pulverizing stems by mortar and pestle. The drawback of RT-PCR is that it is more expensive than ELISA and DTBIA. However, it is easy, rapid, and can be used for quarantine and budwood certification programs, which need reliable, sensitive, and rapid techniques.

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