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Quantitative detection of *Citrus tristeza virus* in citrus and aphids by real-time reverse transcription-PCR (TaqMan®)

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Abstract

A quantitative and multiplex real-time RT-PCR assay was developed to detect *Citrus tristeza virus* (CTV) along with plant mRNA, which serves as an internal control to ascertain RNA extraction quality. The real-time technique was validated against 39 CTV strains from around the world as well as with the aphid vector, *Aphis gossypii*, given a 48 h acquisition access period on a CTV source plant. The assay was effective for quantitation of the viral template in infected plants and in single aphids. CTV detection was compared from different plant tissues and for different RNA isolation methods from aphids. Less than 1 fg was consistently detected when RNA transcripts were diluted in extracts from healthy plants while RNA copies carried by single aphids were estimated to be between 12,000 and 13,000,000. The assay was more sensitive and less time consuming than ELISA or traditional RT-PCR. The real-time RT-PCR assay developed is a valuable new tool for detection and titer quantitation of CTV.
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Keywords: Real-time PCR; Closterovirus; Vectors; Disease diagnosis; RNA extraction

1. Introduction

Citrus tristeza virus (CTV), genus C*losterovirus* (Martelli et al., 2005), is the causal agent of tristeza, one of the most economically important viral diseases of citrus. The virus has a host range restricted to most species of the family Rutaceae (Roistacher, 1991) and can be disseminated long distances by movement of virus-infected plant material and locally by several aphid species in semi-persistent mode (Lee and Bar-Joseph, 2000). Types and severity of symptoms induced by CTV are associated with different viral strains. Mixtures of strains occur naturally, which can affect symptom phenology according to the population of the major CTV genotype present or to the interaction between the strains (i.e., cross-protection). Mild isolates do not produce noticeable damage on most commercial scion/rootstock combinations nor decline on sour orange. However, severe isolates cause important diseases such as decline, an incompatibility of various scions grafted on sour orange rootstock. The most virulent isolates cause stem pits in wood of twigs, small and large lateral branches and the main trunk. They also reduce growth of the tree accompanied by a decline in fruit yield, fruit size and quality in severe cases (Lee and Bar-Joseph, 2000).

CTV consists of flexuous particles ∼2000 nm × 11 nm in size, which contain a single-stranded positive-sense RNA (∼19 kb) containing 12 open reading frames (Karasev et al., 1995). Sequence comparisons indicated a relatively high level of conservation among isolates toward the 3′ end and divergence up to 40% toward the 5′ terminal (Mawassi et al., 1996; Hilf et al., 2005).

Several techniques have been developed for the detection and differentiation of CTV isolates. Indexing on a standard host range (Garnsey et al., 2005) detects and categorizes the different biological types of CTV, but it is laborious and expensive for large-scale tests. Serological techniques have been used since the 1970s for detecting CTV (Bar-Joseph et al., 1979; Garnsey et al., 1993). Enzyme-linked immunosorbent assay (ELISA) and direct tissue blot immunoassay (DTBIA) are the most common because of their reliability, rapidity and low relative cost. More recently, polymerase chain reaction (PCR) has been adapted for detection of CTV: two-step reverse-transcription RT-PCR

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(Metha et al., 1997; Hilf and Garnsey, 2000; Huang et al., 2004); one-step RT-PCR (Hung et al., 2000); immunocapture (IC)-RT-PCR (Cambra et al., 2000b, 2002); and multiplex RT-PCR (Roy et al., 2005).

Although PCR techniques described above can detect a low titer of virus, they are not quantitative. Real-time PCR allows rapid detection of target-specific amplicons and accurate quantification when used with a standard curve. Real-time RT-PCR has been reported for the detection of different woody plant-infecting viruses (Marbot et al., 2003; Schneider et al., 2004; Varga and James, 2005,2006; Osman and Rowhani, 2006; Osman et al., 2007) as well as for viruses in different insect vectors (Boonham et al., 2002; Fabre et al., 2003; Olmos et al., 2005).

The objective of this study was to develop highly sensitive, real-time PCR detection of CTV. Primers and TaqMan® probes were selected for simultaneous detection of CTV and plant mitochondrial mRNA as internal control to assay the quality of RNA extracted from the sample. This procedure was applied as a multiplex one-step RT-PCR protocol which was effective in detecting and quantifying CTV in infected plant tissues and aphids.

2. Materials and methods

2.1. Virus sources

The CTV isolates used in this report are shown in Table 1. *Citrus sinensis* var. Madame Vinous seedlings infected with CTV isolates P29 (AF001623) and P81 (AY995567) were used to develop and optimize the real-time RT-PCR assay. To validate the assay, CTV-infected tissue was collected in 2006 from plants in the following collections: Exotic Pathogen Collection (USDA, ARS) in Beltsville, MD (Garnsey et al., 2005); Central California Tristeza Eradication Agency in Tulare, CA (Polek et al., 2005) and Crop Diseases, Pest and Genetics Research Unit (USDA, ARS) in Parlier, CA (Yokomi and Deborde, 2005). In addition, tissue from CTV-infected field trees near Lindsay, CA was collected in May 2006. Bark tissue or leaf petioles were used for total RNA isolation or stored at −20 °C or desiccated with silica gel (Garnsey et al., 1996).

2.2. Aphid vectors

A local biotype of *Aphis gossypii* collected in Sanger, CA (Yokomi and Deborde, 2005) and reared on cotton, was used in this study. Young adult apterae (wingless) aphids were given a 48 h acquisition access period (AAP) on CTV-infected Madame Vinous donor plants and subjected to the various nucleic acid extraction methods. These extracts were tested then by nested RT-PCR and real-time RT-PCR.

2.3. Isolation of total RNA

2.3.1. Plants

Total RNA from CTV-infected plants was recovered from 0.2 g of plant tissue (fresh or frozen bark tissue; fresh or lyophilized leaf petioles) by homogenization with a MiniBeadBeater-96 (Bio-Spec Product, Bartlesville, OK) in 1 ml of grinding buffer (4 M guanidine isothiocyanate, 0.2 M sodium acetate, pH 5.2, 25 mM EDTA, 1 M potassium acetate; 2.5% (w/v) PVP-40 and 2% sodium metabisulfite). The suspension was mixed with 200 μl of 10% *N*-lauroylsarcosine and incubated at 70 °C for 10 min, chilled on ice for 5 min and centrifuged at 13,400 rpm for 10 min. Next, the supernatant was recovered and processed using either the RNeasy Plant Mini Kit (Qiagen) or the procedure described by Foissac et al. (2001). Finally, total RNA was recovered in 50 μl (Qiagen) or 150 μl (Foissac method) of RNase free water. The concentration of total RNA was determined using a NanoDrop® ND-1000 UV–vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.3.2. Aphids

Five variations of total RNA extraction were tested using single viruliferous *A. gossypii* per extraction. In cases where CTV was trapped to nitrocellulose membranes (NCM) (Nitrobind, Micron Separations Inc., Westborough, MA, USA), total RNA was eluted from the membrane by incubation with 30 μl RNase free water at 80 °C for 10 min. In those cases where the aphid was homogenized in buffer, total RNA was recovered from the suspensions as follows:

- Aphid was crushed onto a spot on 3 MM CHR paper (Whatman International Ltd., Maidstone, England) and the spot bathed in 50 μl of 0.5% Triton X-100, vortexed and incubated for 5 min at room temperature (Olmos et al., 2005). Triton extracts were used for nested immunocapture (IC)-RT-PCR or nested RT-PCR.
- The whole aphid was crushed onto a NCM and the total RNA eluted from the crushate with RNase free water as described above.
- Aphid was homogenized in a glass pestle with 25 μl of sodium sulfite containing buffer (SSB) (10 mM Tris–HCl, pH 7.4; 0.65% Na_2SO_3; 0.5% Triton). One 10 μl drop of the supernatant was spotted on a NCM and then after a drying period of 30 min, another 10 μl drop was added to the same spot per aphid (Singh et al., 2004). The total RNA was eluted as previously described.
- Aphid was homogenized in a glass pestle in 100 μl of PBS–0.05% Tween–2% PVP40 followed by chelex extraction (Fabre et al., 2003).
- Aphid was homogenized with a Mini BeadBeater-96 in 500 μl of Trizol reagent (Invitrogen) following the manufacturer's protocol. One hundred microlitters of chloroform were added to the homogenate and tubes were incubated at room temperature for 5 min. This was followed by centrifugation at 12,000 × *g* for 15 min. The supernatant was recovered and mixed with 250 μl of isopropyl alcohol. After incubation at room temperature for 15 min, the total RNA was recovered by centrifugation, and dissolved in 15 μl of RNase free water.

Total RNA recovered using protocols 2, 3, 4 and 5 were used for nested RT-PCR or one-step real-time RT-PCR.

Table 1
CTV isolates tested by multiplex real-time reverse transcription polymerase chain reaction (RT-PCR)

Isolate ID	Origin	Description	Tissue	Total RNA (ng/μl)	Real-time PCR C_t value	
					CTV	Internal control
Central California Tristeza Eradication Agency (CCTEA), Tulare, CA[a]						
CCTEA 114	Tulare Co., CA	Moderate stem pitting (SP) on Madame Vinous (MV), moderate quick decline (QD), reacts with monoclonal antibody MCA13		9.9	21.94	0
CCTEA 192	Kern Co., CA	Mild		17.5	19.51	0
CCTEA 146	Kern Co., CA	Severe QD		17.0	20.10	29.12
CCTEA 95120	Tulare Co., CA	Moderate SP on MV		15.1	17.15	0
CCTEA 170	Kern Co., CA	No data	Bark tissue (frozen)	11.3	18.75	0
CCTEA 117	Tulare Co., CA	QD		13.8	16.30	0
CCTEA 197	Kern Co., CA	Moderate SP on MV		15.1	17.94	0
CCTEA 180	Kern Co., CA	Moderate SP on MV		10.6	18.69	0
CCTEA 155	Kern Co., CA	Seedling yellows (SY) on sour orange (SO)		15.1	17.24	0
CCTEA 160	Fresno Co., CA	Severe QD		34.7	18.68	0
CCTEA 143	Kern Co., CA	Severe QD		8.7	18.65	0
Exotic citrus pathogen collection, USDA, ARS, Beltsville, MD[a]						
B28	Florida	Strong SY, strong decline SP on grapefruit (GF) (T68)		25.8	17.05	42.98
B152	California	Californian SY (SY 576). VT genotype according Hilf and Garnsey, 2005.		70.6	20.03	26.39
B30	Japan	Severe SY		26.6	19.89	30.36
B255	Indonesia	Severe SP in sweet orange (SwtO)		40.8	19.72	25.87
B23	Israel	SY		45.8	18.30	28.33
B119	Hawaii	T30 + natural CTV isolate in CTV cross-protection experiment, Hilo, HA	Leaf petioles desiccated with silica gel	23.5	18.10	42.58
B391	Puerto Rico	QD		27.1	20.22	27.24
B192AT	Corsica	Aphid transmitted (AT) from B192, severe, MCA 13 positive		44.0	14.72	0
B211	Taiwan	Mild isolate selected as potential protecting isolate. T3 genotype according Hilf and Garnsey, 2005.		24.7	20.06	28.14
B405	Spain	SP on SwtO (T318)		54.4	17.31	0
USDA, ARS, Parlier, CA[a]						
			Fresh leaf petioles	100.2	12.14	30.15
P29	Riverside, Co., CA	Severe SP on GF, severe QD, (SY 568)	Leaf petioles desiccated with silica gel	50.1	19.17	34.36
			Frozen bark	85.1	14.08	0
			Fresh bark	91.8	13.49	29.95
P35 (CCTEA 92)	Kern Co., CA	SP on MV and GF		94.6	17.17	24.78
P51	Kern Co., CA	Mild on SwtO		81.7	17.63	22.50
P100	Ventura Co., CA	QD		45.9	14.61	22.60
P21	Kern Co., CA	Mild on SwtO		110.0	17.71	23.28
P36 (CCTEA 94)	Kern Co., CA	SP on MV and GF		82.5	15.57	25.90
P60	Tulare Co., CA	Severe SP on GF, QD	Fresh leaf petioles	66.3	17.45	23.30
P25	Kern Co., CA	Mild on SwtO		94.2	16.94	21.96
P28	Kern Co., CA	Mild on SwtO		100.8	17.26	25.20
P81	Kern Co., CA	Mild on SwtO		120.1	15.24	25.24
P43	Kern Co., CA	SP on MV and GF		80.3	18.32	24.55
P109	Fresno Co., CA	Severe SY on Eureka lemon (EL) and SO, SP on MV		118.7	14.47	22.80
P108	Fresno Co., CA	Severe SY on EL, SP on MV		68.2	15.24	22.85
SY 553	UC Riverside	Meyer lemon isolate		70.5	20.43	24.27
Field samples, Lindsay, CA						
17–11	Tulare Co., CA	Unknown		26.9	22.34	25.61
17–12	Tulare Co., CA	Unknown	Fresh leaf petioles	50.5	21.61	26.44
18–8	Tulare Co., CA	Unknown		71.5	17.85	26.21
19–11	Tulare Co., CA	Unknown		40.2	20.10	24.90

[a] Isolates consisted of infected *Citrus sinensis* var. Madame Vinous seedlings.

Table 2
Sequence of forward and reverse primers and probes used for real-time reverse transcription PCR and conventional RT-PCR

Primer/Probe	Sequence 5′–3′	Expected amplicon size (bp)	Primer location
Real-time RT-PCR			
P25F (forward)	AGCRGTTAAGAGTTCATCATTRC		16,376–16,399[a]
P25R (reverse)	TCRGTCCAAAGTTTGTCAGA	101	16,457–16,477[a]
CTV-CY5[b]	CY5- CRCCACGGGYATAACGTACACTCGG		16,412–16,437[a]
NADF (forward) (Menzel et al. (2002))	GATGCTTCTTGGGGCTTCTTGTT	115	968–987 and 1,836–1,838[c]
NADSR (reverse)	GCGGATCCTCGGACATATATGA		1908–1930[c]
NAD-TET[d]	TET-TGTTAATTGTGGTTACATCCAT-MGB		1859–1881[c]
Conventional RT-PCR			
T36CP-F (forward)	ATGGACGACGAAACAAAGAAATTG	672	16,152–16,176[e]
T36CP-R (reverse)	TCAACGTGTGTTGAATTTCCCA		16,802–16,823[e]
S-CTV FW (forward)	CGCAATTTGAGTTATGGCGGA	119	16,578–15,598[a]
S-CTV REV (reverse)	ATGTACACAGCACATTCTAAATCA		16,709–16,685[a]
NADRS used with the primer NADF	CTCCAGTCACCAACATTGGCATAA	148	19,839–19,863[c]

[a] Primer location based on GenBank accession number AF260651.
[b] Probe labeled with the reporter Cy5 (Cyanine 5).
[c] Primer location based on GenBank accession number D37958.
[d] Minor groove binding (MGB) probe labeled with the reporter TET (tetrachloro-6-carboxyfluorescein).
[e] Primer location based on GenBank accession number AY170468.

2.4. Taqman® probe and primers design

Primers and a Cy5-labeled probe for CTV detection were designed within the CP region using Primer Express software (Applied Biosystems) (Table 2). Multiple alignment of the CP gene from various isolates was performed using ClustalX (Thompson et al., 1997) and GeneDoc (Version 2, 1991). Degenerate nucleotides were included in the primers and probe to make sure that the assay was made as broad spectrum as possible.

An internal control assay designed to amplify the mRNA of the mitochondrial gene NADH dehydrogenase (nad5) was included. The sense primer was located over the splice junction as reported by Menzel et al. (2002), while the reverse primer and the Minor Groove Binding (MGB) TaqMan® TET-labeled probe were designed using the software mentioned above.

2.5. TaqMan® one-step RT-PCR assay

TaqMan® RT-PCR reactions were performed in a final volume of 25 μl using the iScript one-step RT-PCR Kit for probes (Biorad) according to the manufacturer's instructions. The optimum primer and probe concentrations in singleplex and multiplex real-time RT-PCR were determined as the one, which gave the highest reporter fluorescence (end RFUs) and the lowest threshold cycle (C_t). The reactions, carried out with 2 μl of total RNA recovered from plant tissue or aphid, were setup in 8 strip-tubes or 96-well reaction plates and performed on an iQ5 Real-Time PCR Detection System (Biorad). The one-step cycling parameters consisted of 2 min incubation at 55 °C followed by two-step PCR: 5 min incubation at 95 °C followed by 40 cycles at 95 °C for 15 s and 59 °C for 30 s. Each run included two reps each for the unknown sample, the CTV-negative control, the positive CTV-infected control and the non-template control (NTC).

A viral RNA transcript, prepared as described below, and total RNA from Madame Vinous infected with isolate P29 were used in 10-fold serial dilution to generate standard curves and determine the assay efficiency and the quantitation of viral target in the unknown samples.

Data acquisition and analysis were performed with the iQ5 Optical System software version 2.0.

2.6. Preparation of viral RNA transcript for standard curves

A partial CTV coat protein gene from isolate P29, was amplified with T36CP primers (T36CP-F/T36CP-R, Hilf and Garnsey, 2000), purified using the QIAquick PCR Purification Kit (Qiagen), ligated into pGEM-T Easy Vector (Promega) and cloned into *E. coli* JM-109 (Promega). Colonies with the recombinant plasmid were selected by ampicillin resistance and purified using the Wizard Plus SV Miniprep Kit (Promega). The presence of the fragment was confirmed by PCR and its orientation determined by sequencing. The plasmid was linearized with Apa I and RNA transcription performed at 37 °C for 2 h using the T7 RNA polymerase (Roche) and rNTPs (Promega). Plasmid DNA was then digested with RQ1 DNase (Promega) and the transcript isolated by lithium chloride and ethanol precipitation. The concentration was determined using the ND-1000 UV–vis spectrophotometer. Assuming the average molecular weight of a ribonucleotide (340 Da) and the number of bases of the transcript (N_b), the conversion of a microgram of single-stranded RNA to picomole was performed using the formula: pmol of ssRNA = μg (of ssRNA) × (10^6 pg/1 μg) × (1 pmol/340 pg) × ($1/N_b$), and the Avogadro constant (6.023×10^{23} molecules/mol) was used to estimate the number of copies of CTV-RNA (Olmos et al., 2005). Eight 10-fold serial dilutions were prepared by mixing the RNA transcript with healthy Madame Vinous total RNA and used in singleplex and multiplex real-time one-step RT-PCR to generate the standard curve and to determine the CTV-RNA concentration in the unknown samples.

2.7. Comparison of sensitivity between ELISA, conventional RT-PCR and real-time RT-PCR

A pool of three leaf petioles from Madame Vinous plants infected with isolates P29 and P81 were used to evaluate the sensitivity of the various PCR assays. In addition, leaf petioles were extracted in carbonate buffer and processed using the standard ELISA protocol routinely used in the laboratory (Yokomi and Deborde, 2005).

For conventional RT-PCR two protocols were evaluated: (i) two-step RT-PCR; (ii) one-step RT-PCR. In both cases, total RNA was extracted as described by Foissac et al. (2001) and primers specific for the CP region of CTV (Hilf and Garnsey, 2000) and to nad5 were used. For the two-step RT-PCR, 3 μl of total RNAs were used to generate the cDNA in presence of 0.5 μg of random hexamers (Roche), 500 μM of each dNTP, 200 U of M-MLV reverse transcriptase (Promega), 20 U of RNasin ribonuclease inhibitor (Promega). Reactions were performed in a final volume of 20 μl in 1× M-MLV RT reaction buffer (Promega), at 37 °C for 45 min, followed by 10 min at 65 °C. Eight 10-fold serial dilutions of the synthesized cDNA were prepared and 2.5 μl of each was amplified in a 25 μl reaction volume containing a 1× concentration of GoTaq reaction buffer (Promega), 200 μM dNTPs, 0.2 μM of each primer and 1 U of GoTaq DNA polymerase (Promega). Amplification profiles were 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 56 °C for 60 s and 72 °C for 60 s. The reactions were incubated for an additional 10 min at 72 °C. To amplify the partial nad5 gene, the following amplification profile was used: 94 °C for 5 min, followed by 35 cycles at 94 °C for 20 s, 55 °C for 30 s and 72 °C for 20 s. Reaction products were analyzed by electrophoresis in 1% Tris–acetate–EDTA (TAE)–agarose gels and DNA bands visualized by staining the gel in ethidium bromide.

One-step RT-PCR was carried out using eight 10-fold dilutions obtained by mixing 1 μl of total RNA from infected source with 9 μl of total RNA extracted from healthy plant. One microlitter of each dilution was then amplified in 25 μl reaction volume containing 1× GoTaq reaction buffer (Promega), 200 μM dNTPs, 0.2 μM of each primer (S-CTV FW/S-CTV REV; NADF/NADSR), 65 U of M-MLV (Promega) and 1 U of GoTaq DNA polymerase (Promega). Amplification profiles were 42 °C for 1 h, 70 °C for 10 min, 94 °C for 5 min, followed by 35 cycles at 94 °C for 20 s, 55 °C for 20 s and 72 °C for 20 s. The one-step real-time RT-PCR was carried out using the same dilutions used for the one-step RT-PCR previously described.

2.8. CTV detection in aphids

In preliminary tests, nested RT-PCR was used to detect CTV in the aphids and to determine the most effective extraction procedure. cDNA synthesis and first PCR amplification were carried out with 5 μl of each extract, following the same procedure described for plant tissue.

For IC-RT-PCR (protocol 1), cDNA synthesis was performed after incubation of the extracts (50 μl) for 2 h at 37 °C in precoated PCR-tubes. The CTV polyclonal 1212 (Plant Pathology Dept., Univ. Florida, Gainesville, FL) was used at a dilution of 1:1000 to coat PCR-tubes. Following one round of RT-PCR, 1 μl of the generated PCR product was added as the template for nested PCR for further amplification using T36CP-F/P25R primers yielding a 362 bp amplicon. In both cases, PCR consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. PCR was completed by a final extension at 72 °C for 5 min. PCR products were analyzed in 1% TAE–agarose gel and visualized with ethidium bromide stain.

The one-step real-time RT-PCR was optimized using 1 μl of total RNA extracted using the Trizol protocol.

3. Results

3.1. Total RNA isolation from plants

Extracts from healthy, P29 and P81 infected plants using the RNeasy Plant Mini Kit and the Foissac et al. (2001) procedure yielded high quality RNA. No differences were observed in $A_{260/280}$ values, amplicon resolution in agarose gels or C_t values for real-time RT-PCR (data not shown) between the two extraction methods. Consequently, the Foissac et al. (2001) procedure was used to extract the total RNA from all of the infected plants tested.

Differences were observed, however, in amount of RNA recovered from fresh, frozen, and desiccated tissues (Table 1). Desiccation and freezing resulted in low amounts of total RNA extracted (Fig. 1). In spite of this, CTV detection by both conventional and real-time RT-PCR was not affected (Fig. 1). Although fresh tissue gave the lowest C_t in real-time RT-PCR, all CTV isolates tested were detected successfully using the various methods of tissue processing (Table 1). In comparison to fresh or frozen samples infected with CTV, desiccation resulted in lower ELISA A_{405} values, which in some cases resulted in a negative serological test as reported by Hung et al. (2000). In this case, the successful PCR detection was assumed to be due

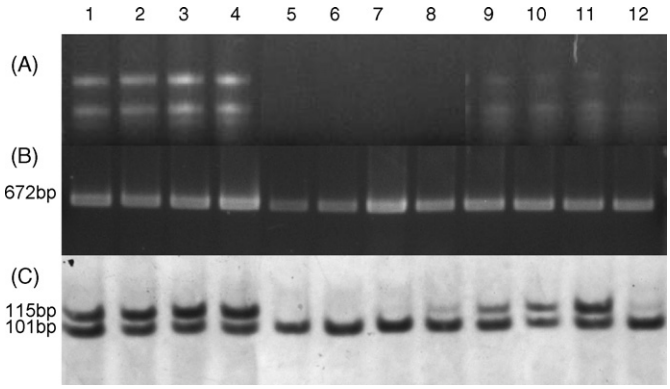

Fig. 1. Electropherograms showing total RNA and CTV coat protein amplicons after conventional reverse transcription polymerase chain reaction (RT-PCR) and multiplex real-time RT-PCR of different infected plant tissues. Samples 1–4 fresh leaf petioles; samples 5–8 desiccated leaf petioles; samples 9–12 frozen bark. (A) Agarose gel electrophoresis of total RNA extracts; (B) agarose gel electrophoresis of CP amplicon obtained by conventional RT-PCR; (C) polyacrylamide gel analysis after multiplex real-time RT-PCR, upper band is the internal control amplicon (115 bp), lower band is the CTV amplicon (101 bp).

to the presence of double stranded RNAs. On the other hand, the nad5 mRNA was not detectable in most of the frozen tissues tested, and, based on A_{260} and C_t values, less total RNA was recovered from desiccated tissue than fresh tissue. Thus, fresh tissue proved to be the best choice for reliable detection of CTV by the multiplex assay. If samples cannot be processed in a short time and need to be stored by freezing or desiccation, the assay is still accurate for CTV detection but the internal control may be not accurate or useful to validate the CTV-negative samples.

3.2. Development of multiplex TaqMan® assays for CTV detection in plant

CTV TaqMan® assays successfully detected CTV isolates P81 and P29. Furthermore, the assay detected CTV in all infected samples from greenhouse-kept plants as well as from CTV-infected field trees (Table 1). All 39 CTV isolates, which included mild, stem pitting, seedling yellows and decline isolates were successfully detected, demonstrating that the primers used detected a broad spectrum of CTV isolates.

The combination of forward 400 nM, reverse 800 nM and probe 80 nM gave the lowest C_t and the highest end relative fluorescence units (RFUs) in singleplex reactions (data not shown).

Multiplex assays included TaqMan® primers and the probe for nad5 mRNA detection. A primers/probe concentration for internal control was selected which resulted in no loss of efficiency in CTV detection. The final primers/probe concentrations for the multiplex assay were: (i) CTV: forward 400 nM, reverse 800 nM and probe 80 nM; (ii) internal control: forward 400 nM, reverse 400 nM and probe 320 nM. Fig. 2 shows a comparison of the efficiency of singleplex versus multiplex for CTV detection. When the concentration of CTV RNA was high, the internal control was less efficient in multiplex than in singleplex (Fig. 3). The multiplex assay consistently detected less than 0.5 femtogram (fg) of CTV RNA transcript when diluted in healthy plant extract (Fig. 2). Regression analysis of the CTV standard curve demonstrated that the assay is highly efficient ($r^2 = 0.987$ and 0.996 (Fig. 2B)) indicating that it can be used with a standard curve to quantify/estimate CTV-RNA concentration (Fig. 4).

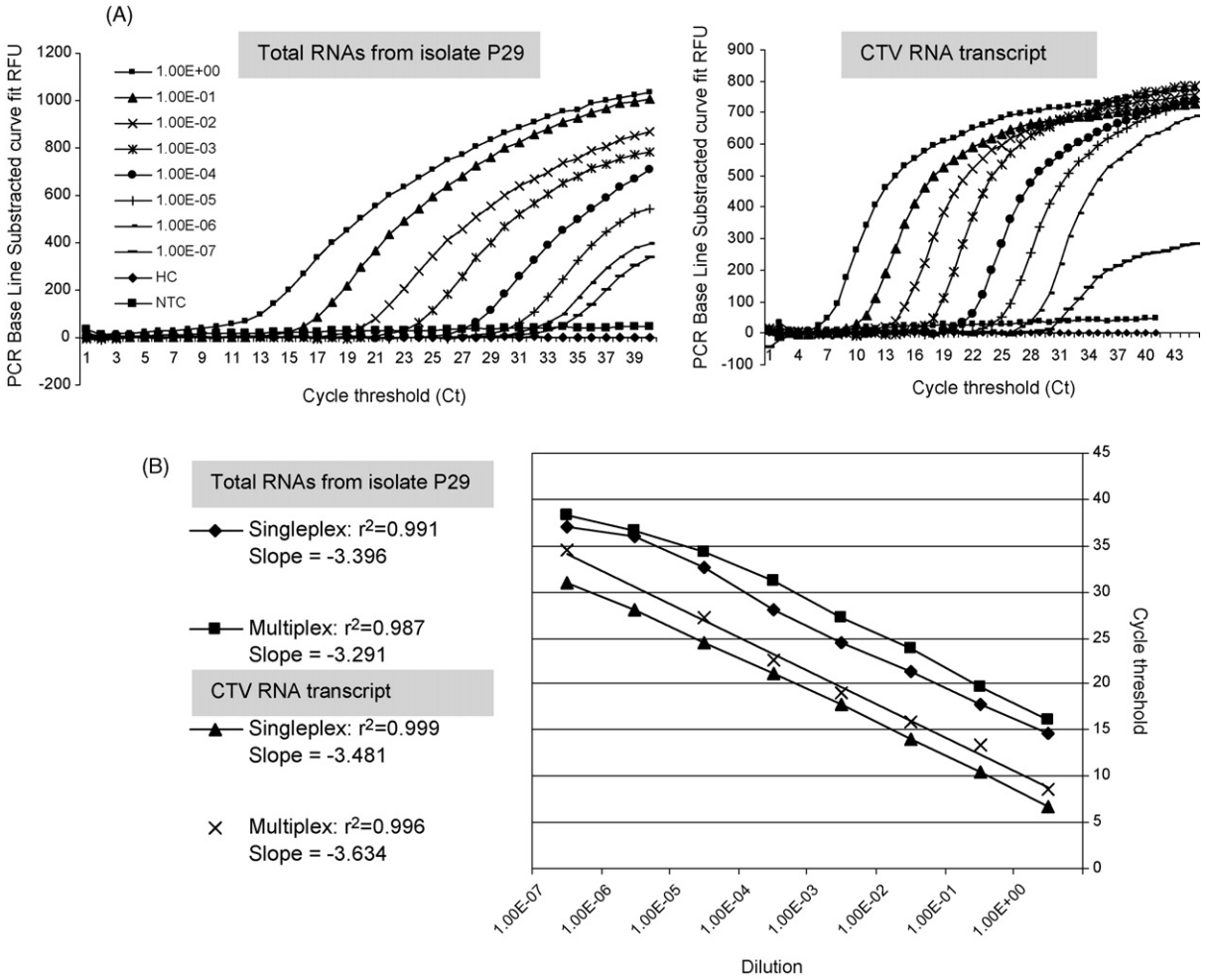

Fig. 2. Real-time reverse transcription polymerase chain reaction (RT-PCR) for CTV. (A) CTV singleplex amplification of serial dilutions (from 1.00E+00 to 1.00E−7) of total RNA from isolate P29 and of CTV RNA transcript (starting concentration 1.8 ng), healthy control (HC) and no template control (NTC). (B) Linear regression for the threshold cycles value vs. dilution of total RNAs and CTV RNA transcript in singleplex and multiplex reactions.

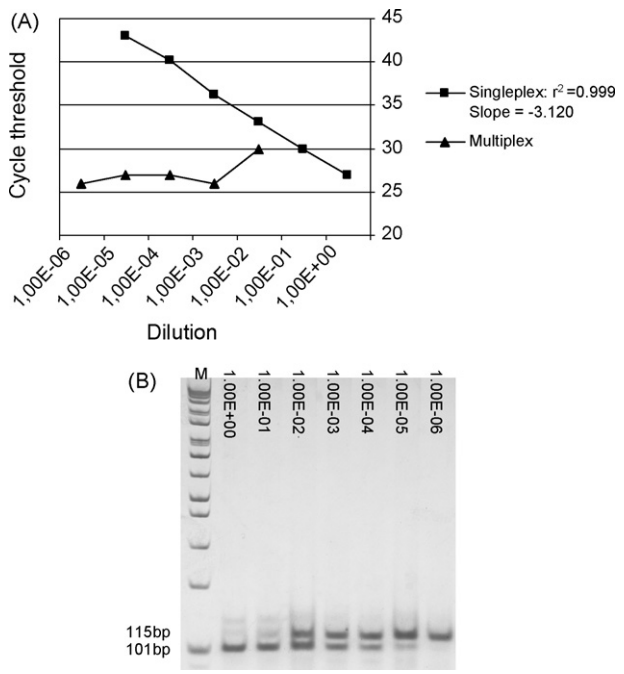

Fig. 3. Real-time reverse transcription polymerase chain reaction (RT-PCR) of the mRNA internal control. (A) Linear regression for the threshold cycles (C_t) values vs. 10-fold dilutions. For singleplex reactions, total RNA was diluted in RNase free water. Multiplex reactions were performed using the CTV RNA transcript diluted in total RNA extracted from healthy plant. (B) Polyacrylamide gel electrophoresis of multiplex real-time RT-PCR products showing the competition between CTV (101 bp) and internal control (115 bp) amplification. M = 1 kb ladder (Invitrogen).

3.3. Sensitivity comparison between ELISA, conventional and real-time RT-PCR

In tests to determine the end-point of CTV detection between the different assays, CTV isolates P29 and P81 were consistently detected when extracts were diluted 10^{-2} by ELISA; and by dilutions 10^{-1} and 10^{-3}, respectively, by one- and two-step RT-PCR (Fig. 5). In contrast, the multiplex one-step real-time RT-PCR consistently gave C_t values lower than 35 when extracts were diluted 10^{-5} (Fig. 2). Thus, real-time RT-PCR was 10^3 times more sensitive than ELISA, as well as 10^4 and 10^2 times more sensitive than one- and two-step conventional RT-PCR, respectively. Conventional one- and two-step RT-PCR for the internal control showed the same end-point dilution limits as for CTV (Fig. 5).

3.4. CTV detection in aphids

Total RNA extracted from single viruliferous aphids by Trizol was the most suitable template for CTV detection by nested RT-PCR (Table 3; Fig. 6). Thereafter, Trizol extracts were chosen for CTV detection in single aphids by one-step real-time RT-PCR. The number of viruliferous aphids detected by real-time RT-PCR indicated that the assay was much more sensitive than conventional nested RT-PCR (Table 3). Quantitative analyses indicated that between 12×10^3 and 13×10^6 of CTV RNA molecules were in a single aphid after a 48 h AAP under test conditions.

4. Discussion

A number of methods have been described to detect CTV. Indexing on Mexican lime seedlings (Wallace and Drake, 1951) is the conventional procedure. For routine detection of CTV, ELISA and DTBIA using polyclonal, monoclonal, recombinant antibodies to provide rapid, economical and reliable results (Bar-Joseph et al., 1979; Cambra et al., 1991, 2000a; Garnsey et al., 1993; Rocha-Pena and Lee, 1991; Nikolaeva et al., 1996; Terrada et al., 2000).

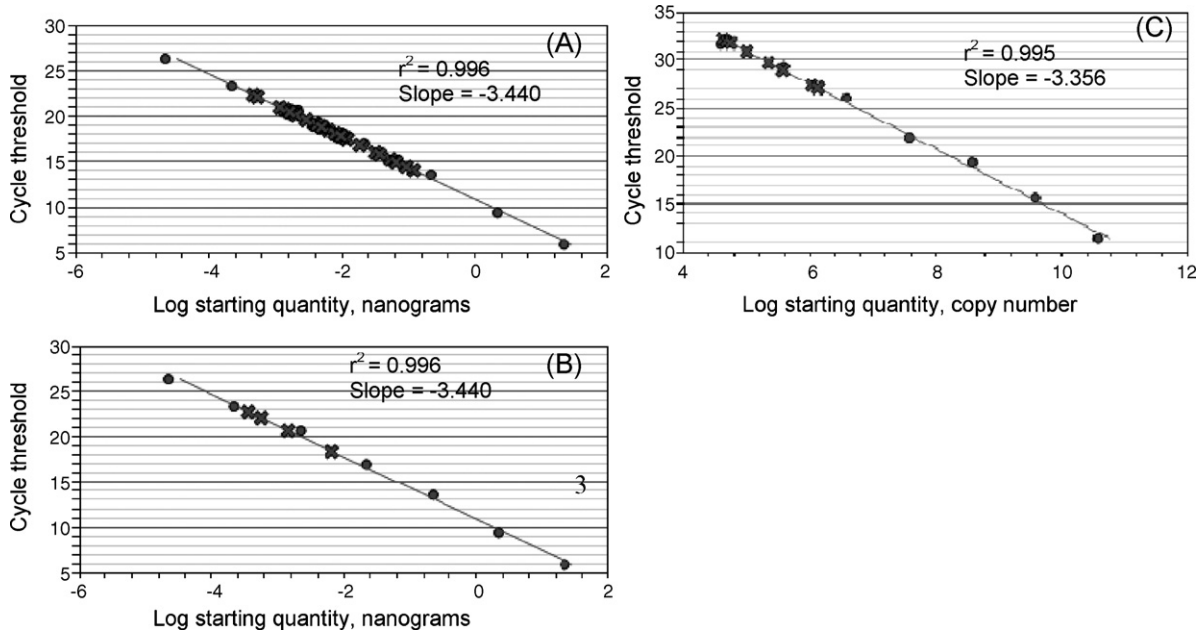

Fig. 4. Quantification of CTV RNA (✖) in infected plants (nanograms, A and B) and in single aphids (copy number, C) based on the standard curve obtained with 10-fold dilution of CTV RNA transcript (●). (A) Samples from the CTV collections, (B) infected samples collected in the field and (C) viruliferous aphids.

(A)

ELISA results								
Dilution	1.00E+00	1.00E-01	1.00E-02	1.00E-03	1.00E-04	1.00E-05	1.00E-06	1.00E-07
*A_{405}	1.240	1.167	0.649	0.267	0.178	0.212	0.161	0.191

* measured after 2h

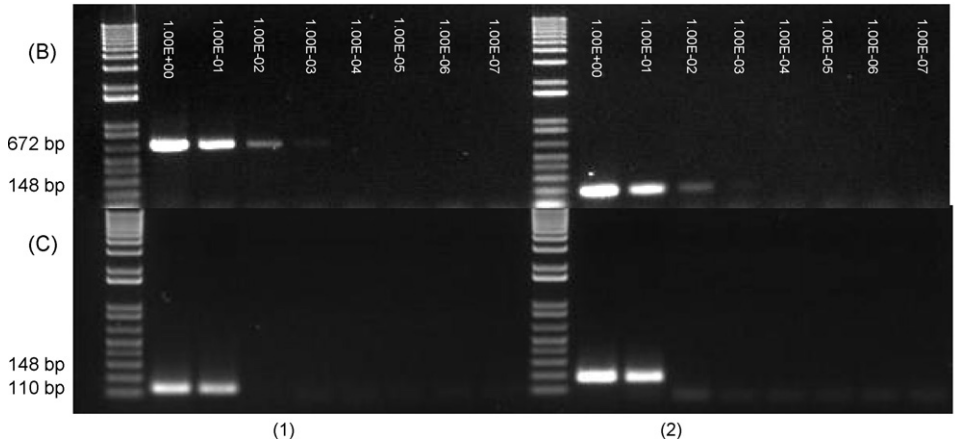

Fig. 5. Sensitivity of ELISA assay, two- and one-step conventional reverse transcription polymerase chain reaction (RT-PCR) using 10-fold serial dilutions of P29 isolate. (A) ELISA results (A_{405}), reference values: positive control (1.722), healthy control (0.162). (B) and (C) Agarose gel electrophoresis of two- and one-step RT-PCR products generated by CTV CP primers (1) and by nad5 primer (2).

Table 3
CTV detection in single *A. gossypii* aphids using different extraction protocols (viruliferous aphids/total aphids tested)

Extraction protocol used on CTV viruliferous aphids	Nested RT-PCR	Real-time RT-PCR
1. Crushed on 3 MM chromatography paper[a]	0/40	n.t.[b]
2. Crushed on nitrocellulose membrane	1/20	n.t.
3. Homogenized in sodium sulfite	13/48	n.t.
4. Homogenized in PBS–Tween-PVP	2/18	n.t.
5. Homogenized in Trizol	16/38	17/20

The aphid was given a 48 h acquisition access period on a CTV-infected Madame Vinous sweet orange donor plant.
[a] 20 aphids processed by IC- and 20 directly in RT-PCR.
[b] Not tested.

PCR-based and molecular hybridization techniques have been developed for CTV detection (Mathews et al., 1997; Hung et al., 2000; Cambra et al., 2000b; Roy et al., 2005; Barbarossa and Savino, 2006) and strain differentiation (Cevik et al., 1996; Niblett et al., 2000; Hilf and Garnsey, 2000; Hilf et al., 2005; Halbert et al., 2004; Sieburth et al., 2005; Huang et al., 2004). These methods are more sensitive than ELISA and are useful for detecting the virus from field samples even when the titer is not optimum for serological tests. IC-RT-PCR, RT-PCR and nested RT-PCR have been also described to detect CTV in vector and non-vector aphid species (Metha et al., 1997; Cambra et al., 2000b; Marroquin et al., 2004).

Real-time PCR has gained popularity over conventional PCR because it uses reduced cycle times and steps and there is no need for electrophoresis, staining and gel documentation. It also

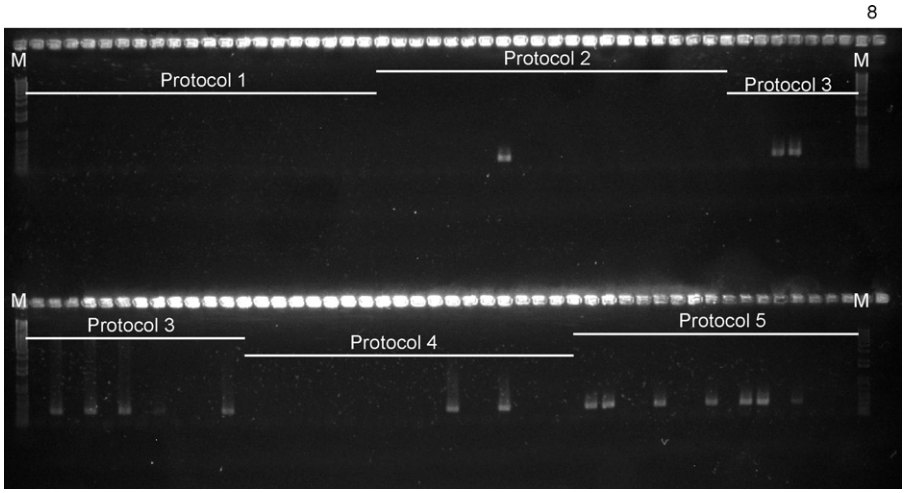

Fig. 6. Agarose gel electrophoresis of nested RT-PCR of total RNA recovered from single aphid using different extraction protocols. M = 1 kb ladder (Invitrogen).

has increased sensitivity, reproducibility and the reduced risk of carry-over contamination since the amplicon production is monitored in a closed tube. Amplicon production is monitored during amplification by labeling the primers, probes or amplicons with fluorogenic molecules (Mackay et al., 2002). TaqMan® probes represent one of the main chemistries used for real-time PCR assays. The successful use of real-time RT-PCR using TaqMan® probes have been reported for the detection of different woody plant-infecting viruses (Marbot et al., 2003; Schneider et al., 2004; Osman and Rowhani, 2006; Osman et al., 2007) as well as to detect viruses in different insect vectors (Boonham et al., 2002; Fabre et al., 2003; Olmos et al., 2005).

In the present study, primers and TaqMan® probes for CTV detection *in planta* and in single aphids by multiplex real-time one-step RT-PCR assay were developed. The TaqMan® RT-PCR assay incorporated an internal control based on the mRNA of the mitochondrial gene NADH dehydrogenase (nad5). This provided a check on the quality of extracted RNA and helped to assess if it was sufficient, hence, reducing chances for false negative results (Menzel et al., 2002).

The primers and TaqMan® probe detected all geographically and biologically different CTV isolates tested. The degeneracy included in the CTV primers and TaqMan® probe assured that the assay was broad spectrum. In contrast, serological methods based on the recognition of specific epitopes by monoclonal and some polyclonal antibodies may not detect some isolates due to coat protein variability among strains (Wang et al., 2007). In these cases, a mixture of monoclonal or polyclonal antibodies is usually needed (Cambra et al., 1990).

The multiplex real-time RT-PCR assay described in this report was more sensitive than ELISA and conventional RT-PCR and consistently detected less then 1 fg of CTV RNA transcript mixed into extracts from healthy tissue. This suggested that the method could detect a very low level of CTV-RNA in infected field tissue as was demonstrated by the detection of CTV from naturally infected trees near Lindsay, CA. This high sensitivity allowed its use even when the virus titer was low and not readily detectable by serological methods.

The real-time RT-PCR assay was able to detect CTV in frozen or desiccated plant tissues; whereas ELISA failed to detect CTV in some desiccated or dried tissue (data not shown) as was reported by Hung et al. (2000). Furthermore, compared with ELISA and conventional RT-PCR, the one-step multiplex assay is fast, relatively easy to perform and cost effective.

Relative or absolute quantitation of target nucleic acids is an essential feature of real-time PCR assay (Mackay et al., 2002). The CTV real-time assay was based on absolute quantitation (nanograms/copies) of CTV-RNA in the unknown samples in either plant tissues or aphids. This approach is useful for monitoring the progress of infection (i.e., after vector transmission/graft inoculation, viral replication in resistant or tolerant citrus cultivars), and transcription of exogenous genes in transgenic plants and to assess the large diversity in the transmissibility of CTV isolates by aphid vectors.

The method used to extract total RNA from viruliferous *A. gossypii* was found to be critical. Detection of CTV by nested RT-PCR was directly related to the extraction procedure used (Fig. 6). Specifically, under test conditions of RT-PCR here: (i) CTV was detected and quantified when total RNA was extracted from aphids by Trizol; (ii) no positive reactions were obtained after the first RT-PCR with any of the other protocols tested; and (iii) no CTV was detected from aphids squashed on 3 MM CHR paper. The CTV quantitation obtained from test aphids were greater than those reported from aphids carrying plum pox virus (Olmos et al., 2005), a non-persistent aphid transmitted virus and similar to that reported for aphids carrying barley yellow dwarf virus (Fabre et al., 2003), a persistent aphid transmitted virus. The Trizol extraction and real-time PCR was simple and more reliable for CTV detection in aphids than using nested RT-PCR as reported by Cambra et al. (2000b) and Marroquin et al. (2004).

In conclusion, real-time RT-PCR was used successfully to detect CTV in infected plants and viruliferous aphid vectors. Rapid and accurate detection of CTV achieved by real-time RT-PCR can now be used as a tool to support control measures to limit CTV disease spread. In addition, it can be used in quarantine, eradication and certification programs.

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References

Barbarossa, L., Savino, V., 2006. Sensitive and specific digoxigenin-labelled RNA probes for routine detection of *Citrus tristeza virus* by dot-blot hybridization. J. Phytopathol. 154, 329–335.

Bar-Joseph, M., Garnsey, S.M., Gonsalves, D., Moscovitz, M., Purcifull, D.E., Clark, M.F., Loebenstein, G., 1979. The use of enzyme-linked immunosorbent assay for detection of *Citrus tristeza virus*. Phytopathology 69, 190–194.

Boonham, N., Smith, P., Walsh, K., Tame, J., Morris, J., Spence, N., Bennison, J., Barker, I., 2002. The detection of tomato spotted wilt virus (TSWV) in individual thrips using real-time fluorescent RT-PCR (TaqMan). J. Virol. Methods 101, 37–48.

Cambra, M., Garnsey, S.M., Permar, T.A., Henderson, C.T., Gumpf, D., Vela, C., 1990. Detection of *Citrus tristeza virus* (CTV) with a mixture of monoclonal antibodies. Phytopathology 80, 103.

Cambra, M., Camarasa, E., Gorris, M.T., Garnsey, S.M., Carbonell, E., 1991. Comparison of different immunosorbent assays for *Citrus tristeza virus* (CTV) using CTV-specific monoclonal and polyclonal antibodies. In: Brlansky, R.H., Lee, R.H., Timmer, L.W. (Eds.), Proceedings of the 11th Conference of International Organization of Citrus Virologists. IOCV, Riverside, CA, pp. 38–45.

Cambra, M., Gorris, M.T., Román, M.P., Terrada, E., Garnsey, S.M., Camarasa, E., Olmos, A., Colomer, M., 2000a. Routine detection of *Citrus tristeza virus* by direct immunoprinting-ELISA methods using specific monoclonal and recombinant antibodies. In: da Graça, J.V., Lee, R.F., Yokomi, R.K. (Eds.), Proceedings of the 14th Conference of International Organization of Citrus Virologists. IOCV, Riverside, CA, pp. 34–41.

Cambra, M., Olmos, A., Gorris, M.T., Marroquín, C., Esteban, O., Garnsey, S.M., Llauger, R., Batista, L., Peña, I., Hermoso de Mendoza, A., 2000b. Detection of *Citrus tristeza virus* by print capture and squash capture-PCR in plant tissue and single aphids. In: da Graça, J.V., Lee, R.F., Yokomi, R.K. (Eds.), Proceedings of the 14th Conference of International Organization of Citrus Virologists. IOCV, Riverside, CA, pp. 42–49.

Cambra, M., Gorris, M.T., Olmos, A., Martínez, M.C., Román, M.P., Bertolini, E., López, A., Carbonell, E.A., 2002. European diagnostic protocol (DIAGPRO) for *Citrus tristeza virus* in adult trees. In: Duran-Vila, N., Milne, R.G., da Graça (Eds.), Proceedings of the 15th Conference of International Organization of Citrus Virologists. IOCV, Riverside, CA, pp. 69–77.

Cevik, B., Pappu, S.S., Pappu, H.R., Benscher, D., Irey, M., Lee, R.F., Niblett, C.L., 1996. Application of bi-directional PCR to *Citrus tristeza virus*: detection and strain differentiation. In: da Graça, J.V., Moreno, P., Yokomi, R.K. (Eds.), Proceedings of the 13th Conference of International Organization of Citrus Virologists. IOCV, Riverside, CA, pp. 17–24.

Fabre, F., Kervarrec, C., Mieuzet, L., Riault, G., Vialatte, A., Jacquot, E., 2003. Improvement of barley yellow dwarf virus-PAV detection in single aphids using a fluorescent real-time RT-PCR. J. Virol. Methods 110, 51–60.

Foissac, X., Svanella-Dumas, L., Dulucq, M.J., Candresse, T., Gentit, P., 2001. Polyvalent detection of fruit tree tricho, capillo and foveaviruses by nested-PCR using degenerated and inosine containing primers. Acta Horticolturae 550, 37–44.

Garnsey, S.M., Permar, T.A., Cambra, M., Henderson, C.T., 1993. Direct tissue blot immunoassay (DTBIA) for detection of *Citrus tristeza virus* (CTV). In: Moreno, P., da Graça, J.V., Timmer, L.W. (Eds.), Proceedings of the 12th Conference of International Organization of Citrus Virologists. IOCV, Riverside, CA, pp. 39–50.

Garnsey, S.M., Gottwald, T.R., Borbón, J.C., 1996. Rapid dissemination of mild isolates of *Citrus tristeza virus* following introduction of *Toxoptera citricida* in the Dominican Republic. In: da Graça, J.V., Moreno, P., Yokomi, R.K. (Eds.), Proceedings of the 13th Conference of International Organization of Citrus Virologists. IOCV, Riverside, CA, pp. 92–103.

Garnsey, S.M., Civerolo, E., Gumpf, D.J., Paul, C., Hilf, M.E., Lee, R.F., Brlansky, R.H., Yokomi, R.K., Hartung, J.S., 2005. Biological characterization of an international collection of *Citrus tristeza virus* (CTV) isolates. In: Hilf, M.E., Duran-Vila, N., Rocha-Peña, M.A. (Eds.), Proceedings of the 16th Conference of International Organization of Citrus Virologists. IOCV, Riverside, CA, pp. 75–93.

Halbert, S.E., Genc, H., Cevik, B., Brown, L.G., Rosales, I.M., Manjunath, K., Pomerinke, M., Davison, D.A., Lee, R.F., Niblett, C.L., 2004. Distribution and characterization of *Citrus tristeza virus* in south Florida following establishment of *Toxoptera citricida*. Plant Dis. 88, 935–941.

Hilf, M.E., Garnsey, S.M., 2000. Characterization and classification of *Citrus tristeza virus* isolate by amplification of multiple molecular markers. In: da Graça, J.V., Lee, R.F., Yokomi, R.K. (Eds.), Proceedings of the 14th Conference of International Organization of Citrus Virologists. IOCV, Riverside, CA, pp. 18–27.

Hilf, M.E., Mavrodieva, V.A., Garnsey, S.M., 2005. Genetic marker analysis of a global collection of isolates of *Citrus tristeza virus*: characterization and distribution of CTV genotypes and association with symptoms. Phytopathology 95, 909–917.

Huang, Z., Rundell, A.P., Guan, X., Powell, A.C., 2004. Detection and isolate differentiation of *Citrus tristeza virus* in infected field trees based on reverse transcription-polymerase chain reaction. Plant Dis. 88, 625–629.

Hung, T.H., Wu, M.L., Su, H.J., 2000. A rapid method based on the one-step reverse transcriptase-polymerase chain reaction (RT-PCR) technique for detection of different strains of *Citrus tristeza virus*. J. Phytopathol. 148, 469–475.

Karasev, A.V., Boyko, V.P., Gowda, S., Nikolaeva, O., Hilf, M.E., Koonin, E.V., Niblett, C.L., Cline, K., Gumpf, D., Lee, R.F., Garnsey, S.M., Levandowski, D.J., Dawson, W.O., 1995. Complete sequence of the *Citrus tristeza virus* RNA genome. Virology 208, 511–520.

Lee, R.F., Bar-Joseph, M., 2000. Tristeza. In: Timmer, L.W., Garnsey, S.M., Graham, J.H. (Eds.), Compendium of Citrus Diseases. APS Press, St. Paul, MN, pp. 61–63.

Mackay, I., Arden, K., Nitsche, A., 2002. Real-time PCR in virology. Nucleic Acids Res. 30, 1292–1305.

Marbot, S., Salmon, M., Vandrame, M., Huwaert, A., Kummert, J., Dutrecq, O., Lepoivre, P., 2003. Development of real-time RT-PCR assay for detection of prunus necrotic virus in fruit trees. Plant Dis. 87, 1344–1348.

Marroquin, C., Olmos, A., Gorris, M.T., Bertolini, E., Martinez, M.C., Carbonell, E.A., Hermoso de Mendoza, A., Cambra, M., 2004. Estimation of the number of aphids carrying *Citrus tristeza virus* that visit adult citrus trees. Virus Res. 100, 101–108.

Martelli, G.P., Agranovsky, A.A., Bar-Joseph, M., Boscia, D., Candresse, T., Coutts, R.H.A., Dolja, V.V., Falk, B.W., Gonsalves, D., Hu, J., Jelkmann, Karasev, A.V., Minafra, A., Namba, S., Vetten, H.J., Wisler, C.G., Yoshikawa, N., 2005. Virus taxonomy. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego, CA.

Mathews, D.M., Riley, K., Dodds, J.A., 1997. Comparison of detection methods for *Citrus tristeza virus* in field trees during months of nonoptimal titer. Plant Dis. 81, 525–529.

Mawassi, M., Mietkiewska, E., Gofman, R., Yang, G., Bar-Joseph, M., 1996. Unusual sequences relationships between two isolates of *Citrus tristeza virus*. J. Gen. Virol. 77, 2359–2364.

Menzel, W., Jelkmann, W., Maiss, E., 2002. Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. J. Virol. Methods 99 (1/2), 81–92.

Metha, P., Brlansky, R.H., Gowda, S., Yokomi, R.K., 1997. Reverse-transcription polymerase chain reaction detection of *Citrus tristeza virus* in aphids. Plant Dis. 81, 1066–1069.

Niblett, C.L., Genc, H., Cevik, B., Halbert, S., Brown, L., Nolasco, G., Bonacalza, B., Manjunath, K.L., Febres, V.J., Pappu, H.R., Lee, R.F., 2000. Progress on strain differentiation of *Citrus tristeza virus* and its application to the epidemiology of citrus tristeza disease. Virus Res. 71, 97–106.

Nikolaeva, O.V., Karasev, A.V., Powell, A.C., Gumpf, D.J., Garnsey, S.M., Lee, R.F., 1996. Mapping of epitopes for *Citrus tristeza virus*-specific monoclonal antibodies using bacterially expressed coat protein fragment. Phytopathology 86, 974–979.

Olmos, A., Bertolini, E., Gil, M., Cambra, M., 2005. Real-time assay for quantitative detection of non-persistently transmitted *Plum pox virus* RNA targets in single aphids. J. Virol. Methods 128, 151–155.

Osman, F., Rowhani, A., 2006. Application of a spotting sample preparation technique for the detection of pathogens in woody plants by RT-PCR and real-time PCR (TaqMan). J. Virol. Methods 133, 130–136.

Osman, F., Leutenegger, C., Golino, D., Rowhani, A., 2007. Real-time RT-PCR (TaqMan®) assays for the detection of *Grapevine Leafroll associated viruses* 1–5 and 9. J. Virol. Methods 141, 22–29.

Polek, M., Gumpf, D.J., Wallen, C.M., Riley, K.M., 2005. Biological characterization of naturally occurring *Citrus tristeza virus* strains in California. In: Hilf, M.E., Duran-Vila, N., Rocha-Peña, M.A. (Eds.), Proceedings of the 16th Conference of International Organization of Citrus Virologists. IOCV, Riverside, CA, pp. 68–74.

Rocha-Pena, M.A., Lee, R.F., 1991. Serological technique for detection of *Citrus tristeza virus*. J. Virol. Methods 34, 311–331.

Roistacher, C.N., 1991. Graft-Transmissible Diseases of Citrus: Handbook for Detection and Diagnosis. IOCV and FAO, Rome, p. 286.

Roy, A., Fayad, A., Barthe, G., Brlansky, R.H., 2005. A multiplex polymerase chain reaction method for reliable, sensitive and simultaneous detection of multiple viruses in citrus trees. J. Virol. Methods 129, 47–55.

Schneider, W.L., Sherman, D.J., Stone, A.L., Damsteegt, V.D., Frederick, R.D., 2004. Specific detection and quantification of plum pox virus by real-time fluorescent reverse transcription-PCR. J. Virol. Methods 120, 97–105.

Sieburth, P.J., Nolan, K.G., Hilf, M.E., Lee, R.F., Moreno, P., Garnsey, S.M., 2005. Discrimination of stem-pitting from other isolates of *Citrus tristeza virus*. In: Hilf, M.E., Duran-Vila, N., Rocha-Peña, M.A. (Eds.), Proceedings

of the 16th Conference of International Organization of Citrus Virologists. IOCV, Riverside, CA, pp. 1–10.

Singh, R.P., Dilworth, A.D., Singh, M., McLaren, D.L., 2004. Evaluation of a simple membrane-based nucleic acid preparation protocol for RT-PCR detection of potato viruses from aphid and plant tissues. J. Virol. Methods 121, 163–170.

Terrada, E., Kerschbaumer, R.J., Giunta, G., Galeffi, P., Himmler, G., Cambra, M., 2000. Fully "recombinant enzyme-linked immunosorbent assay" using genetically engineered single-chain antibody fusion proteins for detection of *Citrus tristeza virus*. Phytopathology 90, 1337–1344.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876–4882.

Varga, A., James, D., 2005. Detection and differentiation of plum pox virus using real-time multiplex PCR with SYBR green and melting curve analysis: a rapid method for strain typing. J. Virol. Methods 123, 213–220.

Varga, A., James, D., 2006. Real-time RT-PCR and SYBR green I melting curve analysis for the identification of plum pox virus strains C, EA, and W: effect of amplicon size, melt rate, and dye translocation. J. Virol. Methods 132, 146–153.

Wallace, J.M., Drake, R.J., 1951. Recent developments in studies of quick decline and related diseases. Phytopathology 41, 785–793.

Wang, C., Ni, H., Wang, G.P., Zhang, J.K., Hui, L., 2007. Molecular analysis of a Chinese *Citrus tristeza virus* isolate with differential serological reactivity. J. Plant Pathol. 89, in press.

Yokomi, R.K., Deborde, R.L., 2005. Incidence, transmissibility, and genotype of *Citrus tristeza virus* (CTV) isolates from a CTV eradicative and a non-eradicative district in central California. Plant Dis. 89 (8), 859–866.