Rapid Differentiation and Identification of Potential Severe Strains of *Citrus tristeza virus* by Real-Time Reverse Transcription-Polymerase Chain Reaction Assays

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ABSTRACT

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A multiplex Taqman-based real-time reverse transcription (RT) polymerase chain reaction (PCR) assay was developed to identify potential severe strains of *Citrus tristeza virus* (CTV) and separate genotypes that react with the monoclonal antibody MCA13. Three strain-specific probes were developed using intergene sequences between the major and minor coat protein genes (CPi) in a multiplex reaction. Probe CPi-VT3 was designed for VT and T3 genotypes; probe CPi-T36 for T36

genotypes; and probe CPi-T36-NS to identify isolates in an outgroup clade of T36-like genotypes mild in California. Total nucleic acids extracted by chromatography on silica particles, sodium dodecyl sulfate-potassium acetate, and CTV virion immunocapture all yielded high quality templates for real-time PCR detection of CTV. These assays successfully differentiated CTV isolates from California, Florida, and a large panel of CTV isolates from an international collection maintained in Beltsville, MD. The utility of the assay was validated using field isolates collected in California and Florida.

Additional keywords: decline, seedling yellows, stem pitting, strain differentiation.

Citrus tristeza virus (CTV) (Closteroviridae) is a phloem-limited, semipersistent aphid-transmitted virus. The virus exists in infected trees as heterogeneous populations, often with more than one genotype along with variants and defective RNAs (1,8,15,17,24,32). This genetic complexity results in different symptoms among citrus cultivars. Strains characterized in a citrus host range index test are generally categorized as: mild, decline inducing death of scions grafted onto sour orange (SO) rootstock; and sweet orange stem pitting (OSP) and grapefruit stem pitting (GSP). OSP and GSP are damaging CTV isolates that cause defective phloem development resulting in greatly reduced growth and production. Isolates that cause stunting and chlorosis of grapefruit, sour orange, or lemon seedlings in the greenhouse are referred to as seedling yellows (SY) (5,18). Thus, the ability to differentiate CTV strains is practical and important for the management of diseases caused by CTV.

Spread of CTV in Central California has increased significantly over the past few years (33,34) and local efforts to suppress the virus by survey and eradication have become too costly and extremely difficult. Routine surveys and monitoring of CTV in California have been based on enzyme-linked immunosorbent assay (ELISA) using universal CTV antiserum, whereas Florida used MCA13 antibody which generally detects all CTV strains except T30 (19,20). However, MCA13 does not distinguish isolates that cause only decline from isolates that also may cause stem pitting or mild symptoms, creating a need for more selective

probes to rapidly discriminate CTV strains among MCA13 reactive isolates.

Other detection procedures have been developed to differentiate strains which include, but are not limited to, single-strand conformation polymorphism (23,25); restriction fragment polymorphism length analysis (7); reverse transcription (RT)-polymerase chain reaction (PCR) assay with selective primers (4,11,13); and hybridization with specific probes (6,9). Saponari and Yokomi (29) developed a quantitative real-time RT-PCR (qRT-PCR) assay to identify some CTV isolates based on 3' end sequences. Ruiz-Ruiz et al. (27) described a qPCR assay to differentiate CTV strains using TaqMan LNA probes designed from sequences in the 5' portion of the CTV genome.

Rapid identification of genetic diversity of CTV by qPCR can aid efforts to manage CTV by identifying potentially virulent isolates that growers and regulatory agencies can target for CTV disease management decisions. We developed a qPCR assay using specific TaqMan probes for simultaneous detection and differentiation of CTV strains within 2 h which allows rapid assessment of potential virulent CTV strains. This method is under consideration for use by the Central California Tristeza Eradication Agency (CCTEA), Tulare, CA, to select CTV-infected trees that should be removed to eliminate potential severe strain inoculum sources. The Bureau of Citrus Budwood Registration (BCBR), Florida Department of Agriculture and Consumer Services, Winter Haven, FL, is also considering using this qPCR method to identify severe CTV strains found contaminating registered budwood source trees. This is particularly important in Florida because the efficient CTV vector, *Toxoptera citricda*, is well established and accelerates spread of virulent CTV strains (9). This work was designed to validate this procedure.

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MATERIALS AND METHODS

CTV isolates. The assays were developed and assessed with 56 California CTV isolates maintained by the Agricultural Research Service (ARS), Parlier, CA (33) and the CCTEA (21), 82 isolates from a collection maintained by ARS in Beltsville, MD (5), and 60 isolates maintained by the BCBR (30). Florida and Beltsville isolates were obtained as desiccated leaf and bark tissue from infected Madam Vinous sweet orange (*Citrus sinensis* L.). These isolates are listed and described in Table 1 and identified using the following suffixes: CCTEA-, CA-, and P- for California isolates; B- for ARS Beltsville isolates; and FL-, FS-, and T- for Florida isolates.

Selection of strain-specific TaqMan probes. Sequences between P25 (CP) and P27 (CPm) coat protein genes of various isolates were used as previously reported to design a selective 6'-carboxy-fluorescein (6-FAM)-labeled TaqMan probe (CPi-VT3, minor groove binding [MGB]-ACGGKGRATTCGC) for the identification of isolates containing VT and T3 genotypes (29). Based on an alignment of multiple CP-CPm intergene sequences, two additional strain-specific TaqMan probes, CPi-T36 (MGB-ACGGTAACATTACTATCCCC) and CPi-T36NS (MGB-ACG GTARTATYATRCATCCT), were designed at the same nucleotide positions as the CPi-VT3 probe and were used with the primers P27F/P27R (29). The alignment of the multiple sequences and analysis of conserved sequences was carried out using ClustalX (31), followed by the neighbor-joining method in the MegAlign program of DNASTAR (Lasergene, 7.2) to determine phylogenetic relationships.

The CPi-VT3 and CPi-T36 MGB probes were both labeled with 6-FAM and used in Parlier, whereas assays performed in Florida were labeled with NED (Applied Biosystem) and VIC (Applied Biosystem), respectively. The CPi-T36NS probe was labeled with tetrachloro-6-carboxyfluorescein (TET). These strain-specific probes were tested in multiplex reactions with the generic CP25f/r primers and Cyanine 5 (CY5)-labeled TaqMan probe CP-CY5 (28).

Comparison of extraction procedures for qPCR templates. Young leaves, midribs, and bark infected with CTV isolates P29 and P81 were used to evaluate methods for qPCR template preparation. Total nucleic acids (TNA) from ≈ 200 mg of fresh tissue or 50 mg of desiccated tissue were isolated by chromatography on silica particles (28) or by sodium dodecyl sulfate (SDS)-potassium acetate (14) and used directly in one-step real-time RT-PCR assay. Immunocapture with CTV rabbit polyclonal antiserum 1212 (Plant Pathology Department, University of Florida, Gainesville) followed by RT-PCR (12) was tested as an alternative to TNA isolation. In this case, extraction was performed with PBS-PVP-Tween buffer (1:5, wt/vol) and PCR using tubes precoated with the CTV antiserum at a dilution of 1:1,000. Samples processed in Parlier were ground in 2-ml tubes using the Mini-Beadbeater 96 (BioSpec Products, Inc., Bartlesville, OK), whereas in Florida, a Homex 6 with tissue in the manufacturer's plastic bag (Bioreba, Switzerland) was used. Aliquots of each nucleic acid preparation were used to set up qPCR reactions using the CTV generic primer/TaqMan probe set (CP25f/r-CTV-CY5) (28). The resultant cycle threshold (Ct) values were then compared to evaluate the efficacy of each extraction procedure tested.

Single and multiplex real-time RT-PCR assays. One and a half microliters of plant TNA containing ≈ 0.3 to $0.5 \mu\text{g}$ of total RNA or cDNA prepared as described above was used in a final volume of $12.5 \mu\text{l}$ containing $1 \times$ iScript one-step RT-PCR buffer and iScript reverse transcriptase (for one-step qRT-PCR) (Bio-Rad Laboratories, Hercules, CA) or $1 \times$ iQ Supermix buffer (for qPCR) (Bio-Rad Laboratories). Primer and probe concentrations and cycling conditions for P25F/R-CTV-CY5; P27F/R-CPi-VT3, CPi-T36, and CPi-T36NS probes were as previously reported for single and multiplex reactions (29). Reactions were performed

with the following conditions: 80 nM for CTV-CY5 probe; 160 nM for each CPi probe; 160 nM for P25R; and 320 nM for P25F, P27R, and P27F. The amplification profile was one cycle at 55°C for 2 min and then 5 min at 95°C followed by 40 cycles at 95°C for 15 s and 56°C for 40 s. The first cycle was skipped when the qPCR followed the cDNA synthesis (two-step qPCR).

Interference of nontarget CTV isolates was tested by spiking a constant quantity of TNA from the P81 isolate (mild, T30 genotype) to each serial dilution of the CPi target isolate. The quantity (μl) of P81 added was determined by its Ct value with CTV-CY5 (generic) probe in a way that the first dilution of each target isolate contained an equimolar amount of both isolates (target and nontarget).

The treatments in the multiplex assays were as follows: (A) TNA from P29 (VT genotype) and B3 (T36 genotype) isolates tested with a mixture of CPi-VT3, CPi-T36, and CP-CY5 probes; (B) TNA from B3 and CA-P116 (T36-NS genotype) isolates tested with a mixture of CPi-T36, CPi-T36NS, and CP-CY5 probes; (C) same as treatment A except the TNA from the isolate CA-P116 was added to the mixture along with the CPi-T36NS probe; and (D) all three target strains (P29, B3, and CA-P116) with all CPi probes without the generic probe.

Treatments A and B were used to test a panel of selected isolates (Table 1); assays were conducted with two replications for each sample, plus a CTV-negative control, a CTV-positive control, and a nontemplate control. Samples from CCTEA and ARS-Beltsville and California field isolates were processed in one-step qRT-PCR, while a two-step qPCR was performed on Florida samples.

RESULTS

Single and multiplex real-time RT-PCR assays. Excellent CTV detection and strain differentiation were achieved with the probes developed. All nucleic acid preparations tested as templates for qPCR assays were effective. Based on Ct values, however, the best procedure for nucleic acid extraction was by chromatography on silica particles (range 13.3 to 18.1). The SDS-potassium acetate method resulted in slightly higher Ct values (range 15.3 to 19.6) but was still effective. qPCR using viral cDNA synthesized after immunocapture of virus particles resulted in a higher Ct value indicating it was less effective than the other two methods.

Standard curves generated in single assays with the CPi-T36 and CPi-T36NS probes had correlation coefficients (R^2) of 0.989 and 0.998, respectively, indicating high reproducibility and amplification efficiency in the optimal range of 90 to 120%. When these probes were multiplexed with the universal CP-CY5, the R^2 values of the standard curves generated using the same 10-fold dilutions ranged from 0.990 for the CP-CY5 to 0.996 for the FAM-labeled CPi-T36 and 0.999 for the TET-labeled CPi-T36NS, thus indicating high reproducibility and no reduction in the amplification efficiency. Specifically for the CPi-T36, the efficiency for the single was 91.8 versus 91.7 for the multiplex assays and that for the CPi-T36NS was 95.0 versus 93.2, respectively. The amplification efficiency obtained with the CP-CY5 in these multiplex assays was 120% versus 111%, respectively.

When the five 10-fold dilutions prepared with the reference isolates B3 and P29 were artificially mixed and used as a template for the multiplex assays, both isolates were clearly detected by the CPi-VT3 and CPi-T36 probes as well as by the generic CP-CY5 probe up to a dilution of 10^{-4} . No interference was observed when the two probes were labeled with the 6FAM or individually labeled with NED and VIC (data not shown). Similarly, CPi-T36, CPi-T36NS, and CP-CY5 probes simultaneously identified the isolates B3 and CA-P116 in all five mixed 10-fold dilutions. The only exception in performance found was when the CPi-T36NS

TABLE 1. Isolates used to optimize and validate the multiplex assays^a

Isolate ^b	Results of the real-time RT-PCR (Cycle threshold)				Symptom category ^c	MCA13 reactivity ^d	Reference for biocharacterization and MCA13 reaction
	CP25-CY5	CPi-VT3	CPi-T36	CPi-T36NS			
VT or T3 genotype in single or mixed infection							
B23	19.00	19.04	0	0	GSP (0.5–3) & GVIS (1–2.2)	Positive	Hilf et al. (12); Garnsey et al. (5)
B31	22.09	22.81	0	22.31		Positive	Hilf et al. (12); Garnsey et al. (5)
B46	21.95	20.38	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)
B188	20.36	24.74	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)
B248	17.94	22.47	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)
B343	19.86	20.86	20.33	0		Positive	Hilf et al. (12); Garnsey et al. (5)
B384	23.83	24.56	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)
FL0039	22.07	20.22	23.14	nt ^e		Positive	Sieburth et al. (30)
FL194	22.32	19.65	22.62	nt		Positive	Sieburth et al. (30)
FL197	23.63	20.83	25.75	nt		Positive	Sieburth et al. (30)
FL205	29.61	27.11	0	0		Positive	Sieburth et al. (30)
B8	20.51	19.87	0	0	SY (2–3)	Positive	Hilf et al. (12); Garnsey et al. (5)
B119	18.97	21.97	0	0	GSP (1.5–2.5)	Positive	Hilf et al. (12); Garnsey et al. (5)
B131	18.94	24.81	0	0	GVIS (2–2.8)	Positive	Hilf et al. (12); Garnsey et al. (5)
B136	27.97	27.17	0	0	TBS-WB (1–3)	Positive	Hilf et al. (12); Garnsey et al. (5)
B152	20.17	20.51	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)
B211	21.16	21.78	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)
CCTEA106	18.95	18.45	0	0		Positive	Polek et al. (21)
CCTEA107	18.65	19.56	0	0		Positive	Polek et al. (21)
CCTEA99416	17.98	22.35	0	20.65		Positive	Polek et al. (21)
B6	18.34	18.17	0	0	GVIS (1.5–3)	Positive	Hilf et al. (12); Garnsey et al. (5)
B28	17.88	20.18	0	0	SY (0.7–3)	Positive	Hilf et al. (12); Garnsey et al. (5)
B30	20.47	20.55	0	0	GSP (0.3–2)	Positive	Hilf et al. (12); Garnsey et al. (5)
B37	18.03	19.31	0	0	OSP (0.7–2.5)	Positive	Hilf et al. (12); Garnsey et al. (5)
B58	21.38	22.87	0	21.03		Positive	Hilf et al. (12); Garnsey et al. (5)
B65	17.63	22.31	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)
B71	17.31	17.54	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)
B77	19.77	23.46	0	24.56		Positive	Hilf et al. (12); Garnsey et al. (5)
B165	20.72	20.86	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)
B185	20.07	18.61	0	24.22		Positive	Hilf et al. (12); Garnsey et al. (5)
B192AT	15.04	15.27	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)
B195	18.88	18.58	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)
B199	22.70	22.01	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)
B219	20.37	18.31	0	19.25		Positive	Hilf et al. (12); Garnsey et al. (5)
B253	18.87	24.26	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)
B255	20.54	26.40	0	24.94		Positive	Hilf et al. (12); Garnsey et al. (5)
B270	20.13	23.28	0	19.26		Positive	Hilf et al. (12); Garnsey et al. (5)
B316	19.28	21.70	0	25.78		Positive	Hilf et al. (12); Garnsey et al. (5)
B405	17.88	20.00	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)
B417	19.55	21.16	0	18.88		Positive	Hilf et al. (12); Garnsey et al. (5)
P29	15.65	17.32	0	0		Positive	R. K. Yokomi, <i>unpublished data</i>
P60	16.23	16.32	0	0		Positive	R. K. Yokomi, <i>unpublished data</i>
P108	19.36	21.32	0	0		Positive	R. K. Yokomi, <i>unpublished data</i>
P108-1Bat	16.32	18.56	0	0		Positive	R. K. Yokomi, <i>unpublished data</i>
P108-39at	17.84	18.64	0	0		Positive	R. K. Yokomi, <i>unpublished data</i>
P109	17.18	18.39	0	0		Positive	R. K. Yokomi, <i>unpublished data</i>
P109-2at	18.96	23.96	0	0		Positive	R. K. Yokomi, <i>unpublished data</i>
P109-4at	19.45	15.63	0	0		Positive	R. K. Yokomi, <i>unpublished data</i>
FL007	22.86	20.65	0	nt	SY (0.7–3)	Positive	Sieburth et al. (30)
FL208	29.73	25.79	26.49	nt	GSP (0.3–2)	Positive	Sieburth et al. (30)
FS043	32.87	27.25	0	nt	GVIS (1.5–3)	Positive	Sieburth et al. (30)
T66H548	28.35	22.37	0	0	OSP (0.7–2.5)	Positive	Sieburth et al. (30)
T068	26.88	24.40	0	0		Positive	Sieburth et al. (30)
T068-1	25.29	27.6	0	0		Positive	Sieburth et al. (30)

(Continued on next page)

^a Isolates in bold were used as reference genotypes to optimize the qRT-PCR protocols and test the strain specificity of the primer/probe sets.^b In each category isolates were listed in the following order: Agricultural Research Service (ARS), Beltsville; Central California Tristeza Eradication Agency (CCTEA) & ARS, Parlier; and Florida BCBP (Bureau of Citrus Budwood Registration), Florida Department of Agriculture and Consumer Services, Winter Haven, FL.^c Biological assays data are from Garsney et al. (5), Polek et al. (21), Sieburth et al. (30), and R. K. Yokomi (*unpublished data*). Reactions induced in sour orange (SO), Duncan grapefruit (DGF) and Madam Vinous (MV); GSP = stem-pitting on DGF; OSP = stem-pitting on MV; SY = seedling yellows on SO and/or DGF; GVIS = severe stunting on DGF; TBS-WB = thick bark syndrome with wood bristles on DGF (5,30). Numbers in parenthesis indicate the range of the severity scored on a scale of 0 to 3. For CCTEA isolates, the scored reported have been adjusted from a scale 0 to 15 to a scale of 0 to 3 (21).^d MCA13 reactivity data were from Garsney et al. (5), Hilf et al. (12), Polek et al. (21), Sieburth et al. (30), and R. K. Yokomi (*unpublished data*).^e nt = not tested.^f NA = data not available.^g Infected Madam Vinous plant coinoculated.

TABLE 1. (Continued from previous page)

Isolate ^b	Results of the real-time RT-PCR (Cycle threshold)				Symptom category ^c	MCA13 reactivity ^d	Reference for biocharacterization and MCA13 reaction	
	CP25-CY5	CPi-VT3	CPi-T36	CPi-T36NS				
B24	20.32	20.73	0	0	SY (1.5)	Positive	Hilf et al. (12); Garnsey et al. (5)	
B36	28.73	27.97	0	0	NA ^f	Positive	Hilf et al. (12); Garnsey et al. (5)	
B148	22.17	20.99	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)	
B166	21.11	19.71	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)	
B246	23.56	23.65	0	20.73		Positive	Hilf et al. (12); Garnsey et al. (5)	
B412	20.07	23.63	0	22.65		Positive	Hilf et al. (12); Garnsey et al. (5)	
FL206	30.51	23.54	25.39	nt		Positive	Sieburth et al. (30)	
FL216	28.85	27.41	0	0		Positive	Sieburth et al. (30)	
T003AT10	28.44	21.45	0	nt		Positive	Sieburth et al. (30)	
B22	21.94	22.01	0	0	Symptomless	Positive	Hilf et al. (12); Garnsey et al. (5)	
B25	19.67	19.76	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)	
B79	22.93	23.59	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)	
FL187	26.75	21.14	21.86	nt		Positive	Sieburth et al. (30)	
FL192	25.75	19.62	20.91	nt		Positive	Sieburth et al. (30)	
FL207	31.39	22.47	25.26	nt		Positive	Sieburth et al. (30)	
FS595	28.61	19.93	0	nt		Positive	Sieburth et al. (30)	
Mixture of different genotypes								
B192	24.78	24.21	0	0	Symptomless	Positive	Hilf et al. (12); Garnsey et al. (5)	
B340	21.31	20.33	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)	
FL084	20.00	0	19.67	nt		Positive	Sieburth et al. (30)	
FL123	20.46	0	20.36	nt		Positive	Sieburth et al. (30)	
FL129	23.32	0	21.00	nt		Positive	Sieburth et al. (30)	
FS540	22.33	0	21.26	nt		Positive	Sieburth et al. (30)	
FS545	21.61	0	22.74	nt		Positive	Sieburth et al. (30)	
FS625	23.39	0	22.04	nt		Positive	Sieburth et al. (30)	
T067	27.52	0	22.24	nt		Positive	Sieburth et al. (30)	
B271	19.13	0	18.57	0	GSP (0.3–1.2)	Positive	Hilf et al. (12); Garnsey et al. (5)	
FL079	18.54	0	18.01	nt		Positive		Sieburth et al. (30)
FL086	17.45	0	18.43	nt		Positive		Sieburth et al. (30)
FL149	16.81	0	17.05	nt		Positive		Sieburth et al. (30)
FL173	23.66	0	23.06	nt		Positive		Sieburth et al. (30)
FL188	23.57	0	22.13	nt		Positive		Sieburth et al. (30)
FS252	27.61	0	24.67	nt		Positive		Sieburth et al. (30)
FS329	23.81	0	22.97	nt		Positive		Sieburth et al. (30)
FL145	18.98	0	19.19	nt	OSP (0.5) & GSP (1.3)	Positive	Sieburth et al. (30)	
FL169	22.52	0	21.49	nt		Positive	Sieburth et al. (30)	
FS425	27.57	0	25.19	nt	NA	Positive	Sieburth et al. (30)	
FS442	21.67	0	21.13	nt		Positive	Sieburth et al. (30)	
FS480	19.84	0	19.84	nt		Positive	Sieburth et al. (30)	
FS642	30.30	32.68	29.73	0		Positive	Sieburth et al. (30)	
FS627	22.56	0	21.82	nt		Positive	Sieburth et al. (30)	
P81+P109^g	19.11	21.89	0	0		Positive	R. K. Yokomi, <i>unpublished data</i>	
Nonstandard genotype								
B337	28.76	0	0	0	Symptomless	Negative	Hilf et al. (12); Garnsey et al. (5)	
B339	24.94	0	0	0		Negative	Hilf et al. (12); Garnsey et al. (5)	
B158	26.01	0	0	24.62		Positive	Polek et al. (21); R. K. Yokomi, <i>unpublished data</i>	
CA-P116	15.80	0	0	15.65		Positive	Polek et al. (21); R. K. Yokomi, <i>unpublished data</i>	
CCTEA 4	21.34	0	0	18.96		Positive	Polek et al. (21); R. K. Yokomi, <i>unpublished data</i>	
CCTEA114	23.02	0	0	19.63		Positive	Polek et al. (21); R. K. Yokomi, <i>unpublished data</i>	
CCTEA115	19.08	0	0	18.52		Positive	Polek et al. (21); R. K. Yokomi, <i>unpublished data</i>	
CCTEA108	23.04	0	0	19.64		Positive	Polek et al. (21); R. K. Yokomi, <i>unpublished data</i>	
CCTEA 89	20.36	0	0	17.46		Positive	Polek et al. (21); R. K. Yokomi, <i>unpublished data</i>	
CCTEA 65	18.96	0	0	18.96		Positive	Polek et al. (21); R. K. Yokomi, <i>unpublished data</i>	
FL53	26.01	0	0	28.16		Positive	Sieburth et al. (30)	
B26	26.53	0	0	0	GSP (0.2–2.8)	Positive	Hilf et al. (12); Garnsey et al. (5)	
B129	24.56	0	0	0		Positive	Hilf et al. (12); Garnsey et al. (5)	
P108-35at	22.36	0	0	0		Positive	R. K. Yokomi, <i>unpublished data</i>	
B234	23.20	0	0	20.49	NA	NA	Hilf et al. (12); Garnsey et al. (5)	
B371	21.27	0	0	26.52		NA	Hilf et al. (12); Garnsey et al. (5)	

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TABLE 1. (Continued from previous page)

Isolate ^b	Results of the real-time RT-PCR (Cycle threshold)				Symptom category ^c	MCA13 reactivity ^d	Reference for biocharacterization and MCA13 reaction
	CP25-CY5	CPI-VT3	CPI-T36	CPI-T36NS			
		T30 genotype					
B29	26.12	0	0	0	Symptomless	Negative	Hilf et al. (12); Garnsey et al. (5)
B32	22.41	0	0	0		Negative	Hilf et al. (12); Garnsey et al. (5)
B252	21.81	0	0	0		Negative	Hilf et al. (12); Garnsey et al. (5)
B277	19.33	0	0	0		Negative	Hilf et al. (12); Garnsey et al. (5)
B305	25.60	0	0	0		Negative	Hilf et al. (12); Garnsey et al. (5)
B334	20.24	0	0	0		Negative	Hilf et al. (12); Garnsey et al. (5)
CCTEA116	17.46	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 165	17.87	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 192	19.22	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
P1	15.36	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
P100	21.33	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
FL186	23.59	0	0	nt		Negative	Sieburth et al. (30)
FL193	26.13	0	0	0		Negative	Sieburth et al. (30)
FL230	25.68	0	0	nt		Negative	Sieburth et al. (30)
FL248	25.73	0	0	nt		Negative	Sieburth et al. (30)
FL277	24.57	0	0	nt		Negative	Sieburth et al. (30)
FL311	21.32	0	0	nt		Negative	Sieburth et al. (30)
FL328	23.52	0	0	nt		Negative	Sieburth et al. (30)
FS253	26.48	0	0	0		Negative	Sieburth et al. (30)
T026	21.93	0	0	nt		Negative	Sieburth et al. (30)
T030	26.52	0	0	0		Negative	Sieburth et al. (30)
T55-1	25.56	0	0	nt		Negative	Sieburth et al. (30)
T069	32.38	0	0	nt		Negative	Sieburth et al. (30)
T069-1	29.97	0	0	nt		Negative	Sieburth et al. (30)
B314	20.13	0	0	0	Negative	Hilf et al. (12); Garnsey et al. (5)	
B391	21.37	0	0	0	Mild DGF reactions (GSP 0.1–0.8); OSP (0.3–0.5)	Negative	Hilf et al. (12); Garnsey et al. (5)
B397	20.83	0	0	0		Negative	Hilf et al. (12); Garnsey et al. (5)
CCTEA 117	15.32	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 118	21.39	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 120	19.87	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 127	18.75	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 128	23.56	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 137	16.49	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 139	16.89	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 141	18.19	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 146	19.23	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 150	18.53	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 154	17.69	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 155	18.33	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 160	20.06	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 161	17.58	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 162	20.01	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 163	22.31	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 170	19.64	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 172	18.46	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 175	21.84	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 180	18.32	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 183	15.26	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 197	18.52	0	0	0		Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>
CCTEA 193	16.00	0	0	0	Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>	
CCTEA 199	17.03	0	0	0	Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>	
CCTEA 96235	18.65	0	0	0	Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>	
CCTEA 96112	18.91	0	0	0	Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>	
P81	18.90	0	0	0	Negative	Polek et al. (21), R. K. Yokomi, <i>unpublished data</i>	
FL204	25.02	0	0	nt	Negative	Sieburth et al. (30)	
FL209	27.23	0	0	0	Negative	Sieburth et al. (30)	
FL217	25.67	0	0	nt	Negative	Sieburth et al. (30)	
FL223	22.82	0	0	nt	Negative	Sieburth et al. (30)	
FL224	25.44	0	0	nt	Negative	Sieburth et al. (30)	
FL227	28.01	0	0	nt	Negative	Sieburth et al. (30)	
FL237	22.48	0	0	nt	Negative	Sieburth et al. (30)	
FL245	22.92	0	0	nt	Negative	Sieburth et al. (30)	
FL251	23.54	0	0	nt	Negative	Sieburth et al. (30)	
FL267	27.14	0	0	nt	Negative	Sieburth et al. (30)	
FL272	23.92	0	0	nt	Negative	Sieburth et al. (30)	
FL298	27.50	0	0	nt	Negative	Sieburth et al. (30)	

(Continued on next page)

and CPi-VT3 probes were combined (treatments C and D), creating an interference which led to the inability to detect isolate CA-P116 (data not shown). This, however, was not the case for the other target isolates. The sensitivity of the multiplex assays (treatments A and B) was as good as that obtained in single reactions. The limit of detection of the multiplex assays were comparable to that of the single (up to 10^{-4}), and the overall Ct value difference of the multiplex versus the single ranged between +0.8/+1.6 Ct. In addition, the three CPi probes clearly identified the target sequences up to a ratio of 1:1,000 in the presence of the nontarget P81 mild isolate (Fig. 1).

The three CPi probes effectively detected VT, T3, T36, and T36NS genotypes in single and natural mixed infections from reference collections (Table 1). Panel tests using various CPi probes were in good agreement with the previous work describing the biocharacterization and sequence grouping using MMM markers of each isolate and genotype of this panel (12). All isolates that reacted with the CPi probes were also found to be positive with the monoclonal antibody MCA13 (12,20) in double-antibody sandwich indirect (DASI)-ELISA.

Five budwood source trees from the Florida BCBR tested positive with the CPi-T36 probe but failed to react with MCA13 in DASI-ELISA (Table 2) (P. J. Sieburth, *unpublished data*) and suggests a higher sensitivity of the qPCR assays compared with ELISA.

CPi-T36NS probe. CPi-T36NS probe detected isolates which did not react with any of the MMM markers; however, for a few isolates faint amplicons and inconsistent results were obtained with T30POL primers. These isolates were also associated with lower MCA13 reactivity (OD_{405}) than T36 or VT isolates in DASI-ELISA (R. K. Yokomi, *unpublished data*). The low MCA13 reactivity of these isolates resulted in occasional false negatives in detecting field infections by serology compared with their reactivity with the CPi-T36NS probe (Table 2). For some of these

isolates the presence of the MCA13 epitope was confirmed by sequence analysis of the CP gene (Genbank accession nos. EU878380, EU325938, EU325936, EU325930, and GQ131683). This group included five isolates in the CCTEA collection (CCTEA65, 89, 108, 114, and 115) and some more widely distributed isolates from southern California (Table 2). CCTEA biological indexing of representative isolates indicated that these isolates were mild in all indicator plants (21). A few of these isolates were detected in the Beltsville collection where they were primarily in mixed infections with severe VT or T3 strains. This isolate type was also found among the Florida isolates (FL053).

The CPi probes did not react with any T30 genotype isolates of a panel in Table 1 (12) or with any from an outgroup of isolates including but not limited to P108-35at, B26, B129, and 99416-9 (Fig. 1).

DISCUSSION

Although there is great genetic diversity and variability between and among various CTV isolates (1,16,32), no clear association of specific CTV-induced symptoms exists with specific genotypes. Variable pathogenicity occurs among isolates sharing the same genotype (11,12). However, mild to moderate symptoms are typically associated with T30 genotypes while T3, VT, and T36 genotypes are associated with more severe symptoms (1,12). Our objective was to develop a reliable and sensitive assay to identify isolates that have potential economic impact in commercial citrus groves. Three qRT-PCR TaqMan probes called CPi-T36, CPi-T36NS, and CPi-VT3 were designed to target isolates in three distinct phylogenetic groups (Fig. 1). Although qPCR reactivity was based on short sequences of the MGB-TaqMan probe (15 to 21 nt), differentiation of strains of a large panel of genetic diverse isolates was confirmed.

TABLE 1. (Continued from previous page)

Isolate ^b	Results of the real-time RT-PCR (Cycle threshold)				Symptom category ^c	MCA13 reactivity ^d	Reference for biocharacterization and MCA13 reaction
	CP25-CY5	CPi-VT3	CPi-T36	CPi-T36NS			
FL302	17.40	0	0	nt	OSP (1.5) GSP (3)	Negative	Sieburth et al. (30)
T4	17.13	0	0	0		Negative	Sieburth et al. (30)
FL278	27.38	0	0	nt		Negative	Sieburth et al. (30)
T36 genotype							
B3	15.32	0	14.58	0	SY (1.5–2)	Negative	Hilf et al. (12); Garnsey et al. (5)
CCTEA96337	18.36	0	16.19	0		Negative	Polek et al. (21)
CCTEA96339	20.50	0	18.89	0		Negative	Polek et al. (21)
FL092	19.39	0	19.16	nt		Negative	Sieburth et al. (30)
FL139	22.00	0	21.02	nt		Negative	Sieburth et al. (30)
T036	24.79	0	20.17	0		Negative	Sieburth et al. (30)
T066	29.62	0	24.65	0		Negative	Sieburth et al. (30)
T066-1	23.09	0	20.10	0		Negative	Sieburth et al. (30)
T067-1	30.08	0	23.76	nt	GSP (0.3–1)	Negative	Sieburth et al. (30)
FL154	20.13	0	19.99	nt		Negative	Sieburth et al. (30)
FL165	20.33	0	18.17	nt		Negative	Sieburth et al. (30)
FL191	22.18	0	20.83	nt		Negative	Sieburth et al. (30)
FS577-1	24.38	0	21.89	nt		Negative	Sieburth et al. (30)
T6671P1A	23.47	0	21.76	nt		Negative	Sieburth et al. (30)
B83	24.32	0	0	0		Negative	Hilf et al. (12); Garnsey et al. (5)
FL104	20.87	0	20.64	nt	OSP (0.5–1.5) &	Negative	Sieburth et al. (30)
FL141	17.41	0	18.67	nt	GSP (0.5)	Negative	Sieburth et al. (30)
FL090	19.58	0	25.48	nt	NA	Negative	Sieburth et al. (30)
FL252	18.56	0	19.65	nt		Negative	Sieburth et al. (30)
FS377	22.70	0	19.36	nt		Negative	Sieburth et al. (30)
FS393	22.62	0	21.24	nt		Negative	Sieburth et al. (30)
FS420	23.68	0	20.10	nt		Negative	Sieburth et al. (30)
FS577	24.91	0	22.08	nt		Negative	Sieburth et al. (30)

Real-time PCR is a powerful tool for rapid and sensitive detection of plant pathogens and its use for CTV detection and genotype differentiation has been recently reported (2,22,26–28). Ruiz-Ruiz et al. (27) used real-time RT-PCR to differentiate between mild and severe CTV strains. Their method allowed the detection and the identification of the CTV strains by performing four independent reactions. In contrast, the method described herein is based on a multiplex assay that using different primers/probes with amplification protocols that allowed single tube, simultaneous differentiation of CTV isolates containing VT, T3,

or T36 genotypes. Strain differentiation was also extended within the T36 clade to separate a subgroup of MCA13 reactive isolates which are biologically mild and not previously recognized. The assays herein described were effective in identifying target isolates in a mixture with nontarget isolates. Ruiz-Ruiz et al. (27) were able to detect the severe target genotypes (VT, T3, and T36) in mixtures containing 2.5% of the homologous and 97.5% of the heterologous transcripts. Our CPi probes were able to detect the homologous viral RNA up to a ratio of 1:1,000 when mixed with the mild P81 isolate. An example of this sensitivity is the reaction

Fig. 1. Standard curves obtained in multiplex real-time reverse transcription-polymerase chain reaction assays with five 10-fold dilutions (from 1 to 10⁻⁴) of total nucleic acids (TNA) from *Citrus tristeza virus* infected plant P29, CA-P116, and B3, each dilution included a standard amount of TNA recovered from a P81-infected plant. **A**, Standard curve generated with the 10-fold dilutions of P29 TNA using the FAM-labeled CPi-VT3 and the CY5-labeled CP-CY5 probes. **B**, Standard curve generated with the 10-fold dilutions of B3 TNA using the FAM-labeled CPi-T36 and the CY5-labeled CP-CY5 probes. **C**, Standard curves generated with the 10-fold dilution of CA-P116 TNA using the FAM-labeled CPi-T36NS and the CY5-labeled CP-CY5 probes. The standard curves are indicated in the graphs by the fluorophore labeling the respective probe. As shown, all three CPi probes detected the target strains up to a dilution of 1.00E-03. Since all dilutions contained an equal amount of the TNA of P81 isolate, constant Ct values were obtained for all dilutions with the generic CP-CY5 probe.

TABLE 2. Field samples collected in different California plots and from the budwood source trees in Florida during 2008 to 2009^a

Plot	Location	Collection date	No. of samples tested	DASI-ELISA (no. positive samples)			Real time RT-PCR (no. positive samples)		
				STD ^b	MCA13	CP-CY5	CPi-VT3	CPi-T36	CPi-T36NS
California									
1	Tulare Co.	2008–2009	44 ^c	44	3	44	0	0	3
2	Tulare Co.	2008	20 ^c	20	0	20	0	0	0
3	Tulare Co.	2009	21 ^d	15	0	15	0	0	0
4	Ventura Co.	2008	21 ^d	21	8	21	0	0	10
5	Riverside Co.	2009	64 ^d	39	14	39	0	0	20
		Total	170	139	25	139	0	0	33
Florida									
	BCBR ^e	2008	670 ^d	72	0	75	0	5	nt ^f

^a All samples were tested both in double-antibody sandwich indirect enzyme-linked immunosorbent assay (DASI-ELISA) and real-time reverse transcription-polymerase chain reaction (RT-PCR).
^b DASI-ELISA performed using a broad-spectrum polyclonal antiserum.
^c Samples collected from only known *Citrus tristeza virus* (CTV)-infected trees.
^d Samples collected from blocks of trees of unknown CTV status.
^e BCBR: Bureau of Citrus Budwood Registration, Florida Department of Agriculture and Consumer Services, Winter Haven, FL.
^f Not tested.

of CPi-VT3 probe with the isolate B192, a mixture of mild and VT strains (3). This VT subisolate was not detectable using the SP LNA TaqMan probe (27).

Ct values based on serial dilutions of TNA from T36, T36NS, and VT genotype isolates had comparable sensitivity by multiplex and single assays. This indicates the assays were optimized and that only minimal interference and/or competition occurred between primer/probe sets.

CPi-T36NS probe successfully detected MCA13-reactive isolates in the collections (CCTEA89, CCTEA65, CCTEA4, CCTEA115, CCTEA114; CCTEA108; B158; FL053) which induced mild symptoms in the indicator plants (5,21,30) and those in field samples which remained undetectable using the MCA13. Nucleotide sequence homology was found among this group of isolates and New Zealand CTV strains recently characterized as *Poncirus trifoliata*-resistance breaking (RB) isolates (10; S. J. Harper, *personal communication*) (i.e., NZRB-TH30; Fig. 1). Based on these similarities, RB isolates could be detected by the CPi-T36NS probe. Because the homology of the putative California and New Zealand RB isolates is based on a very small genome region, these California isolates need to be tested for the ability to replicate in *P. trifoliata*.

In conclusion, the TaqMan RT-PCR assays described herein allow detection of CTV in sample extracts from citrus trees. Furthermore, a positive reaction with one or more of the CPi probes identifies the presence of potential virulent virus strains. If a multiplex qRT-PCR test is used for CTV strain differentiation, the CTV-CY5 probe can serve as a CTV-positive internal control to insure that template preparation and qPCR conditions were correct. Finally, the CPi probes differentiated MCA13-reactive isolates into at least three distinct groups which will be useful to separate MCA13-reactive isolates into potentially mild versus potentially more severe isolates.

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