Genetic Differentiation and Biology of *Citrus tristeza virus* Populations Spreading in California

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ABSTRACT. Incidence of *Citrus tristeza virus* (CTV)-infected trees in Central California by 2009 reached a point where eradication of infected trees was no longer feasible. Representative CTV strains collected from 2008-2010 were selected from a collection of isolates from Central and Southern California citrus trees and molecular and biological properties were determined. Characterizations included serology with MCA13, real-time Reverse Transcription (RT) quantitative (q) Polymerase-Chain Reaction (PCR) (RT-qPCR) assays with strain-specific markers, and PCR-Single Stranded Conformation Polymorphism (SSCP) analysis of coat protein (CP) gene region products. Variants identified by PCR-SSCP were cloned and sequenced in the CP and P20 gene regions to characterize each strain and evaluate their phylogenetic relationships. Central California CTV strains typically were MCA13-negative with a T30-like genotype and mild or asymptomatic on indicator plants. Southern California isolates and a few from Central California were mostly T30-like strains in single or mixed infections with a non-standard (NS-) and/or a T3-like genotype. All NS and T3-like strains reacted with MCA13. CP and P20 gene sequences from NS strains clustered in the same major clade as T36 and were named T36NS. Gene sequence analysis of the T36NS strains indicated a close relationship to the CTV T36-decline strain from Florida and CTV resistance-breaking strains from New Zealand. Sequences from T3 strains were related to the seedling yellows (SY) NUaga strain from Japan. These California strains were bioindexed in greenhouse tests. T36NS strains were mild on Mexican lime and asymptomatic on other citrus indicators. T3-like strains in single or mixtures with T30-like strains induced strong SY in indicator plants. Thus, California CTV strains grouped into three classes: i) mild with a mild genotype sequence profile which did not react with MCA13; ii) T36NS-like genotype which reacted with MCA13 but were mild in bioindex tests; and iii) severe strains with a T3-like genotype and MCA13 positive which produced strong SY in indicator plants. These data support and validate the use of genotype-specific probes to test CTV samples collected from field surveys to identify citrus trees infected by virulent CTV strains which should be eradicated as soon as possible.

In 1995-96, several Pest Control Districts in Central California stopped removing *Citrus tristeza virus* (CTV) - infected trees (12) due to rapid local spread of the virus by aphid vectors (19,20). Over the past 10 years, high inoculum reservoirs developed and CTV eradication in Central California became unfeasible. In 2009, the Citrus Pest Detection Program (CPDP) (previously known as the Central California Tristeza Eradication Agency), Tulare, CA adopted a revised policy to identify citrus trees infected with virulent strains of CTV and arrange for their prompt removal from the field while leaving trees infected with mild or benign strains in place (1). This program was based on recent characterizations of Central California CTV strains which showed that the vast majority were mild in citrus planted on CTV-tolerant or -resistant rootstocks (8, 10, 13, 15, 19, 20).

To identify putative severe strains of CTV, the CPDP tests extracts from citrus trees by ELISA using the detecting antiserum, MCA13, a CTV monoclonal antibody (MAb) which reacts with most severe strains of CTV (11). MCA13-reactive strains are further evaluated by real-time Reverse Transcription (RT) quantitative (q) Polymerase Chain Reaction (PCR) (RT-qPCR) assays using sequence-specific probes (14, 16, 17, 21). Confirmatory tests for severity are

performed by bioindexing tests in the greenhouse or screenhouse (1, 22). This report characterizes representative CTV strains collected over the past few years in California.

MATERIAL AND METHODS

Field surveys and virus strains. CTV strains were collected from trees in commercial citrus groves in Central and Southern California (Table 1). CTV surveys were conducted at each sample site to estimate CTV incidence. Samples consisted of 8 to 12 petiole leaves from the most recent mature flush growth from four ordinate quadrants around the tree canopy at ~3 m height.

Serology. Young stems and petioles from field trees were blotted on 0.45 µm nitrocellulose (BioRad 162-0115) and processed by direct tissue print immunoassay (DTBIA) (2, 5) with MCA13 (Nokomis Corp) and a broad-spectrum CTV cocktail of MAbs (Agdia or Plant Print) as described by Yokomi et al. (22). Extracts from greenhouse-grown citrus as healthy, MCA13-positive and MCA13-negative controls were included in the serological tests. DTBIA results were confirmed by double antibody sandwich indirect enzyme-linked immunosorbent assays (DAS-I-ELISA) performed as described by Garnsey and Cambra (3) using microtiter plates (Immulon 4 HBX, Thermo Scientific, Rochester, N.Y.) coated with goat-anti CTV and virus detected with antiserum from expressed protein of the CTV coat protein gene (9). CTV polyclonal antibodies were kindly provided by the Citrus Clonal Protection Program, Dept. Plant Pathology and Microbiology, University of California, Riverside.

Multiplex RT-qPCR. Virions were immunocaptured in PCR tubes pre-coated with CTV polyclonal antiserum 1212 (Dept. Plant Pathology, Univ. Florida, Gainesville, FL) (19). Templates were subjected to RT-qPCR using broad-spectrum primers P27F/P27R and strain discriminating Taqman® probes CPi-VT3, CPi-T36 and CPi-T36NS (21).

Cloning and sequencing. Cloning in pGEMT-easy (Promega, USA) was performed on PCR products from the coat protein (CP) gene region and, in some cases, from the P20 gene or obtained using the strain-selective primers T3K17f/r (7). Nucleotide sequencing was performed with at least three recombinant clones per isolate (L152, EX348 and EX355).

Single strand conformation polymorphism (SSCP). SSCP analysis was conducted for the CP gene amplicon to determine if an isolate was a single strain or a mixture of CTV strains. The methodology used was previously described (17).

CTV bioindexing. CTV strains were graft inoculated in a standard citrus host range as described by Garnsey et al. (4). Plants were examined for symptoms and plant height measurements were taken 2, 4, and 12 mo post inoculation. Composite scores for stunting and foliar symptoms (0-6) and stem pitting (0-6) symptoms in Mexican lime (ML) (*Citrus aurantiifolia*) and Madam Vinous (MV) (*C. sinensis*); and stunting and seedling yellows (SY) reaction (0-6) and woody alteration, cheesy bark and wood bristles) (0-6) in Duncan grapefruit (DGF) (*C. paradisi*). Sour orange (SO) (*C. aurantium*) and Eureka Lemon (EL) (*C. limon*) were rated for stunting and seedling yellows (0-6). Sweet orange/sour orange was rated for stunting 0-6. In all cases zero was no reaction and 6 was severe. The symptom reaction score for each host plant was the average of three replications. Severity per isolate was the rated as the sum of individual host reaction scores.

TABLE 1
MOLECULAR ANALYSIS OF CALIFORNIA *CITRUS TRISTEZA VIRUS* ISOLATES COLLECTED AND ASSAYED FROM 2008 TO 2010

Location	County	No. isolates tested	No. MCA13 positive	MMM[1] analysis	RT-qPCR[2]			Accession number
					CPiVT3	CPiT36 NS	CPiT36	
Reedley	Fresno	40	0	T30	0	0	0	GQ424352-GQ424353
Strathmore	Tulare	17	0	T30	0	0	0	EU878379; EU878380-EU878384
Porterville	Tulare	6	0	T30	0	0	0	Not available
Lindsay	Tulare	1528	13	T30	0	13	0	EU878379; GQ424350-GQ424351; Banklit 1456923
Exeter JG[4]	Tulare	18	18	T30 & T3	18	10	0	JN082700-JN082704
Exeter[5]	Tulare	2450	15[3]	T30 & T3[3]	5[3]	10[3]	0[3]	
Pauma	Riverside	62	0	T30	0	0	0	Not available
UCR[6]12B	Riverside	99	52	T30	0	52	0	GQ424356-GQ424359; GQ131683
Fillmore	Ventura	252	44	T30	0	44	0	GQ424346-GQ424349

[1]Multiple Molecular Marker analysis (7).
[2]Real time Reverse Transcription (RT)-quantitative (q) Polymerase Chain Reaction Assay (RT-qPCR) (21).
[3]Molecular assays conducted from samples taken from hierarchical surveys.
[4]Field location ~6 km west of the Lindcove Research and Extension Center (LREC), Exeter, CA
[5]Field locations in a ~1.6 km radius of the LREC, Exeter, CA
[6]University of California Riverside.

TABLE 2

BIOINDEXING RESULTS OF SEVEN *CITRUS TRISTEZA VIRUS* (CTV) ISOLATES COLLECTED IN CALIFORNIA

Location	Isolate	Genotype	MCA13	Severity rating (4)						Total	Notes
				Bioindex host							
				Mexican lime[1]	Madam Vinous[2]	Sour orange	Duncan grapefruit[3]	Eureka lemon	Swt/ SO[4]		
	Ex-350	T3	Yes	10	3	6	7	4	5	35	Severe SY
	Ex-355	T3+T30	Yes	11	3	6	8	6	6	40	Severe SY
Fillmore	F1-5	T36NS+T30	Yes	1	0	1	0	0	1	3	-
	F7-2	T30	No	1	0	1	0	0	1	3	-
Lindsay	P08.6	T30	No	1	0	1	0	0	1	3	-
	P08.4	T36NS+T30	Yes	1	0	1	0	0	3	5	-
	L-152	T36NS	Yes	1	0	5	1	0	2	9	-
Green-house controls	SY568	VT	Yes	11	10	5	10	4	5	45	Severe SY & SP
	RH	T3	Yes	11	5	6	10	5	5	42	Severe SY/mild OSP
	S1	T36NS	Yes	2	0	1	0	0	1	4	-
	P81	T30	No	1	2	0	0	0	2	5	-

[1] Composite scores reflecting stunting and foliage symptoms (0-6) & stem pitting (SP) (0-6);
[2] Composite scores reflecting stunting and vein clearing (0-6) & orange SP (OSP) (0-6);
[3] Composite scores reflecting stunting and seedling yellows reaction (0-6) & woody alteration (SP, cheese bark, wood bristles) (0-6).
[4]Sweet orange scion propagated on sour orange rootstock.

RESULTS

SSCP analysis showed that the majority of the strains from eradicative districts in Central California had a simple SSCP profile; whereas strains from non-eradicative districts near Exeter and southern California frequently showed a complex pattern which was confirmed as mixtures of different strains or genotypes by Multiple Molecular Marker (MMM) analysis (7). Based on RT-qPCR marker analysis, strains grouped into three categories: i) T30-like genotype with no reaction with either MCA13 or CPi-probes; ii) T36NS-like genotype strains which were MCA13-positive and reacted with the CPi-T36NS probe; and iii) T3-like genotype strains which were positive by MCA13 and the CPi-VT3 probe (Tables 1 & 2). T36-genotype strains were not found but strains such as L152 which reacted with the CPi-T36NS probe were found. These strains reacted with T30POL markers but CP and P20 gene sequence analysis showed these strains were distinct from T30-like genotypes and genetically closer to the T36 genotype and strains with the NZRB-G90-like genotype (GenBank acc. num. FJ525232) (Table 3). The latter strains have been associated to *Poncirus trifoliata* CTV-resistance-breaking strains (6). CP gene sequence analysis confirmed presence of the MCA13 epitope, however, lower reactivity in DTBIA and DASI-ELISA was observed with T36NS genotype strains than T3-like strains.

TABLE 3
NUCLEOTIDE PERCENT IDENTITY COMPARISON BETWEEN THE COAT PROTEIN (LOWER LEFT) AND P20 (UPPER RIGHT) GENES OF *CITRUS TRISTEZA VIRUS*

P20 gene

	EX348	L152	NUagA	NZRB-G90	T30	T36	VT
EX348		96.0	97.8	92.9	92.3	93.0	96.0
L152	92.4		91.4	**95.5**	91.4	**94.2**	92.0
NUagA	98.5	91.1		93.2	93.0	92.7	96.6
NZRB-G90	92.7	**95.9**	92.5		92.0	93.3	92.9
T30	92.9	88.7	93.1	92.9		93.2	92.6
T36	92.3	**93.3**	91.4	90.2	88.5		92.9
VT	97.1	90.2	97.1	88.5	91.3	90.5	
	EX348	L152	NUagA	NZRB-G90	T30	T36	VT

Coat protein

Reference genotypes: NUagA =AB046398; NZRB-G90 = FJ525432; T30=EU937520; T36 = EU937521; VT = EU937519. The highest similarities of T3-like genotype EX348 strain with reference strains shown with grey background. Highest similarities of T36NS strain L152 with reference strains are shown with bold font.

CP gene nucleotide sequence analysis of T3-like strains EX348 and EX355 showed 98.5% homology with SY reference isolate NUagA (GenBank acc. num. AB046398) (Table 3) and were also related to T3 and VT reference genotypes. Similarly, nucleotide sequences from the P20 gene of the Ex348 strain showed 97.8% homology with the reference genotypes NUagA and VT (GenBank Acc. No. EU937519).

Mild stem pitting on MV and severe SY reaction on SO, DGF and EL were induced by all Exeter T3-like genotype strains alone or in a mixture with T30-like genotypes (Table 2). These strains also induced severe stunting, vein clearing and yellowing in ML. T30-like genotype strains induced only mild vein-clearing, leaf-cupping and few pits on ML and occasionally on MV (e.g. isolate P81). Strains associated to T36NS-like genotype were generally mild or symptomless on all indicators, except for isolate P08.4 which caused mild stunting in sweet/sour indicators.

DISCUSSION

Results reported here confirm the earlier reports (19,20) that the dominant CTV strain in California were mild T30-like genotypes which induce mild symptoms or were asymptomatic in bioindexing tests. Serology differentiated strains on the basis of presence or absence of the MCA13 epitope; sequencing analysis and RT-qPCR assays revealed that most MCA-13 reactive strains were associated to T36NS-like genotype and were mild as determined by bio-indexing. These strains were clearly distinguishable in RT-qPCR assays with the CPiT36NS probe and were called T36NS strains. Investigations are ongoing to fully characterize genome and epidemiology of T36NS strains. A few field strains reacted with the CPiVT3 probe and induced SY reactions in bioindex tests.

The current prevalence of mild CTV strains was supported by CPDP surveys in 2010 which found incidence of MCA13-reactive strains rare (ca. 0.2%) in Central California (1). Since most CTV strains were collected from trees on tolerant rootstocks, no field symptoms were observed. In addition, no virulent strains were found where SY568 was found in the 1970's at Agricultural Operations, University California, Riverside and support results of Velázquez-Monreal et al. (18) that this strain was successfully eradicated.

Thus, California CTV strains were separated into three classes: i) mild with a T30 genotype which did not react with MCA13; ii) T36NS-like genotype strains which reacts with MCA13 but were mild in bioindexing; iii) severe strains with a T3-like genotype which reacts with MCA13 and produced strong SY symptoms. These data support and validate the use of genotype-specific probes to test samples collected in field surveys to identify citrus trees infected by virulent CTV strains which should be eradicated as soon as possible (22).

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