Assessment of the *Citrus tristeza virus* Isolates Detected in Spring 2007 at the Lindcove Research and Extension Center, Exeter, California

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ABSTRACT. *Citrus tristeza virus* (CTV) was detected in at least 50 trees at the 71 ha Lindcove Research and Extension Center (LREC) near Exeter, CA in spring 2007. The purpose of this study was to assess the genetic diversity and aphid transmissibility of these isolates. Examination was performed by enzyme-linked immunosorbent assay, reverse transcription polymerase chain reaction, multiple molecular marker assay, coat protein (CP) gene analysis and *Aphis gossypii* transmission. Nine representative trees of different varieties were sampled on May 21, 2007 prior to tree removals. Aphids transmitted CTV from five of the nine source trees tested at an efficiency >6%. All nine isolates had a T30 genotype. A hierarchical subsample (HS) survey of citrus in a 0.8 km radius of the LREC estimated 1,296 trees may be infected with CTV with an overall incidence of 1.2%. Two hundred and one CTV-positive HS samples were examined and found to have a T30 genotype. Phylogenetic analysis of the CP gene region showed one LREC CTV isolate from an S1 citron tree to have 99.8% identity with central California isolates CCTEA114 and 108, which react with the monoclonal antibody MCA13, and based on the sequence homology of the CP gene, fall on the same clade with the T36 isolate. Single strand conformation polymorphism analysis also identified five samples from the HS survey that contained this same profile as the S1 citron isolate. These data suggest that the LREC and surrounding isolates are mild and readily aphid transmissible. However, molecular diversity is present which merits further investigations before implementation of any management strategy.

Index words: serology, aphid transmission, RT-PCR, SSCP, sequencing

The Lindcove Research and Extension Center (LREC) was established in 1959 by San Joaquin Valley growers and the University of California, Riverside to provide a research site typical of the central California citrus industry. The Center is near Exeter, CA in Tulare County and has 51 ha planted to citrus next to the foothills of the Sierra Nevada Mountains. This site provides an ideal location for the research and evaluation of citrus scions, rootstocks, and management strategies with climate and soils representative of the 76,893 ha of citrus planted in the Central Valley. In addition, the LREC houses the Citrus Clonal Protection Program's (CCPP) Foundation and Evaluation budwood orchard as well as the Protected Foundation Block with approximately 1,700 trees representing more than 300 different selections of citrus. The CCPP provides an average of 30,000 disease tested and true-to-type citrus buds annually to California nurserymen, growers and researchers. In addition, the CCPP often responds to national and international requests for citrus germplasm. The most important citrus varieties are maintained in the CCPP's Protected Foundation Block which is free from insect-vectored diseases. However, 1,200 trees of various cultivars remain in the open field as budwood sources and trueness to type evaluations.

The LREC is located in the Tulare County Pest Control District (TCPCD),

which stopped mandatory eradication of *Citrus tristeza virus* (CTV) in 1996 (3). However, the LREC continues a strict policy of zero tolerance of CTV-infected trees. From 1990 to 2006, the typical number of CTV-infected trees found annually at the LREC was zero to three except in 1999 and 2000 when spikes of 10 and five infected trees were found respectively. A border survey conducted from 1996 to 1999 in commercial citrus orchards in a 0.8 km radius around the LREC estimated CTV incidence at approximately the following levels: 39 orchards with 0%; 42 with 1%; 5 with 3%; 2 with 5%; and 3 with > 5% (each orchard is between 2 and 16 ha in size) for an average incidence of 0.14% and provides a good perspective of the virus reservoir impacting the Center during this period.

In spring of 2007, however, 50 trees were detected with CTV at LREC including four of CCPP's registered budwood source trees. All trees at LREC were removed immediately after the positive ELISA test for CTV was confirmed. This represents an alarming increase in the primary spread of CTV and threatens the Center as a citrus research site and for the maintenance of virus-free budwood trees.

The present study was conducted to provide a rapid assessment of the CTV strains collected at and surrounding the LREC. The outcome of this research is meant to help guide and prioritize control measures.

MATERIALS AND METHODS

Isolates. Nine CTV isolates were collected on May 21, 2007 from selected infected trees from different fields at the LREC to obtain a good representation of the virus isolates found at the Center in spring 2007 (Table 1). These isolates were graft inoculated into Madam Vinous and Mexican lime seedlings in the greenhouse.

Additional samples from a hierarchical subsample (HS) (6) conducted by the Central California Tristeza Eradication Agency (CCTEA), Tulare, CA were also tested for CTV. The LREC trees sampled were confirmed to be infected with CTV by double antibody sandwich indirect- (DASI) enzyme linked immunosorbent assay (ELISA) (8). Some of the isolates were tested for reactivity with the monoclonal antibody MCA13 (7).

Transmission tests. Young citrus flush was collected from CTV-infected trees at the Center and kept moist and cool and transported immediately to the laboratory. A fresh cut was made in the stem end and the flush tissue placed in small vase filled with water. Cotton aphids, *Aphis gossypii* Glover reared on cotton seedlings in an insectary were placed on the leaves and covered by a clear plastic cage for a virus acquisition access period of 24 h. The aphids were then transferred to Mexican lime seedlings in groups of 5 to 10 aphids per receptor plant. Replications varied from five to 25 since some trees had poor flush conditions. An inoculation access period of 24 h was used. The surviving aphids were counted and the test plants were sprayed with insecticide and moved to an insect-free greenhouse for a virus incubation period of 2-mo (10). Receptor plants were assayed by ELISA to determine their CTV infection status.

LREC 0.8 km survey. To assess the potential incidence of CTV around the LREC, the CCTEA conducted a hierarchical subsampling survey (HS) (6) in all commercial citrus within a 0.8 km radius of the Center. The survey was conducted in June 2007 and the samples were processed and analyzed by ELISA by the CCTEA. Extracts from HS CTV positive samples from the border survey were also included for CTV evaluation. It should be noted here that for HS, samples from four

adjacent trees (quadrat) are combined as one sample (6).

Immunocapture-reverse transcription polymerase chain reaction (IC-RT-PCR) amplification. IC-RT-PCR for marker amplifications were conducted as previously described (4, 5). Approximately, eight leaf petioles per plant were pulverized in 5 ml buffer using a Kleco 4200 homogenizer (Garcia Manufacturing, Visalia, CA). Virions were immunocaptured in a PCR tube by CTV polyclonal antiserum 1212 (Dept Plant Pathology, Univ. Florida, Gainesville, FL). Reverse transcription and amplification of sequence specific CTV markers were performed using a standard protocol for this multiple molecular marker (MMM) assay (5). The markers used were T36CP, T30POL, and T30K17, VTPOL, T36POL and T3K17. Reaction products were analyzed by electrophoresis on 1% agarose gels and visualized by staining the gel in ethidium bromide.

CTV isolate P81 (AY995567), a typical T30 genotype strain from central California (10) was used as a reference isolate.

SSCP analysis and cloning of the coat protein gene. For single strand conformation polymorphism (SSCP) analysis, 1 µl of the T36CP product (672bp) was added to 9 µl of denaturing mixture (95% formamide and 0.05% bromophenol blue), heated for 10 min at 95°C, chilled in ice and electrophoresed at room temperature in 10% non-denaturing polyacrylamide gel at 100V for 15 min followed by 3 h at 200V. DNA bands were then visualized by staining the gel in silver nitrate.

The CP gene of the S-1 citron isolate was selected for cloning using standard molecular biology protocols. Multiple alignment of the resultant nucleotide sequence was conducted and its phylogenetic relationship with reference

isolates determined using MegAlign software (Lasergene 7, DNASTAR, Madison, WI).

To further confirm the diversity of this isolate, double stranded (ds) RNA was extracted from 3 g bark tissue by phenol-chloroform treatment and CF11 chromatography as described by Dodds (1). The dsRNA profile was then compared to that obtained for the reference isolate P81.

RESULTS

Transmission tests. Five of the nine LREC CTV isolates were transmitted by aphids after virus acquisition in the laboratory from detached field flushes. Overall, transmission was obtained in 10 of 155 replications (6.5%). Transmission rate was highest from trees of Washington Navel (12% to 16%) while one transmission each was obtained from trees of Fukumoto, Xuegan sweet orange, and Cluster Navel (Table 1). No transmission was obtained from trees of Siranui Dekopon, S-1 citron, or Tsunokaori tangor. Replications for this trial were small due to the limited time and access to collect samples due to the need for immediate tree removal to eradicate the CTV infection. No association was found between aphid transmission and virus titer as determined by ELISA (Table 1). Note that one of the Washington Navel source had an OD_{405} of 0.17 which failed to meet test standards as CTV positive, yet, still resulted in 3 transmissions of 25 replications (12%). When the results from only the Navel sources (including the Fukumoto and Cluster Navel) were combined, a transmission rate of 10% was obtained.

Genotype analysis. *LREC isolates.* All nine LREC isolates tested had good to excellent product amplification with the CP primers (Table 1). Hence, the MMM analysis was performed and classified all

isolates as having a T30-like genotype (Table 1).

TABLE 1

GENOTYPE CLASSIFICATION AND APHID TRANSMISSION OF *CITRUS TRISTEZA VIRUS* ISOLATES FROM THE LINDCOVE RESEARCH AND EXTENSION CENTER COLLECTED MAY 21, 2007

Field	Variety	Planting date	DASI-ELISA[1]	IC-PCR[2] CP[3]	MMM[4] genotype	Aphid transmission[5] No. +/no tested	%
1	Washington Navel	2002	1.15	+++	T30	4/25	16
1	Washington Navel	2002	0.17	+	T30	3/25	12
2	Fukumoto	2005	0.40	+++	T30	1/5	20
2	Fukumoto	2005	0.97	+++	T30	0/20	0
3	Siranui Dekopan	2001	0.32	++	T30	0/15	0
4	Xuegan Swt Org.	1996	0.90	+++	T30	1/10	10
4	Cluster Navel	1988	0.81	+++	T30	1/15	6.7
5	S-1 Citron	1997	1.25	+++	T30	0/15	0
5	Tsunokaori Tangor	2003	0.42	+++	T30	0/5	0
					Overall transmission	10/155	6.5

[1]Double antibody sandwich-indirect enzyme-linked immunosorbent assay values expressed as optical density at 405 nm wavelength (OD 405 for the healthy control was 0.082)

[2]Immunocapture reverse transcription polymerase chain reaction assay

[3]Coat protein. + = Amplicon intensity.

[4]Multiple molecular marker analysis of genotype; T30 genotype was assigned based on the positive reaction with T30POL & T30K17 and negative reaction with VTPOL, T36POL and T3K17

[5]CTV donor tissue for vector tests was young succulent tissue excised from field infected trees. The flush was placed immediately in water and brought to the laboratory for a 24 h acquisition access period with *Aphis gossypii*. Groups of 5 to 10 aphids were then transferred to Mexican lime seedlings for a 24 h inoculation access period after which the surviving aphids were counted. Seedlings were sprayed with insecticide and returned to an insect-free greenhouse for a 2 mo incubation period after which confirmation of CTV transmission was based on an ELISA test on each receptor plant.

LREC 0.8 km survey. An estimated 110,795 citrus trees are within 0.8 km of the LREC. A total of 7,248 HS samples were taken of which 322 were found to be positive for CTV. The estimated number of infected trees was 1,296 and the estimated overall infection level was calculated to be 1.2%. Two hundred and fifteen of the HS CTV-positive samples were assayed by RT-PCR and good cDNA was obtained from 201 of these samples. The MMM assay was then conducted on these samples and all were found to have a T30-like genotype.

SSCP. Analysis of the CP gene of the nine LREC isolates revealed that eight had the same SSCP pattern as P8. The LREC S1 citron isolate, however, had a different pattern (Fig. 1A). Different dsRNA profiles were also observed from these two representative samples. Both had the 13.3 $x10^6$ and the $0.8x10^6$ Da band, but differed by the number and/or mobility of additional bands. For example, the S-1 citron isolate had a doublet in correspondence to the $2.0x10^6$ Da band; whereas P81 had a single band (Fig. 1B).
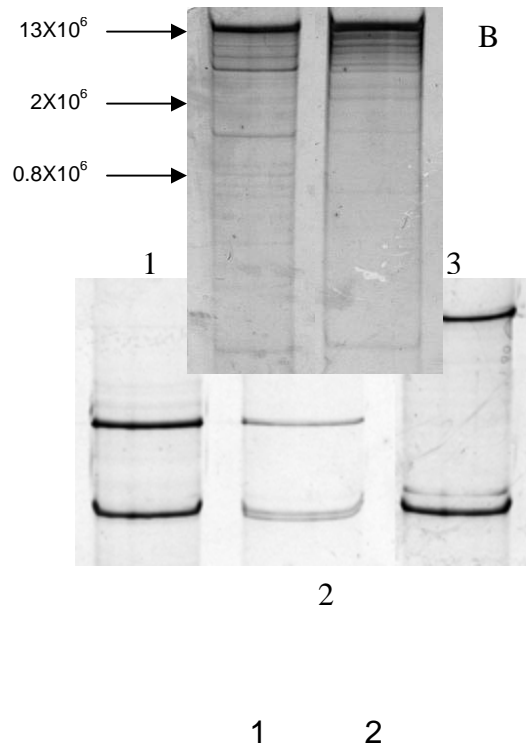

Fig. 1. Comparison of the molecular patterns obtained from the *Citrus tristeza virus* isolates from LREC and the ½ mile survey compared to the T30-like isolate P81. (A) SSCP profiles of the coat protein gene. Lane 1: LREC S-1 citron isolate; lane 2: HS sample showing a mixed infection; lane 3: P81. (B) Double-stranded RNA patterns confirming the diversity among isolates. Lane 1: LREC S-1 citron isolate; lane 2: P81.
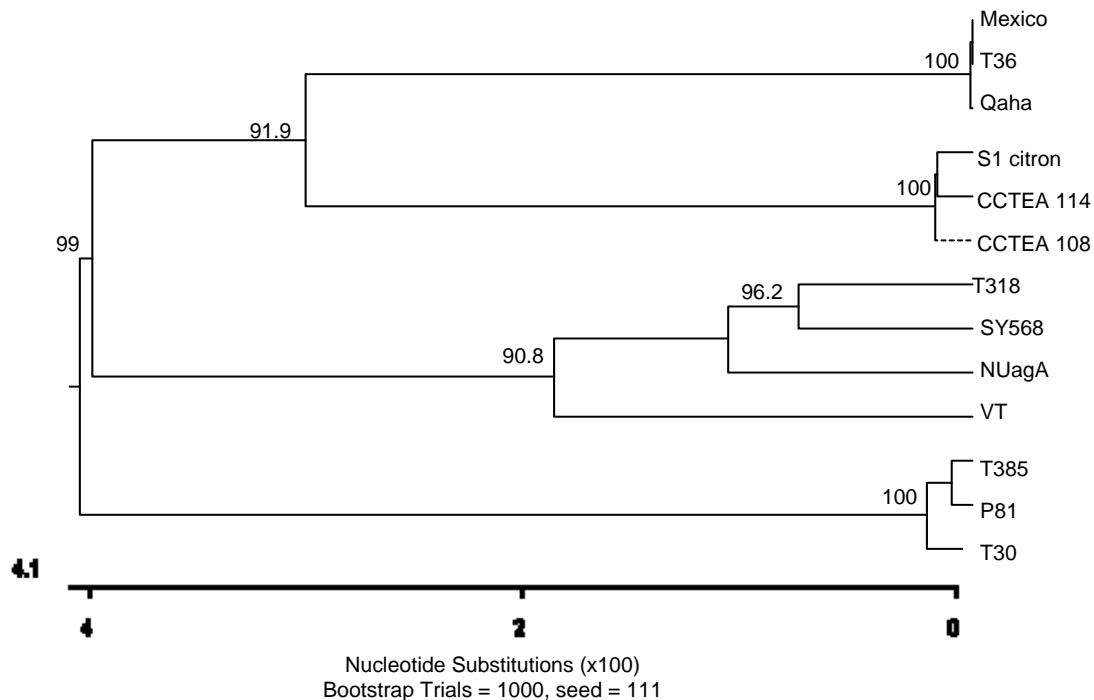
Fig. 2. Phylogenetic relationships among LREC-S1 citron isolate, standard isolates and other Californian isolates (P81: GenBank acc. no. AY995567; CCTEA 114: GenBank acc. no. EU325938; CCTEA 108: GenBank acc. no. EU325936). The dendrogram is derived from the ClustalW multiple alignment of the CP nucleotide sequences using MegAlign software (Lasergene 7, DNASTAR). Bootstrap values for 1,000 replicates are indicated. Branch lengths are proportional to number of nucleotide substitutions.

HS samples were also analyzed by SSCP. Five were found to have the same unique pattern as the S-1 citron isolate. A mixture of this isolate and the mild P81-like isolate was found in several of these samples. This mixture in a HS sample could be explained either as one tree infected by the mixture or two or more of the four trees sampled were infected by the different isolates (Fig. 1A). This relationship was confirmed by obtaining a positive test with MCA13 by the S1 citron isolate and the five HS samples with the same SSCP pattern as the S1 citron strain.

Phylogenetic analysis of the coat protein gene. Since the S-1 citron isolate was different, its CP gene was cloned and sequenced. Phylogenetic analysis of its

sequences was compared with various reference isolates. This isolate showed 99.8% identity with central California isolates CCTEA114 (EU325938) and 108 (EU325936) (11) which were all MCA13 positive and were on the same clade with the T36 strain (Fig. 2).

DISCUSSION

This study was conducted to obtain a rapid characterization and assessment of the CTV isolates found in spring 2007 at the LREC. The overall transmission rate of the nine CTV isolates collected was > 6%. This

likely is an under estimation since virus acquisition was from excised field collected tissue of various cultivars and ELISA indicated high variability in virus titer in these source plants. When the data from the navel varieties was combined, a transmission rate of 10% was obtained. These data agree with previous reports on transmission of central California CTV isolates (10, 12) and suggests secondary spread of CTV would likely occur rapidly since abundant populations of the cotton aphid were observed on citrus in this area (Yokomi and O'Connell, unpublished data).

The MMM analysis indicated that the LREC isolates and those detected in the 0.8 km survey of commercial citrus surrounding the LREC had a T30-like genotype. T30 is a mild isolate described from Florida (4,5). This data suggests that these isolates are mild but this must be confirmed in a greenhouse host range biocharacterization test. This test is now underway by the CCTEA.

SSCP is sensitive enough to discriminate between isolates which differ by one nucleotide (9) and showed that the S-1 citron isolate was different from all the other isolates which were identical to P81. Phylogenetic analysis of the coat protein gene indicated this isolate shared 99.8% identity to central California isolates 114 and 108 which react with MCA13. Biocharacterization scores for these two isolates are 17.7 and 6.0, respectively (8). These isolates and that from S-1 citron have a 94.2% identity with the T36 strain, a CTV decline isolate from Florida (4, 5). Although used in Florida to detect CTV decline isolates, MCA13 reactive isolates are not correlated with quick decline CTV in California (8). Garnsey et al (2) compared MCA13 reaction with biological activity among isolates in an international CTV collection and found a strong positive correlation with serological reaction and the isolate's ability to induce orange stem pitting and seedling yellows. The CCTEA tristeza collection, representative of the isolates found in the Central Valley, contain only a few MCA13-reactive isolates. Two of these isolates were tested in a standard aphid transmission test and resulted in 0% and 1% transmission by the cotton aphid (Yokomi, unpublished data). This may suggest that such isolates have less potential for spread under central California conditions. Although the majority of California CTV isolates are mild, isolates exist with potential to cause stem pitting in sweet orange and grapefruit (8, 11). The data presented show that CTV diversity occurs, even within the mild T30 genotype group, in the Lindcove area. Since this diversity is associated with MCA13 reactivity, vigilance must be continued to limit spread of such strains.

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