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## Characterization of Virus Isolates from a Field that Once Contained an Unusually Severe Strain of *Citrus tristeza virus*

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**ABSTRACT.** A field on the UC Riverside campus where a severe isolate of *Citrus tristeza virus* (CTV) had been found in the late 1970s was revisited twenty years after tree removal and subsequent replanting to look for the reoccurrence of the isolate now known as SY568. This isolate can cause severe stem pitting, stunting, and vein corking in citrus hosts including grapefruit and sweet orange and is readily aphid and graft transmitted. Two hundred and sixty three sweet orange trees were tested and 52% (136 trees) were positive for CTV by ELISA using a polyclonal antibody. Of these 136 CTV positive trees, 45 of them also reacted with the monoclonal antibody MCA13, known to react with decline causing isolates of CTV from Florida and SY568. Forty trees (20 MCA13 negative and 20 MCA13 positive) were selected for further characterization. When peeled shoots and leaves were examined for symptoms, no stem pitting or vein corking were found. Isolates were also analyzed using RNase protection assays to compare several different areas of the genome. Many of the isolates tested were similar to each other, but up to nine distinct patterns were seen depending on the probe used. Most fell into two major groups, however none were similar to SY568 in any genomic region. It appears that eradication of the severe isolate SY568 was successful, although a majority of the trees are infected with mild isolates of CTV.

The prevalent strains of *Citrus tristeza virus* (CTV) in southern California are mild, as are ones that occur in other citrus growing areas such as Florida and Spain (11), and cause no economic loss as long as rootstocks tolerant to quick decline are used. In the field, the California and Florida mild strains typically do not induce stem pitting, stunting, or small fruit size or other CTV-related symptoms such as seedling yellows in scions or severe decline in trees on tolerant rootstocks (4, 18). In the 1970s a severe seedling yellows form of tristeza was being found increasingly in the Citrus Variety Collection in the Agricultural Experiment Station at UC Riverside (UCR-CVC) (2, 11, 12, 13). An in depth survey was conducted at the UCR-CVC, and one CTV isolate which was isolated from a stunted and severely stem pitted Minneola tangelo stood out. This isolate was named "12B" for the field from which it came, but has come to be known as SY568 and has been char-

acterized extensively (15, 17). It causes severe stunting, stem pitting, and vein corking in sweet orange and grapefruit seedlings, and severe vein corking in Mexican lime plants (2, 12). In 1979, an extensive tree eradication program was established in the citrus groves at UCR in an attempt to eliminate all the seedling yellows or stem pitting isolates of CTV (2, 12). Field 12B was completely replanted by 1983 as part of the Citrus Variety Collection with a mixture of varieties of different citrus species, including many sweet orange varieties.

No complete follow-up survey was ever done in the new plantings for the presence of CTV. Therefore, it is not known if isolate SY568 has reappeared or other SY568-like isolates are present there now. The sweet orange trees in UCR field 12B were used for this study to assess the presence of CTV, examine the genetic and biological variation of those CTV isolates, and determine if SY568-like isolates are currently present.

## MATERIALS AND METHODS

**Plant material.** All sweet orange trees (236), represented by 132 different accessions, in field 12B in the Citrus Variety Collection at UCR were chosen for the survey. Varieties were established on Carrizo citrange or C-35 citrange rootstocks. Leaf and stem tissue was collected from around the entire canopy of each tree, examined for symptoms, then petiole and/or bark tissue was used for each assay as noted below.

**ELISA.** All 263 trees were initially screened by double-antibody sandwich indirect (DAS-I) ELISA using a broad spectrum polyclonal antibody for the coat protein of CTV (5). One-half gram of petiole tissue was ground in PBS-Tween containing 2% PVP-40 (1/10 w/v) for use as the antigen samples. Trees that were positive for the presence of CTV using the polyclonal antibody were then assayed by DAS-I ELISA using the monoclonal antibody MCA13 which reacts with decline inducing and stem pitting isolates of CTV from Florida (10), as well as California isolate SY568. After all trees were screened, 40 (20 MCA13 positive, 20 MCA13 negative) were selected for further analysis.

**Nucleic acid characterization.** RNase protection assays (RPA) (7, 16) used radio-labeled RNA transcripts synthesized from

cDNA clones derived from the SY568 sequence to analyze the 5' untranslated region (5' UTR), coat protein (CP), and p65 heat shock protein analog (p65HSP) genomic regions of all 40 selected trees as previously described (17) (Table 1, Fig. 1). Total nucleic acid extracts (TNA) were purified from petiole tissue and processed through the RPA procedure. Fingerprints were recorded by electrophoresing the <sup>32</sup>P-labeled RPA products through 6% polyacrylamide/7 M urea gels which were then dried and exposed to a sheet of X-ray film for 12-48 hr. Large TNA extracts were prepared from a single lot of tissue and stored in aliquots to ensure sample consistency for analyses using multiple probes. After analysis and comparison of the fingerprints, five isolates were selected for additional RPAs using probes for the 5A polyprotein, RNA-dependent RNA polymerase (RdRp), diverged coat protein (dCP), 3' UTR, as well as the p13 and p20 genes. In all RPA analyses, four experimental controls were included: 1) the minus-sense radioactive probe alone without RNase treatment to check the quality and proper size of the RNA transcript; 2) the same minus-sense probe alone with RNase treatment to ensure that complete degradation of the probe would occur if not protected by a homologous sample; 3) the

TABLE 1  
SPECIFICATIONS OF RNASE PROTECTION ASSAY PROBES FROM CDNA CLONES  
OF *CITRUS TRISTEZA VIRUS* ISOLATE SY568

Probe	Size of probe alone in nucleotides (nt)	Size of probe protected by plus-sense transcript (nt)	CTV viral sequences contained in probe (nt)	Position of probe in SY568 genome (nt)
5' UTR	166	130	120	8-128
5A polyprotein	635	599	557	996-1553
RdRp	939	908	885	9230-10115
p65HSP	735	696	655	13225-13878
diverged CP	933	903	854	15403-16257
CP	450	403	361	16256-16616
p13	428	389	384	17224-17607
P20	633	586	552	17737-18288
3' UTR	329	309	257	18992-19249

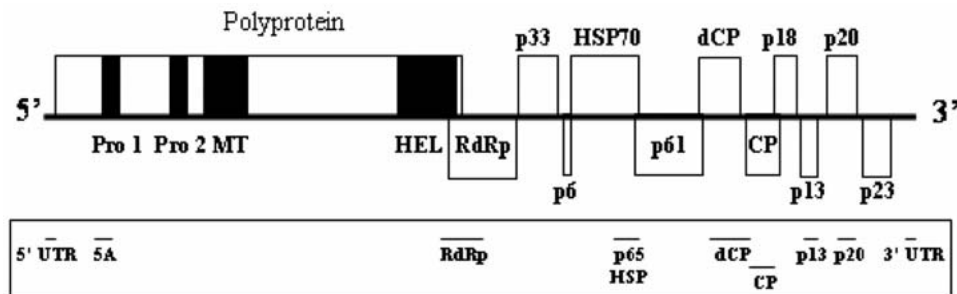


Fig. 1. Schematic of CTV SY568 genome and location of RNase protection assay probes (box below map).

minus-sense probe hybridized to a plus-sense RNA transcript from the same cDNA clone, then exposed to RNase treatment to show that little or no degradation of the probe would occur if protected by an identical sample; and 4) the probe hybridized to purified total nucleic acid from a sweet orange tree infected with CTV isolate SY568 and treated with RNase to record a fingerprint for that isolate for comparison with new field isolates. It is common to see minor digestion products as a result of non-specific digestion (7) and such bands were not taken into account when comparing experimental fingerprint patterns to each other and to those of the controls.

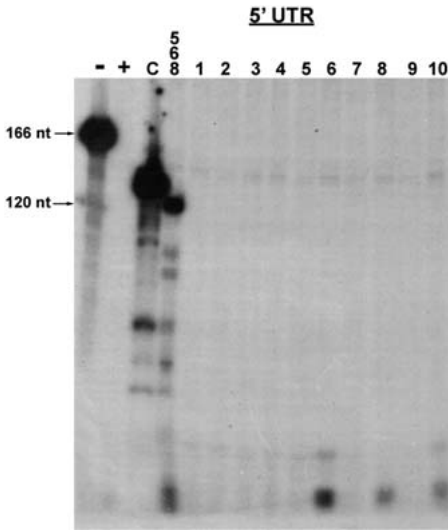
**RESULTS AND DISCUSSION**

**Biological analysis.** Bark was removed from twigs from each of the 40 selected trees and examined for stem pitting, as were twigs from healthy and SY568 infected control sweet orange trees. The SY568 twigs showed moderate to severe stem pitting over the entire surface of each twig, while no stem pitting was seen in any of the twigs from any of the 40 field trees or the healthy control. Leaves from each tree were also examined. No symptoms were detected on any of the leaves from field trees, while the SY568 control leaves had obvious vein corking.

**ELISA.** Of the 263 field trees tested, 136 (52%) were positive for

CTV using a polyclonal antibody for the major coat protein gene. When these 136 CTV positive trees were further screened using the monoclonal antibody MCA13, 45 trees (33% of CTV positive trees, 17% of total trees) reacted positively. These results were not surprising since CTV is endemic in southern California as is its primary vector, *A. gossypii*. The monoclonal antibody MCA13 was developed in Florida for the detection of decline inducing strains (10) and also reacts well with SY568. It does not detect all severe strains however (3), and does occasionally react with some mild strains (6). In this study a relatively small number of trees (45) reacted with MCA13 and did confirm that all of the tristeza isolates were not the same. However, this data did not predict the severity of isolates and did not reliably indicate the presence of an SY568-like isolate. The MCA13 status was used not only to identify isolates from field 12B which might be SY568-like, but also to select isolates for further characterization by RPA.

**RNase protection assays.** Preliminary screening of all 40 isolates was done using RNA probes representing the 5' UTR, CP, and p65HSP regions of the genome of isolate SY568 to determine the genetic variation among them and to detect the presence of SY568-like isolates. The 5' UTR probe was not protected by RNAs from any of the 40 trees (Fig. 2) indicating these 40 isolates

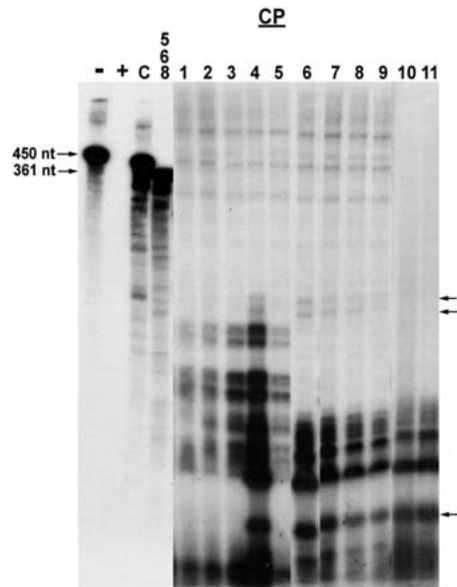


**Fig. 2.** Autoradiograph of RNase protection assay gel using the 5' UTR probe against samples from field 12B. Samples are: probe alone with no RNase (-), probe with RNase (+), probe hybridized to a plus-sense complement RNA transcript, with RNase (C), probe hybridized to SY568 (568) and 10 representative samples from sweet orange trees in field 12B (1-10), with RNase. The positions of the full length probe including non-viral plasmid sequences (166 nt) and actual CTV viral nucleotides protected (120 nt) are shown on the left margin.

share little sequence similarity with SY568 at the 5'-end. This complete digestion of the 5'UTR probe was also a common result when known mild strains were analyzed (D. Mathews, unpublished results). The 5' UTR probe was able to rule out the presence of SY568-like isolates, but was not useful for identifying distinct isolates of CTV. The 5' UTR is known to be the most variable region of the CTV genome (1, 8), which could explain the total lack of protection of that probe with any of the field isolates.

The CP gene probe was partially protected by all 40 field isolates, producing two dominant fragmentation patterns (Fig. 3). Twenty-seven isolates showed the same profile composed of about 15 small to medium sized segments (pattern A), while 13

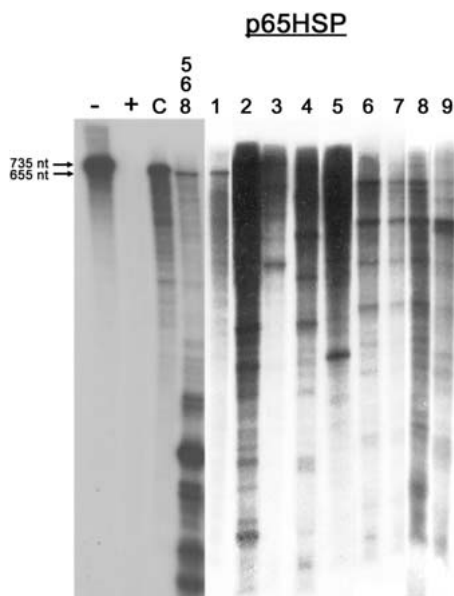
isolates (all MCA13 positive) presented a shorter fragmentation pattern having about 8 bands (pattern B), many of which were common to the two patterns (Fig. 3, pattern A: lanes 1-5; pattern B: lanes 6-11). Two additional fragments migrating above all bands in both patterns A and B were found in 15 isolates, thus appearing independently of those 2 patterns (Fig. 3, lanes 4, 6, 7, 8, and 9). A unique low molecular weight band was seen in 21 isolates, always appearing in pattern B, but rarely occurring with pattern A (Fig. 3, lanes 4, 6-11). At least three distinct fragmentation patterns within the CP gene of CTV were found in this field. CTV isolates generally



**Fig. 3.** Composite autoradiographs of RNase protection assay gels using the coat protein gene probe against samples from field 12B. Samples are: probe alone with no RNase (-), probe with RNase (+), probe hybridized to a plus-sense complement RNA transcript, with RNase (C), probe hybridized to SY568 (568) and 11 representative samples from sweet orange trees in field 12B (1-11), with RNase. The positions of the full length probe including non-viral plasmid sequences (450 nt) and actual CTV viral nucleotides protected (361) are shown on the left margin. Bands noted in the text are indicated by arrows on the right margin.

share greater than 80% homology with each other both at the amino acid and nucleotide level of the coat protein, with differences being most significant between isolates with different biological activities (9).

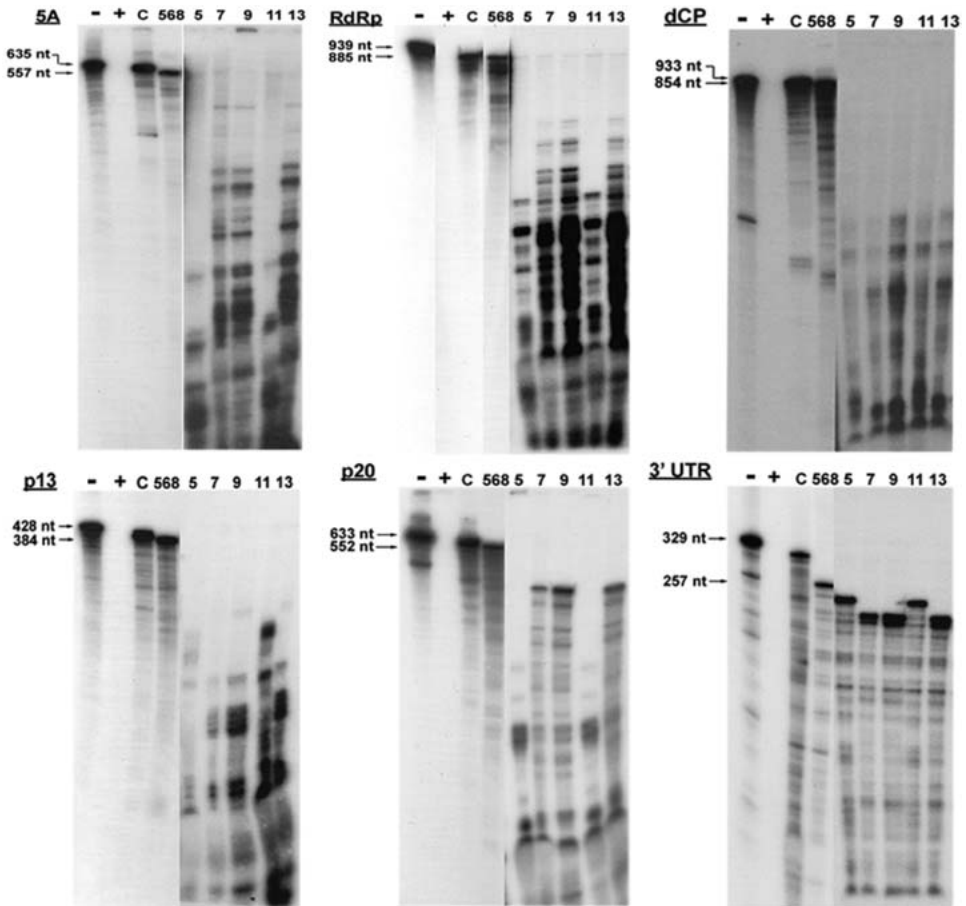
Similar variations were seen using the p65HSP probe (Fig. 4). However this probe is unique in that the cDNA clone used to produce the RNA transcript is homologous to a mild strain within the SY568 isolate (15, 17), so that a fully protected band using this probe indicates the presence of a mild strain. All of the other RPA probes are homologous to one or more of the dominant severe strains



**Fig. 4.** Composite autoradiographs of RNase protection assay gels using the p65 HSP probe against samples from field 12B. Samples are: probe alone with no RNase (-), probe with RNase (+), probe hybridized to a plus-sense complement RNA transcript, with RNase (C), probe hybridized to SY568 (568) and 9 representative samples from sweet orange trees in field 12B (1-9), with RNase. The positions of the full length probe including non-viral plasmid sequences (735 nt) and actual CTV viral nucleotides protected (655) are indicated by arrows on the left margin. Note that results for trees 13-16 (lane 1), 5-2 (lane 4), 9-12 (lane 5), and 11-10 (lane 8) which are fully characterized in Fig. 5 are included here.

of CTV within the SY568 isolate (14, 15, 17). When hybridized against the SY568 control sample, a small amount of fully protected p65HSP probe is seen at the top of the gel (representing the low titre of the mild strain in the mixed infection), and a fragmentation pattern representing the majority of the probe (from the high titre severe strains) is seen in the bottom half of the gel (Fig. 4, lane 568). Field samples that show a high proportion of fully or nearly fully protected probe are predicted to be mild (non stem pitting, non seedling yellows), and those with only smaller fragments are predicted to be more severe. Twenty-five field isolates were able to fully protect the p65HSP probe, some with a small number of variably sized cleavage fragments (Fig. 4, lanes 1-5). Another 12 isolates lacked the fully protected segment, but showed two relatively high MW bands with additional bands in the pattern (Fig. 4, lanes 6-9). Three isolates did not produce clear profiles for this probe and were not further analyzed. At least seven different fragmentation patterns were observed using the p65HSP probe, but none showed the recognizable pattern of the severe strains from isolate SY568.

Based on these results, five isolates (trees 5-2, 7-7, 9-12, 11-10, and 13-16) were selected for full characterization by RPAs with six other probes (5A, RdRp, dCP, p13, p20, and 3'UTR). In addition, RPAs were repeated using the 5'UTR, CP, and p65HSP probes against each of the five isolates with similar results, showing only minor shifts in fragmentation patterns. When the 5A and RdRp probes were used, isolates 7-7, 9-12, and 13-16 had profiles made up of about 17-20 fragments, while a pattern having fewer and smaller fragments (7-12) was obtained using isolates 5-2 and 11-10 (Fig. 5, panels 5A and RdRp). Analysis with the dCP probe produced five similar banding patterns and no discrimination between iso-



**Fig. 5.** Composite autoradiographs of RNase protection assay gels using probes representing the 5A polyprotein (panel 5A), RNA-dependent RNA polymerase gene (panel RdRp), divergent coat protein gene (panel dCP), p13 gene (panel p13), p20 gene (panel p20), and the 3' untranslated region (panel 3' UTR) hybridized to samples 5-2 (5), 7-7 (7), 9-12 (9), 11-10 (11), and 13-16 (13) from field 12B, with RNase. Controls are: probe alone with no RNase (-), probe with RNase (+), probe hybridized to a plus-sense complement RNA transcript, with RNase (C), and probe hybridized to SY568 from sweet orange (568), with RNase. The sizes of each probe alone including non-viral plasmid sequences (upper number), and actual CTV viral sequences protected (lower number) are indicated by arrows on the left margin of each panel.

lates was seen (Fig. 5, panel dCP). Using the p13 and p20 probes, isolates 7-7, 9-12, and 13-16 again exhibited a similar pattern, which was different from that of isolates 5-2 and 11-10 (Fig. 5, panels p13 and p20). Analysis with the 3'UTR probe yielded two somewhat less distinct banding patterns for the same isolates as above (Fig. 5, panel 3'UTR). However these patterns were also similar to the fragmentation pattern seen in the SY568 profile, whereas

none of the other probes showed any similarities between the patterns seen for the field samples and that of SY568. Since the 3' UTR is the most conserved region of the CTV genome (8) this is not an unusual result. The five isolates formed two distinct groups. The first group was represented by isolates 5-2 and 11-10, which were also both MCA13 positive when analyzed by ELISA. Isolates 7-7, 9-12, and 13-16 formed the second group and were all MCA13

negative. The 5A, RdRp, and p20 probes were quite useful for distinguishing between different field isolates although the CP and p13 probes also showed some discrimination. The p65HSP probe detected only minor differences between these field isolates, but all are predicted to be mild based on their RPA patterns. The dCP probe was not useful for discrimination between these isolates. The dCP region of the CTV genome is relatively well conserved 89-99% (14, 17) which may account for all five isolates having identical patterns. In contrast, many of the other genome regions analyzed by RPA are equally well con-

served at the nucleotide level yet still showed discriminatory differences using RPA. All of the probes, except the 3' UTR, were able to strongly distinguish between these field isolates and SY568.

These results show that there are several different variants of CTV present in field 12B at UCR, with two being dominant among those that were fully characterized. SY568 seems to have been successfully eliminated from the field at UCR, and no other severe, stem pitting strains were found providing evidence in support of the concept of tree removal for the long term elimination of specific strains of CTV.

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