Citrus tristeza virus in Florida: A Synthesis of Historical and Contemporary Biological, Serological, and Genetic Data

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ABSTRACT. The decline of trees on sour orange rootstock is the only commercially important disease currently caused by *Citrus tristeza virus* (CTV) in Florida. Virus isolates from commercial citrus plantings in Florida have previously been characterized biologically as mild (do not cause decline on sour orange rootstock) and severe (do cause decline). CTV-induced stem-pitting is not currently a commercially important disease in Florida. Discrimination of severe and mild isolates in Florida has been accomplished previously by biological indexing and by the monoclonal antibody MC13, which does not react to Florida mild isolates. The Florida T30 and T36 isolates are type isolates for the mild and severe groups respectively, and both genomes have been fully sequenced. We developed a PCR-based assay to characterize CTV isolates by amplification of sequence specific molecular markers using primers derived from the nucleotide sequence of the VT, T3, T30 and T36 isolates. The pattern of amplified markers is used to assign to a CTV isolate a specific genotype. When we applied this marker analysis to a collection of over 400 CTV accessions from Florida the T30 and T36 genotypes, either singly or together, were the primary genotypes in commercial citrus in Florida. The VT genotype was found in some Meyer lemon trees, not in commercial sweet orange or grapefruit plantings. The T3 genotype, which is common in many other countries, was not found in commercial trees. Biological indexing of select isolates supported the association of the T36 genotype with induction of a graft incompatibility on sour orange rootstock. The widespread distribution in Florida of two predominant genotypes may reflect the sources of CTV germplasm that have been introduced and subsequently spread via propagation and aphid vectors. The limited genetic variability of CTV currently present in Florida may facilitate the use of genetically based assays to regulate future ingress of other CTV genotypes into Florida citrus budwood.

Citrus tristeza virus (CTV) is distributed widely throughout the world's citrus growing areas and is the most prevalent and damaging viral disease of citrus. Strains of CTV differ in pathogenicity, with some strains causing economically damaging diseases, while other strains are relatively benign, and cause no visible symptoms or loss of tree vigor or yield. Stem-pitting disease caused by specific strains of CTV occurs in both rootstock and scion and can decrease tree growth and vigor, leading to a decline in fruit size, quality and yield. Another important CTV-caused disease is decline, which is a graft incompatibility that develops at the budunion on sour orange rootstock. The development of this incompatibility can lead to the decline and eventual death of the tree.

In Florida, the economically important disease caused by CTV is the decline of sweet orange or grapefruit trees on sour orange rootstock. Isolates of CTV that induce this incompatibility are known as decline strains. Other strains in Florida that do not cause decline or any noticeable disease symptoms are called mild strains. Neither mild nor decline strains in Florida cause economically damaging stem-pitting symptoms in sweet orange or grapefruit.

An effective method of discrimination between decline and mild strains has been enzyme-linked immunosorbent assay (ELISA) using the MC13 monoclonal antibody, which reacts with Florida decline strains (these strains are termed MC13^{+}) but not with Florida mild strains (termed MC13^{-}) (16). In Florida, MC13 is used to certify registered budwood trees for purposes of propagation. Budwood from registered trees that are MC13^{-} can be propagated for distribution, whereas budwood from registered trees that are MC13^{+}

cannot be used for propagation. Although MC13 discriminates well between most Florida mild and decline isolates, it also reacts with CTV isolates in an international CTV collection in Beltsville, MD, and these isolates can cause severe stunting and stem-pitting in sweet orange and grapefruit (S. M. Garnsey, unpublished data). If such isolates were present in Florida, they could not be discerned from Florida decline isolates by MC 13.

Recently, the genomic sequences of five isolates of CTV were determined (1, 13, 14, 17, 18), including the Florida type decline isolate, T36, and the type mild isolate, T30. The nucleotide differences between these sequences were used to develop an immunocapture-RT-PCR assay that discriminates between CTV strains by amplifying sequence specific molecular markers to create a specific marker pattern termed the isolate genotype (11, 12). Specific genotype groups are named for the type isolate giving that particular marker pattern, in particular the isolates T3, T30 and T36 from Florida, and VT from Israel. Using this technique, isolates of CTV can then be grouped based upon a shared genetic marker pattern. An assessment of the exotic CTV collection in Beltsville correlated the most severe symptoms of stunting and stem-pitting with CTV isolates that were assessed with a T3 or VT genotype (Hilf, unpublished data).

The Florida isolates T3, T30 and T36 are part of a standing collection of CTV isolates collected and propagated from commercial and door yard trees over a period of at least 40 years (9, and S. M. Garnsey, unpublished data). In conjunction with the collection and propagation of these isolates, descriptions of the symptoms apparent in the original field trees were recorded. This field symptom data, along with data generated by the serological and genetic characterization techniques now available, can be summarized to provide a comprehensive picture of CTV in Florida. In particular, from a historical and regulatory perspective, we were interested in determining if the population of CTV found to be MC13^{+} and associated with trees exhibiting decline symptoms in the field are genetically homogeneous. Finally, we present this study as a model for integrating biological, serological and genetic data to provide a holistic view of CTV not obtained from any single analysis technique.

MATERIALS AND METHODS

Sources of virus. CTV isolates were collected from commercial and dooryard sites scattered throughout the state of Florida over a period of 33 yr from 1965 to 1998.). Virus isolates were established from budwood obtained from field trees by graft inoculation to citrus plants, primarily Madam Vinous sweet orange, and maintained in the glasshouse at the USDA Horticultural Research Station in Orlando and Fort Pierce, Florida. Isolates were maintained in two collections labeled FL and FS. Virus isolates in the FS collection were collected over the entire period indicated, while the isolates in the FL collection were obtained during the period 1993 to 1998. The isolate T3 predates the FS and FL collections, and was first described by Grant (9) who isolated it from a field Mexican lime tree.

Serological analysis. Serological analysis of CTV was by standard double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) essentially as described in (16). Tests were conducted using glasshouse propagated tissue, not the original field samples. Samples were assayed for reactivity with strain non-specific polyclonal or monoclonal antibodies, and with a strain specific monoclonal antibody, MC13. In general plant samples were considered positive for CTV,

using general and strain specific antibodies in ELISA, when OD_{480} values for samples were 2.5 to 3 times the value of extracts from uninoculated control plants.

Genetic marker analysis of CTV. Analyses were conducted on glasshouse propagated material, not on original field tissue. In summary, RNA in virions captured by CTV polyclonal antibody-labeled magnetic beads was used as template for reverse transcription of cDNA using random hexamers and standard procedures as described (11, 12). The amplification of general and specific CTV molecular markers was with both sense and anti-sense primers at a final concentration of 0.2 µM in a 25 microliter reaction volume containing a 1× reaction buffer concentration, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.625 units of Taq polymerase (Promega Corp., Madison WI). Amplification parameters were 30 or 35 cycles of 94°C for 30 s, 56°C for 60 s, 72°C for 60 s, followed by incubation at 72°C for 10 min. Reaction products were analyzed by standard agarose gel electrophoresis.

Virus sequence and primer development. Primers used for amplification of specific markers for evaluation of isolate genotypes were as described previously (12). The primers used to amplify T36 markers were derived from the published T36 sequence (13), available as Genbank Accession No. U16304. Primers for VT markers were derived from the published sequence of the VT isolate (14) available as Genbank Accession No. U56902. Primers for T30 markers were derived from the published sequence of the T30 isolate (1) available as Genbank accession AF26065. Primers for the T3k17 markers were derived from unpublished sequence data originally provided by A.V. Karasev. In addition to the markers described in ref. 12, an additional VT sequence specific marker was used for isolate characterization. The sense primer for this marker was, 5'-GTACCCTC-CGGAAATCACG-3', and the anti-sense primer was, 5' GGTAGGGTC-TACTCGTTTCAT-3'. These two primers amplified a 564 bp marker corresponding to map positions 19 to 583 of the published VT genomic nucleotide sequence.

The capsid protein (CP) marker is a general CTV marker and was amplified to assure that the immunocapture and reverse transcriptase steps were effective. The sequence specific markers were amplified from the same cDNA reaction as the CP marker. Genotype designations were made after considering the marker profile generated using the indicated marker set, as described previously (12).

RESULTS

Serological and genetic marker analysis of the FS and FL collections. The FS collection represents a historical perspective on CTV isolates collected from infected field trees over a 33-yr period, 1965 to 1998, whereas the FL collection is more contemporary, as these isolates were collected during the 1993-1998 period. Isolates were collected from commercial and non-commercial citrus trees, primarily sweet orange and grapefruit cultivars from 18 counties in Florida. In addition to sweet orange and grapefruit, 36 isolates evaluated were found in Meyer lemon. Table 1 is a summary of results of the serological and molecular analysis of 405 CTV isolates from these collections. Of the 405 isolates studied, 206 or 51% were classed as T30-like based upon marker analysis. Interestingly, 19 (9%) of isolates with a T30 genotype were classed as MC13^{+} by serological analysis. This was unexpected since the type isolate for this group, T30, is MC13^{-}.

The next largest grouping of isolates was that assessed as being mixedly infected, containing both an isolate with a T30 genotype and one

TABLE 1

SEROLOGICAL STATUS AND MOLECULAR MARKER GROUPING OF 405 FLORIDA ISOLATES
OF *CITRUS TRISTEZA VIRUS*

Genotype	No. of isolates	MC13 status of isolate		
		MC13+	MC13-	MC13+ (%)
T30	206	19	187	9
T30+T36	131	114	17	87
T36	32	26	6	81
VT	28	28	0	100
VT+T36	8	8	0	100

with a T36 genotype. This category contained 131 isolates, or 32% of the total. Serological assessment showed that 114 (87%) of the isolates in this group were MC13^{+}, a level that is associated with the presence of the T36 genotype. Seventeen isolates (13%) of mixed infected isolates were MC13^{-}, an unexpected result.

Only 32 isolates (8%) tested in the combined collections were assessed as T36-like only. Isolates with a T36 genotype would be predicted to have a MC13^{+} character, but surprisingly six of the 26 isolates (23%) were MC13^{-}.

Thirty-six of the isolates tested contained VT genotype markers, 28 with VT markers only, and eight with both VT and T36 markers, which are apparently mixed infections. All of these isolates were from Meyer lemon, not from commercial sweet orange or grapefruit trees. These isolates are uniformly MC13 [+].

CTV genotypes, MC13 status and field symptoms. The visual health status of citrus trees on sour orange rootstock in the field was eval-

uated and recorded at the time budwood for propagation was taken for 114 CTV isolates tested in this study. An assessment of diseased was made for trees showing symptoms considered typical of CTV induced decline of trees on sour orange rootstock, as well as trees on sour that were appreciably stunted or showing signs of stem pitting in the rootstock or scion, associated with an apparent diseased appearance.

For 30 trees rated "healthy", 83% (25/30) contained CTV with a T30 genotype, and 17% (5/30) of trees rated healthy contained CTV with a mixture of the T30 and T36 genotypes (Table 2). For 84 trees assessed as diseased, 35% (29/84) contained CTV with a T30 genotype, 12% (10/84) had CTV with a T36 genotype, and 53% (45/84) contained a mixture of the T30 and T36 genotypes. Overall, 65% of trees associated with a diseased phenotype were associated with a T36 genotype. The MC13 serological status was also assessed for these 114 isolates. Table 2 summarizes the results of MC13 evaluation of these

TABLE 2

CITRUS TRISTEZA VIRUS GENOTYPES ASSOCIATED WITH HEALTHY AND DISEASED
FIELD TREES ON SOUR ORANGE ROOTSTOCK

Isolate genotype	Visual assessment of field trees		Serological status MC13+
	Healthy	Diseased	
T30	83%	35%	11%
T36	0%	12%	90%
T30+T36	17%	53%	82%

114 isolates based upon genotype of the isolate, independent of the visual rating of the source tree. For isolates with a T30 genotype, 11% of the isolates were MC13+, whereas this status was 90% and 82% for isolates of the T36 and mixed T30 and T36 genotypes respectively.

DISCUSSION

The current picture of CTV genotypes and distribution is largely consistent with information and observations that have accumulated over the past 50 yr (8), and suggests that the present distribution of CTV in commercial citrus is a product of natural spread by aphids and propagation of CTV-infected budwood. Surveys conducted soon after the first confirmation of tristeza in commercial citrus in 1951 indicated that some scattered infected trees were present in numerous locations, but with the exception of central Florida, there was little evidence that natural spread had occurred. Cohen and Burnett (5) in 1960 observed that three types of reaction were present in central Florida where CTV was most active. They concluded i) that older stunted trees on sour orange that indexed positively for CTV probably had become infected at the time of propagation rather than via natural means; ii) large trees on sour that suddenly began to decline had become infected in the field by aphids; and iii) the presence of trees on sour that had indexed positively for CTV infection by inoculation to Mexican lime, yet remained healthy looking, were infected with mild CTV which did not induce decline but which was being spread.

While the incidence of CTV increased rapidly during the 1960s (2, 3), decline of trees on sour remained mostly localized in certain areas (6), and use of sour orange rootstocks continued at historic levels (25-35%) until more widescale epidemics of decline occurred in the 1980s (4).

Attempts to limit spread of CTV in the voluntary budwood certification program were abandoned in the 1960s (8), and by 1980 most commercial budwood sources were infected (7). Continued successful propagation of nursery trees on sour orange during the 1970s suggests that the majority of these isolates were mild. However, since a majority of the trees propagated have always been on CTV tolerant rootstocks there was ample opportunity for a reservoir of decline isolates to accumulate that would have been undetected by field observation. The initial emphasis of the Florida budwood program to use budwood from highly productive fruiting trees in vigorous health may have contributed to the avoidance of propagating isolates that would be deleterious to the scion.

Evidence indicates that isolates of both the T30 and T36 genotypes were readily spread by either melon aphids or spirea aphids prior to establishment of the brown citrus aphid (3, 19, 20, 21, 22). Occurrence of super infections could be surmised from various field observations and was confirmed experimentally by Yokomi et al. (21). While some protective effects of pre-existing infections of T30 genotypes against challenge by the T36 genotype has been suggested (21) these effects are apparently temporary and stable mixed infections commonly occur. Interestingly, two genetic groups of CTV found commonly in other citrus growing regions, T3 and VT (12), have been found in Florida, but apparently have not spread into areas sampled in this study. The T3 genotype was recovered in a single instance from Florida material in the mid-1950s (9), but the origin of the isolate is unknown. Meyer lemon was introduced into Florida prior to 1920 (8) presumably with CTV. In Florida the VT genotype was found only in Meyer lemon sources, suggesting that this genotype was endemic to

Meyer lemon. Since aphid species differ in their patterns of spread of CTV (10), it is reasonable to speculate that the lack of recovery of this genotype from commercial sweet orange or grapefruit suggests a level of vector specificity, or that unspecified factors were affecting spread of this genotype out of Meyer lemon.

Although the development of the current CTV populations and their distribution may be fairly well deduced, the origins of the two major genotypes remain unknown. Introduction of CTV into Florida prior to 1909 can be inferred from early observations with satsuma mandarins, and certainly occurred with the introduction of Meyer lemon prior to 1920 (8). Introduction of CTV undoubtedly has occurred also by less well documented introductions of infected budwood. The T30 genotype apparently is quite common worldwide and genetically stable (1) and could have had many origins. The T36 genotype is less well distributed (12) and may have a more specific origin. Periodic severe freezes have undoubtedly also had a filtering effect on the CTV genotype reservoir and have promoted large scale shifts to new growing areas which, in turn, have influenced long distance movement of infected trees.

The identification of two major genetic populations of CTV in Florida might have been predicted from the biological and serological data gathered on CTV. However, the identification of MC13^{+} reactive field isolates with T30 genotypes was unexpected, since in Florida this reactivity was thought to be limited to isolates with a T36 genotype. Also surprising were the MC13(-) isolates with T36 genotypes. This may be indicative of the degree of stability of the MC 13 reactive epitope in a given population of CTV (15). The impact of this on the utility of MC13 as a regulatory testing tool in Florida is unclear, but this variability surely needs further study since there is heavy reliance on the use of MC13 to exclude positively reactive trees as sources of commercial budwood.

Despite this discrepancy, the association of recorded field symptoms with genetic and serological data supports the observation that decline symptoms are associated primarily with MC13 reactive CTV, with the presence of the T36 genotype, singly or as part of a mixed infection with the T30 genotype (Table 2). Non-declining field trees yielded primarily MC13 negative isolates with a T30 genotype. Interestingly, 35% of field trees rated as "diseased" contained only a T30 genotype. However, this assessment was made by analyzing virus propagated in the greenhouse from infected field material, not from analysis of tissue directly from these diseased trees. The discrepancy may represent the degree to which only one genotype of a mixed infection was successfully propagated in the greenhouse. Future studies of this type can now include comparative analysis of virus content at the time of collection and after greenhouse propagation.

The clearest message derived from this type of study is that no single current method of evaluation is adequate to effectively measure the variability and dynamics of CTV populations in a given citrus growing area. This coupling of contemporary laboratory analyses with an effective historical record and the availability of a collection of contemporary and historical CTV germplasm can provide a foundation for understanding how populations of CTV (or other citrus pathogens) may have changed over time. Alternatively, this same information provides a comparison with which to detect future changes in these populations, and to provide the incentive to alter regulatory procedures to meet potential future threats. Clearly, for Florida, the CTV situation has been and is relatively stable with regard to CTV populations and disease. With a more informed perspective on how CTV in

Florida compares to other areas, regulatory procedures and tools can be adapted and implemented in a pro active manner, rather than in a reactive one.

ACKNOWLEDGMENT

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