Characterization and Classification of Citrus Tristeza Virus Isolates by Amplification of Multiple Molecular Markers

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ABSTRACT. Nucleotide sequence and hybridization data using cDNA clones from the non-conserved regions of the genomes of the Israeli CTV isolate VT and the Florida isolates T3, T30, and T36 were used to develop sequence specific primers for PCR-based differentiation of CTV isolates of unknown genetic relatedness. Virus isolates from outside Florida were obtained from the exotic CTV collection at Beltsville, MD. Virions were captured from tissue extracts with antibodies, and reverse transcription and PCR were performed on captured virions with random and selective primers, respectively. Isolates were evaluated for the amplification of one common and 11 sequence-specific PCR products (markers) with primers derived from analogous sites within the genomes of the T3, T30, T36, and VT isolates. This set of amplified markers created a distinct marker profile for each isolate and this was termed the isolate "genotype". Analyses of 44 isolates yielded 14 different genotypes, but some clearly were minor variants of the T3, T30, T36, and VT genotypes. While the majority of isolates tested produced a product with at least one selective primer pair, at least three isolates did not produce products with any selective primers, suggesting that there are CTV sequences distinct from those of the isolates already sequenced. Isolates obtained from the same region or country often had the same pattern of markers, suggesting a common origin and possible extensive spread of a common CTV genotype in that location. Similar patterns of markers from isolates from different regions suggested a possible connection, such as movement of plant material between these regions. Isolates from other regions showed more diverse patterns of markers, suggesting greater diversification of CTV in that region or possible multiple introductions of different CTV isolates. We suggest that the use of selective primers in PCR-based assays provides a reliable and rapid method of assessing the degree of genetic variability present in global CTV populations as well as the relatedness of individual isolates from different citrus-growing regions.

Citrus tristeza virus (CTV) is widespread throughout the citrus producing regions of the world and still presents a threat to citrus production from both a disease and quarantine perspective. Isolates of CTV traditionally have been characterized and differentiated from one another by the comparison of symptoms that develop in inoculated host assay plants (1). Isolates also can be differentiated to an extent using serology. Monoclonal antibodies have been used to varying extents to identify regionally severe and mild isolates of CTV (12) or to identify isolates that may induce specific symptoms, such as stem pitting in sweet orange (10). Isolate characterization also has been based upon capsid protein gene sequences (3, 8, 11), and 5'-terminal sequences (7). However, comparison of the sequence of a single gene or region may reflect differences for just that region, but this region may not be reflective of the entire genome. A better understanding of molecular relatedness was obtained by the comparison of the complete genomes of fully sequenced isolates (9, 13). These analyses indicated that the genomes of CTV isolates were most divergent in the 5' proximal eleven kilobases of the genome, and much less divergent in the 3' terminal eight kilobases of the genome. Because it is difficult to completely sequence the 19 kb CTV genome, routine comparison of isolates by this technique is impractical. An alternative approach was developed based upon the amplification of mul-

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tiple molecular markers by the polymerase chain reaction (PCR) using primers derived from divergent regions of the genomes of the Florida isolates T3, T30 and T36. Using the marker profiles (termed the viral "genotype") generated by these primers, we differentiated these three isolates and identified additional isolates similar to each genotype (5). We expanded the number of sequence-specific primers by including ones made from the sequence of the Israeli isolate VT (9) and used these primers in combination with an immunocapture protocol to examine isolates from many citrus regions. We present data here that documents that this is a useful technique to classify CTV isolates that share common molecular markers.

MATERIALS AND METHODS

Sources of virus used for analysis. The Florida CTV isolates were maintained in citrus plants in the glasshouse at the USDA Horticultural Research Station in Orlando, Florida. Details of the origins of these isolates are given elsewhere (5), but in summary, the isolates T30, T30-1, T49-1, T55-1, and T56-1 are field-derived CTV isolates that have similar serological and biological characteristics. They are considered mild isolates in Florida. The isolates T36, T64-1 and T66-1 were isolated from field trees on sour orange rootstock undergoing CTV-induced decline. The isolate T3 was isolated from a Mexican lime tree in the field (4) and T68 was recovered from Ellendale mandarin budwood imported into Florida from Australia without authorization. The suffix (-1) in the isolate designation indicates that the isolate was aphid transmitted from propagations of the original field source. Citrus tissue containing isolates not endemic to Florida was obtained from an international collection of CTV isolates maintained at Beltsville, MD (1). These are designated as "B" isolates.

Virus extraction, immunocapture and reverse transcription. These procedures are presented in a general outline in Fig. 1. The specific parameters for the immunocapture and reverse transcription steps in this work were the same as those previously described (5).

Amplification of molecular markers by PCR. Virions attached to magnetic beads were used as templates for reverse transcription of cDNA using random hexamers and standard procedures. The amplification of general and specific CTV molecular markers has been described in detail (5). In summary, both sense and anti-sense primers were 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 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. The sequences of the primers used for PCR, and the sizes of the markers they amplify are presented in Table 1. The primers for the capsid protein gene (CP) were derived from the sequence of the Florida isolate T36 and were used to measure the effectiveness of the immunocapture step and the synthesis of cDNA by reverse transcription. The CP gene marker is considered to be a general CTV marker and is not used to discriminate between isolates. The sequence specific primers are named according to the source sequence, and the genomic regions these primers amplify are indicated in Fig. 2. The primers used to amplify T36 markers were derived from the published T36 sequence (6), available as Genbank Accession No. U16304. Primers for VT markers were derived from the published sequence (9) available as Genbank Ac-

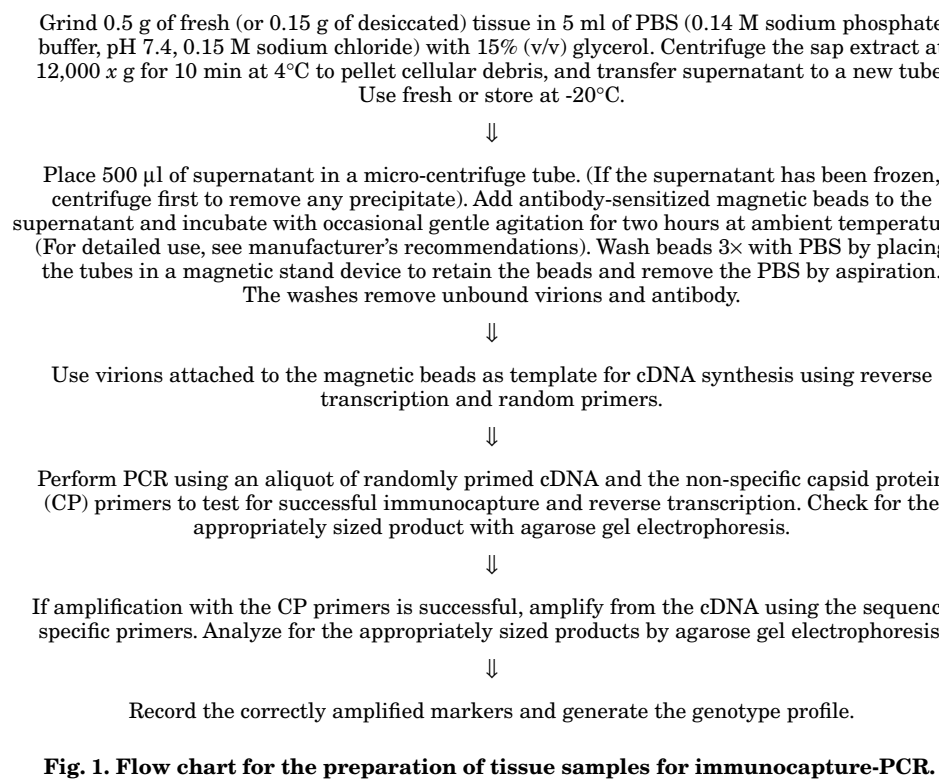

Fig. 1. Flow chart for the preparation of tissue samples for immunocapture-PCR.

cession No. U56902. Primers for the T3 k17 marker and for the T30 markers were derived from unpublished sequence data kindly provided by Drs. A. V. Karasev and M. R. Albiach-Marti.

RESULTS

Amplification patterns of standard isolates. The PCR product profile of an isolate is defined by which markers are amplified from cDNA, and we designated this profile as the isolate genotype. This concept has been described elsewhere (5), and an example is provided in Figs. 3A-G. Figure 3A shows the amplification of the CP marker from 10 Florida isolates that are indicated at the top of Fig. 3A. Figures 3B-D depict the T36pol, T36k17 and the T36-5' markers amplified from the isolates T36, T64-1, and T66-1, identifying these isolates as having a T36 genotype. Figures 3E and 3F show the T30pol and T30-5' markers amplified from the isolates T30, T30-1, T49-1, T55-1, and T56-1. These markers identified isolates with a T30 genotype. Figure 3G shows the T3k17 marker amplified from the isolates T3 and T68, indicating that these isolates have a T3 genotype.

In this study, we included primers derived from the sequence of the Israeli isolate VT that amplify the VTpol, VTk17, and VT-5' markers. We also used primers derived from the isolate T30 to amplify the T30k17 marker in addition to the T30pol and T30-5' markers. A summary of the genotypes of what we termed the four standard isolates, T3, T30, T36 and VT, determined by this method, is presented in Table 2. Unless otherwise stated, all amplification products are the size expected for the primers used (Table 1). Sequence-specific primer pairs derived from the T36 genomic sequence amplified products only from T36 cDNA. No

TABLE 1
SEQUENCE OF PRIMERS USED TO AMPLIFY CTV MOLECULAR MARKERS BY PCR

Isolate/Marker[z]	Sequence of primers (5'→3')	
T36CP (672)[y]	ATGGACGACGAAACAAAGAAATTG	(+)
	TCAACGTGTGTTGAATTTCCCA	(-)
T36POL (714)	TGACGCTAACGACGATAACG	(+)
	ACCCTCGGCTTGTTTTCTTATG	(-)
T36K17 (409)	GTTTTCTCGTTTGAAGCGGAAA	(+)
	CAACACATCAAAAATAGCTAGT	(-)
T36-5' (500)	AATTTCACAAATTCAACCTG	(+)
	CTTTGCCTGACGGAGGGACC	(-)
T30POL (696)	GATGCTAGCGATGGTCAAAT	(+)
	CTCAGCTCGCTTTCTCGCAT	(-)
T30K17 (409)	GTTGTCGCGCCTAAAGTTCGGCA	(+)
	TATGACATCAAAAATAGCTGAA	(-)
T30-5' (594)	CGATTCAAATTCACCCGTATC	(+)
	TAGTTTCGCAACACGCCTGCG	(-)
VTPOL (695)	GACGCTAGCGATGGTCAAGC	(+)
	CTCGGCTCGCTTTCTTACGT	(-)
VTK17 (409)	GTTGTCGCGCTTTAAGTTCGGTA	(+)
	TACGACGTTAAAAATGGCTGAA	(-)
VT-5' (492)	AATTTCTCAAATTCACCCGTAC	(+)
	CTTCGCCTTGGCAATGGACTT	(-)
T3K17 (409)	GTTATCACGCCTAAAGTTTGGT	(+)
	CATGACATCGAAGATAGCCGAA	(-)

[z]Primers for each marker are listed in pairs by sense (+) or antisense (-) orientation.
[y]Numbers in parentheses indicate the size in base pairs expected for the amplified product.

primer pairs derived from the sequence of the three other isolates amplified markers from T36 cDNA. Each of the T30 primer pairs amplified markers of the correct size from T30 cDNA. The VTk17 and VT-5' primers also amplified markers from T30 cDNA, but VTpol primers did not. The VT primer pairs amplified all three markers from VT cDNA. The T30k17 primer pair also amplified from VT cDNA, but the T30pol and T30-5' primers did not. The single T3 primer pair amplified the T3k17 marker only from T3 cDNA. The VTpol and VT-5' primers also

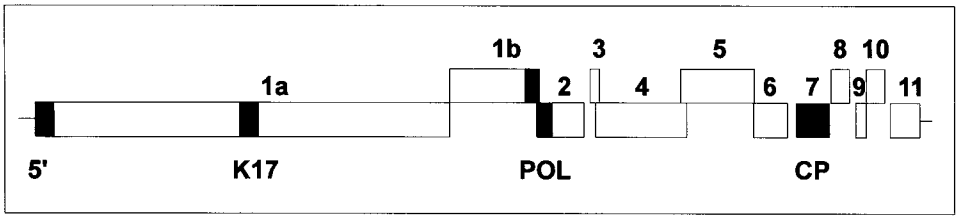

Fig. 2. Schematic diagram of the CTV genome based upon the T36 isolate. The viral open reading frames are indicated by numbers, and are listed in ascending order from the 5' to the 3'-terminus, in the manner of Karasev et al. (5). The approximate genomic locations of the sequence specific markers are indicated by shaded areas and by the name of the amplified marker. Figure 2 was published previously in Hilf et al. (4) and is used here with permission.

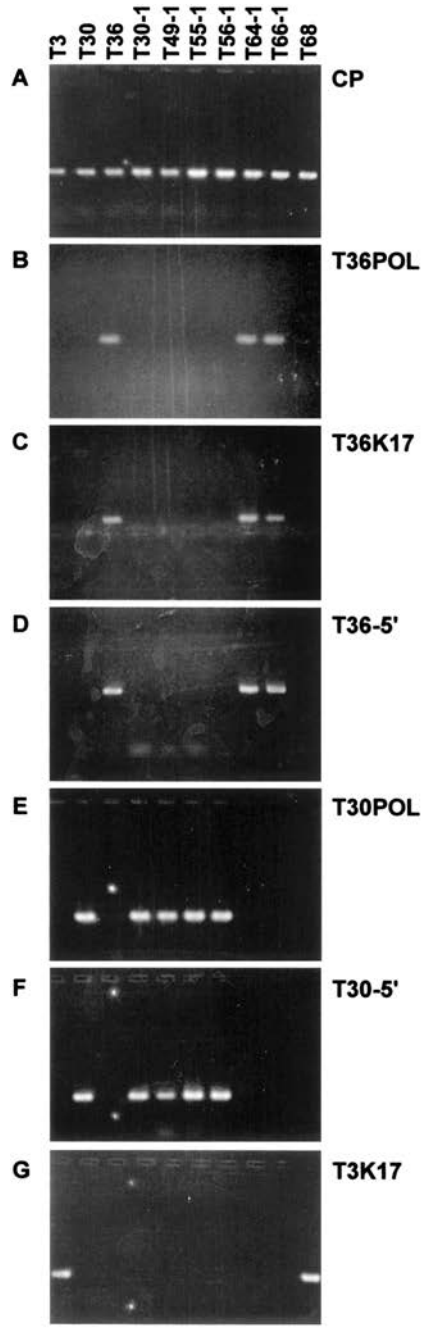

Fig. 3A-G. PCR amplification products from Florida isolates. The individual isolate is indicated at the top of the figure, and the marker amplified in each panel is indicated to the right. Lanes contain ethidium bromide stained DNA amplified from cDNA of the indicated isolate with the primer pair specific for the indicated marker. Figure 3 was published previously in Hilf et al. (4) and is used here with permission.

amplified products from T3 cDNA. While amplification from heterologous sequences occurred with certain isolates, this does not necessarily mean that these isolates were mixed populations. For example, the T30pol, T30k17, T30-5', as well as the VTk17 and VT-5' primers amplified DNA from T30 cDNA. However, sequence obtained from randomly generated cDNA clones derived from T30 RNA did not contain VT-like sequences (Albiach-Marti, unpublished data). The VTk17 and VT-5' primers apparently amplified sequence variants inherent in the T30 RNA population. Other examples of heterologous amplification also may not result from a mixed population.

Characterization of isolates from Florida. Additional Florida CTV isolates were analyzed using these primer sets and the results are presented in Table 3. The isolates T30, T30-1, T49-1, T55-1, and T56-1 originated from field trees in Florida and are considered mild isolates based upon field symptoms and the results of inoculations to standard indicator host plants. All of these isolates had the T30 genotype. The isolates T36, T64-1 and T66-1 caused decline of sweet orange or grapefruit on sour orange rootstock under Florida field conditions, and all three isolates had the T36 genotype. Interestingly, the isolates T3 and T68 were the only Florida isolates analyzed that had a T3 genotype.

Characterization of isolates from the exotic CTV collection. The exotic CTV collection at Beltsville, MD, has over 350 accessions of CTV, and we have characterized the majority of these isolates by this technique (data not presented). A portion of these results is presented here to demonstrate the results obtained by this analysis (Table 4). The marker patterns were used to place CTV isolates into classes corresponding to the isolates T3, T30, T36, or VT. The isolates presented in Table 4 were chosen to

TABLE 2
PCR MARKER PROFILES OF THE T36, T30, T3 AND VT ISOLATES USED AS STANDARDS IN THIS STUDY[z]

		PCR Marker										
Origin	Isolate	T36cp	T36pol	T36k17	T36-5'	T30pol	T30k17	T30-5'	T3k17	VTpol	VTk17	VT-5'
Florida	T36	1	1	1	1	0	0	0	0	0	0	0
Florida	T30	1	0	0	0	1	1	1	0	0	1	1
Florida	T3	1	0	0	0	0	0	0	1	1	0	1
Israel	VT	1	0	0	0	0	1	0	0	1	1	1

[z]Shaded boxes with the number "1" indicate a positive amplification of the marker indicated in the top row. Clear boxes with a "0" indicate no amplification of the indicated marker.

demonstrate the distribution of genotypes corresponding to the standard CTV isolates. For example, in addition to VT, the isolates B59 (South Africa), B1 (Reunion), B357 (Corsica), B257 (Thailand) and B229 (Colombia) also had the VT genotype. Three other Israeli isolates, B199, B79 and B201 also had the VT genotype, and these three are characteristic of 13 of the 15 accessions from Israel (data not presented). Three of the isolates from India, B219, B222, and B225, also had the VT genotype and these are representative of 13 of the 15 isolates from India in the Beltsville collection. Although the isolate VT may represent a genotype common in Israel, the data demonstrate that this CTV genotype also is present in other areas.

The T3 genotype also appeared to be widely distributed. The isolates B12, B211, B119 and B122, from Brazil, Taiwan, Hawaii and Colombia, respectively, all had the T3 genotype (Table 4). Although B119 has the T30 k17 marker in addition to the other markers characteristic of the T3 genotype, the overall assessment was that B119 has a T3 genotype. The isolates B272, B271, B216, B307, B364, and B252 are representative of isolates that have a T30 genotype, even though the isolates come from widely separated regions.

Overall, the T36 genotype was the least common genotype in the entire collection, with fewer than

TABLE 3
MARKER PROFILES OF SELECTED FLORIDA CTV ISOLATES[z]

		PCR Marker									
Isolate	T36cp	T36pol	T36k17	T36-5'	T30pol	T30k17	T30-5'	T3k17	VTpol	VTk17	VT-5'
T30	1	0	0	0	1	1	1	0	0	1	1
T30-1	1	0	0	0	1	1	1	0	0	1	1
T49-1	1	0	0	0	1	1	1	0	0	1	1
T55-1	1	0	0	0	1	1	1	0	0	1	1
T56-1	1	0	0	0	1	1	1	0	0	1	1
T36	1	1	1	1	0	0	0	0	0	0	0
T64	1	1	1	1	0	0	0	0	0	0	0
T66-1	1	1	1	1	0	0	0	0	0	0	0
T3	1	0	0	0	0	0	0	1	1	0	1
T68	1	0	0	0	0	0	0	1	1	0	1

[z]Shaded boxes with the number "1" indicate a positive amplification of the indicated molecular marker, and a clear box with a "0" indicates no amplification of the indicated marker.

TABLE 4
MARKER PROFILES OF SELECTED CTV ISOLATES FROM THE BELTSVILLE EXOTIC CTV COLLECTION[z]

		PCR Marker										
Origin	Isolate	T36cp	T36pol	T36k17	T36-5'	T30pol	T30k17	T30-5'	T3k17	VTpol	VTk17	VT-5'
VT Genotype												
Israel	B199	1	0	0	0	0	1	0	0	1	1	1
Israel	B79	1	0	0	0	0	1	0	0	1	1	1
Israel	B201	1	0	0	0	0	1	0	0	1	1	1
India	B219	1	0	0	0	0	1	0	0	1	1	1
India	B222	1	0	0	0	0	1	0	0	1	1	1
India	B225	1	0	0	0	0	1	0	0	1	1	1
S. Africa	B59	1	0	0	0	0	1	0	0	1	1	1
Reunion	B1	1	0	0	0	0	1	0	0	1	1	1
Corsica	B357	1	0	0	0	0	1	0	0	1	1	1
Thailand	B257	1	0	0	0	0	1	0	0	1	1	0
Colombia	B229	1	0	0	0	0	1	0	0	1	1	1
T3 Genotype												
Brazil	B12	1	0	0	0	0	0	0	1	1	0	1
Taiwan	B211	1	0	0	0	0	0	0	1	1	0	1
Hawaii	B119	1	0	0	0	0	1	0	1	1	0	1
Colombia	B122	1	0	0	0	0	0	0	1	1	0	1
T30 Genotype												
Colombia	B272	1	0	0	0	1	1	1	0	0	1	1
C. Rica	B271	1	0	0	0	1	1	1	0	0	1	1
Tanzania	B216	1	0	0	0	1	1	1	0	0	1	0
Cuba	B307	1	0	0	0	1	1	1	0	0	1	1
Spain	B364	1	0	0	0	1	1	1	0	0	1	1
Taiwan	B252	1	0	0	0	1	1	1	0	0	1	0

[z]Isolates that share a common profile are grouped together, with the origin of the isolates listed in the first column on the left. Shaded boxes containing a "1" indicate the amplification of the marker indicated in the top row, while a clear box with a "0" indicates no amplification of the indicated marker.

TABLE 4 (CONTINUED)
MARKER PROFILES OF SELECTED CTV ISOLATES FROM THE BELTSVILLE EXOTIC CTV COLLECTION[z]

Origin	Isolate	PCR Marker										
		T36cp	T36pol	T36k17	T36-5'	T30pol	T30k17	T30-5'	T3k17	VTpol	VTk17	VT-5'
T36 Genotype												
Spain	B33	1	1	1	0	0	0	0	0	0	0	0
Corsica	B359	1	1	1	1	0	0	0	0	0	0	0
C. Rica	B183	1	1	1	1	0	0	0	0	0	0	0
Guatemala	B343	1	1	1	0	0	0	0	0	0	0	0
Non-standard Genotypes												
Australia	B275	1	0	0	1	0	0	0	0	0	0	0
China	B66	1	0	0	0	0	1	0	1	0	0	1
Venezuela	B249	1	0	0	0	0	0	0	1	0	0	1
Bermuda	B282	1	0	0	0	0	1	0	0	0	1	1
Hawaii	B116	1	0	0	1	0	1	0	0	1	0	0
Spain	B159	1	0	0	0	0	0	0	0	0	0	0
Jamaica	B294	1	0	0	0	0	0	0	0	0	0	0
Japan	B29	1	0	0	0	0	0	0	0	0	0	0

[z]Isolates that share a common profile are grouped together, with the origin of the isolates listed in the first column on the left. Shaded boxes containing a "1" indicate the amplification of the marker indicated in the top row, while a clear box with a "0" indicates no amplification of the indicated marker.

five percent of the accessions showing this genotype (data not presented). Some isolates that had this genotype were B33 (Spain), B359 (Corsica), B183 (Costa Rica), and B343 (Guatemala). Two isolates, B33 and B343, had no 5' marker, but were still considered to be T36 genotypes because of the presence of the T36pol and k17 markers.

Also listed in Table 4 are isolates that have non-standard combinations of molecular markers. Isolate B275 from Australia has only the T36-5' marker. Isolate B116 from Hawaii had an unusual combination of the T36-5', T30k17 and VTpol markers. The isolates B249 (Venezuela), B66 (China) and B282 (Bermuda) also are examples of isolates that did not have a standard set of CTV molecular markers. The isolates B159 (Spain), B294 (Jamaica), and B29 (Japan), are examples of isolates from different regions that had no specific molecular markers, although they contain the general CP marker. There were other isolates which did not show sequence-specific markers, and these isolates constitute approximately five percent of the total CTV accessions in the Beltsville collection (data not presented).

DISCUSSION

We have developed a method to characterize and classify CTV isolates based upon the PCR-based amplification of molecular markers using primers derived from the genomes of four CTV isolates which have been partially or fully sequenced. An assessment of a collection of CTV isolates gathered from over 30 countries suggested that these molecular markers were conserved in the global CTV population. This technique can be used to determine the relatedness of isolates within and between regions as well as provide an initial measure of the overall molecular variability of global CTV populations.

We do not know if the accessions tested from each area represent the entire breadth of molecular variability of indigenous CTV populations. However, data obtained from the collection provided some preliminary information on the distribution of all four standard CTV genotypes. The T3, T30 and T36 genotypes are not restricted to Florida, and the VT genotype is similarly not restricted to Israel. The preponderance of the VT genotypes among the accessions from Israel may indicate a high degree of molecular homogeneity in the CTV population in Israel. A similar statement could be made for the CTV population of India, since 13 of the 15 Indian accessions also had the VT genotype. A direct connection between these two populations is unknown.

The Florida mild isolates investigated were similar to the standard isolate T30, and the Florida decline isolates from the field appeared to be propagations of the isolate T36. These data again suggest a genetic similarity among isolates from the same region. There may have been a limited introduction of CTV into the aforementioned areas, with subsequent spread by insect vectors and infected budwood. By testing a limited number of representative samples from a chosen region, information can be developed on the relative distribution of different genotypes. An example of this approach was demonstrated in a companion paper in these proceedings by Garnsey et al. (2).

While the four standard genotypes were recognizable in the collection, there also were isolates with genotypes that were dissimilar to these four. The isolates that had some but not all the markers characteristic of a standard genotype may be simple sequence variants of the standard genotypes. The existence of isolates that have none of the specific markers indicates these isolates have 5'-proximal sequences different from those already described.

Data on the biological characteristics of some of the isolates in the Beltsville collection indicated no direct correlation between the genotype of an isolate and symptoms that resulted after inoculation into the appropriate indicator host (data not presented). Some symptoms were more characteristic of one group, but a symptom such as decline was not limited to a particular CTV genotype.

Overall, we feel that careful application of this technique will be valuable in characterizing and classifying CTV isolates. We foresee its application toward understanding CTV population structure (such as the determination of the prevalence of mixed infections), isolate movement between and within citrus production regions, and selection of isolates for cross-protection based upon both molecular and biological properties. As more sequence information becomes available for different CTV isolates and populations, the number and specificity of markers can be increased to provide more accurate genotypes of new CTV accessions.

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