Incidence and Characterization of Mild and Severe Isolates of Citrus Tristeza Virus From Colombia

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ABSTRACT. Surveys of the major citrus-producing regions, nurseries and germplasm collections in Colombia indicated widespread presence of severe isolates of citrus tristeza virus (CTV). Nearly 90% of the samples tested were infected with CTV, and over 60% reacted positively with the MCA13 monoclonal antibody, which reacts to most severe strains of CTV. The capsid protein (CP) sequences of MCA13 reactive isolates were similar to known stem pitting strains, and the isolates did cause stem pitting on grapefruit and lime in biocharacterization tests. Thus, these isolates pose a threat to citrus production in Colombia and this region. However, the isolates from Mompox did not react with MCA13, their CP sequences were similar to known mild isolates, and they caused no symptoms on grapefruit or lime. Further research on Mompox CTV isolates may provide information useful for controlling CTV in Colombia and other countries by cross-protection.

Index words. Citrus tristeza virus, sequencing, multiple sequence analysis, Latin America.

Citrus tristeza closterovirus (CTV) is a serious pathogen of citrus throughout the world (1, 22), including Colombia (9, 16). It exists as many strains (22), and is expected to become more important in the Caribbean countries and Central and North America with the recent northward movement and establishment in this region of its most efficient vector, *Toxoptera citricida* (Kirkaldy), the brown citrus aphid (BrCA) (23). The development of a standardized host range (7), monoclonal antibodies capable of detecting severe isolates of CTV (20) and characterization of their reactive epitopes (18), and sequencing of many capsid protein genes (CPGs) (19) as well as the entire CTV genome (11) now enable more rapid detection and characterization of CTV and its many isolates (17).

There are approximately 32,000 ha of citrus planted in Colombia: 83% are oranges and 10% are limes which are susceptible to CTV; and 7% are mandarins which are CTV-tolerant. The purposes of this research were to determine the incidence of CTV in the major citrus-producing regions of Colombia and to determine the biological and molecular diversity among Colombian isolates of CTV. This information provides a more complete understanding of the CTV situation in Colombia, as well as a knowledge of the characteristics of those CTV isolates present in that region which might be carried northward by the BrCA.

MATERIALS AND METHODS

Disease surveys and sample collection. Twenty-two locations encompassing approximately 50% of the citrus producing area of Colombia were surveyed. These included the regions near Bogotá, and the eastern Llanos (Fig. 1), the Cauca River Valley from Cali to Medellín, and Mompox, which is a large island-like land mass of several hundred square miles in the lowland tropics and isolated from the rest of the country by the Cauca and Magdalena Rivers (Fig. 1). Samples were collected mainly from commercial citrus groves, but three nurseries and two germplasm collections also were sampled. Leaves generally were taken from random field

Fig. 1. Map of Colombia indicating major cities, Mompox and the sites (*) of collection of citrus samples.

trees, but sometimes were taken from those showing obvious tristeza symptoms such as vein clearing on limes or stem pitting on grapefruits and limes. Several young flushes were collected from each tree, placed in a labeled plastic bag, and stored in an ice chest or refrigerator at 4°C until processed 1-10 days later. Samples were either ground fresh for direct serological analysis, cut into small pieces and placed in 50% glycerol and stored at -20°C, or desiccated and ground into a fine powder, which was stored over desiccant at room temperature. Budwood samples were taken from trees of particular interest and shipped under quarantine permit to the Exotic Citrus Pathogen Collection (ECPC) at the USDA facility, Beltsville, MD, for biological indexing as described by Garnsey et al. (7).

Sample Analyses. Enzyme linked immunosorbent assays (ELISA) were performed as described by Permar et al. (20), using fresh samples of leaf laminae or midribs. All samples were tested with polyclonal antibodies to CTV and the majority also were tested with the monoclonal antibody MCA13, which reacts predominantly with severe strains of CTV (20). Reactions were considered as positive when the OD_{405} values were greater than three times the mean of the healthy controls. Desiccated samples of Florida strains T30 (mild) and T36 (severe) served as positive controls. Western blot analyses were performed with midrib or lamina samples according to Benscher et al. (2), except that the disruption buffer of Laemmli (12) was used. Double stranded RNA (dsRNA) extractions were performed according to Dodds et al. (5). Samples were analyzed by electrophoresis using 6% polyacrylamide gels and visualized by staining with ethidium bromide or silver nitrate.

Molecular Characterization. The CTV CPGs were reverse transcribed, amplified by polymerase chain reaction (PCR), cloned and sequenced essentially as described by Cevik et al. (3) and Pappu et al. (19), using either fresh or desiccated samples. Sequence analyses were performed by computer, using the Seqaid II (21), Clustal V (10), and GCG Pileup (4) sequence analysis programs.

RESULTS AND DISCUSSION

Leaves were taken from a total of 626 trees. They were kept either as separate samples or combined into composites, for a total of 434 samples. Polyclonal antibodies indicated that 387 of 434 (89.2%) of the samples tested from Colombia were infected with CTV (Table 1). MCA13 reacted with 247 of 385 samples (64.2%) (Table 1), suggesting that many of the Colombian samples were infected with severe isolates of CTV. The epitope determinant for MCA13 is located in a phenylalanine residue at amino acid position 124 of the CP (19), rather than tyrosine, as in most mild isolates. However, it is important to indicate that nucleotide

TABLE 1
SEROLOGICAL DETECTION OF CTV IN CITRUS SAMPLES FROM COLOMBIA USING POLYCLONAL ANTIBODIES (PCA) OR MONOCLONAL ANTIBODY 13 (MCA13)

	Antibody			
	PCA	%[z]	MCA13	%[z]
All Colombian samples	387/434[y]	89.2	247/385[y]	64.2
Mompox samples alone	79/120[y]	66.0	0/120[y]	0
Without Mompox samples	308/314[y]	98.1	247/265[y]	93.2

[z]Percent of samples testing positive by ELISA
[y]Numerator is the number of samples testing positive and denominator is the total number of samples tested.

and predicted amino acid sequences of the CP and CPG (15) show that some mild CTV isolates react to MCA13. Although there are exceptions, MCA13 is still a good indicator of CTV severity. Interestingly, the data from Mompox alone showed that 66% of the 120 samples reacted with the polyclonal antibody, but none reacted with MCA13 (Table 1), suggesting that none of the samples from Mompox were infected with severe CTV isolates. When the Mompox samples are not included, then 247 of 265 (93.2%) samples from the other regions of Colombia reacted with MCA13 (Table 1), suggesting an extremely high incidence of severe isolates of CTV in all other regions of Colombia. However, it must be noted that our surveys were not totally random, hence, the incidence may be somewhat overestimated.

Western blots, performed on 30 selected samples with both the polyclonal antibody and MCA13, confirmed the presence of the CTV CP and its two degradation products (8). No reactions were observed with uninfected control samples. Analysis of dsRNA from 132 infected trees revealed at least 35 different dsRNA profiles similar to those described by Moreno et al. (14) (data not shown). Samples reacting with MCA13 consistently yielded 4 to 5 times more dsRNA than MCA13 negative samples. A 0.5×10^6 MW subgenomic dsRNA, previously found to be associated with severe isolates (5), also was absent in all the samples from Mompox.

Of the isolates established at the ECPC, 14 of 16 reacted with MCA13. Eight selected isolates were bioassayed on the standard indicator hosts (7). Six of them (B125, B128, B129, B131, B228 and B229) caused all or most of the following symptoms: obvious vein clearing on Mexican lime; a decline of sweet orange grafted on sour orange rootstock; stem pitting on grapefruit or sweet orange; and a seedling yellows reaction on grapefruit or sour orange. All six reacted with MCA13. Isolates B272 and B274 caused no visible reactions on any of the indicator plants and did not react with MCA13. Their presence in host plants was confirmed by polyclonal antibodies and PCR amplification of their CPGs.

For molecular analyses, we selected four of the biocharacterized isolates, B128, B129, B272 and B274. Isolate B128 was obtained from a severely stem pitted grapefruit tree near Cali, and its severe nature was confirmed in the biocharacterization. Isolate B129 was obtained from a fast growing, symptomless branch of the B128-infected tree and was expected to be mild. However, it also reacted with MCA13 and induced similar symptoms as B128 in the biocharacterization. Isolates B272 and B274, both from Mompox, were selected as mild isolates because they caused no

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T36     MDDETKKLKNKNKETKEGDDVVAAESSFSSVNLHIDPTLITMNDVRQ LS
T30     -------------------E--------G------------------ --
B272    -------------------N--------G------------------ --
B274    -------------------N--------G------------------ --
B128    --------------A-------------G-L-F-------A------ --
B185    ----------------------------G-L---------A------ --
B165    ----------------------------GPL---------A------ -G
B249    ----------------------------G-L---------A------ -G
B129    ----------------------------G-L---------A------R-G
        **************.****.********...*.*******.****** *.

T36     TQQNAALNRDLFLTLKGKHPNLPDKDKDFRIAMMLYRLAVKSSSLQSDDD
T30     ------------A----Y----------H--------------------
B272    ------------A----Y----------H--------------------
B274    ------------A----Y----------H--------------------
B128    ------------------Y---S------HL-------------------
B185    ------------------Y---S------HL-------------------
B165    ------------------Y---S------H--------------------
B249    ------------------Y----------H--------------------
B129    S-----------------Y----------H--------------------
        .************.**** ***.******..********************

T36     ATGITYTREGVEVDLSDKLWTDVVFNSKGIGNRTNALRVWGRTNDALYLA
T30     T---------------------I-Y-------------------------
B272    T-----------------------Y-------------------------
B274    T---------------------I-Y-------------------------
B128    T--V-----------------------------------------S-------
B185    T--------------------------------------------S-------
B165    T--------------------------------------------S-------
B249    T--V---------E-----------------------------------------
B129    T--V---------E-----------------------------------------
        .**.*********.********.*.*****************.*******

T36     FCRQNRNLSYGGRPLDAGIPAGYHYLCADFLTGAGLTDLECAVYIQAKEQ
T30     ------------------------------------------------
B272    ----------AR----------------------------------------
B274    ------------------------------------------------
B128    ------------------------------------------------
B185    ------------------------------------------------V-----
B165    ------------------------------------------------
B249    ------------------------------------------------L-----
B129    ------------------------------------------------L-----
        **********. ********************************.*****

T36     LLKKRGADDVVVTNVRQLGKFNTR
T30     --------E---------------
B272    --------E---------------
B274    --------E---------------
B128    --------EI--------------
B185    --------E---------------
B165    --------E---------------
B249    --------E---------------
B129    --------E---------------
        ********..**************
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Fig. 2. Multiple alignment of deduced amino acid sequences of CTV isolates B128, B129, B272 and B274 from Colombia and previously characterized isolates (6, 13, 19) with T36 from Florida. The alignment was produced using Clustal V. The asterisks and dashes indicate identical amino acids. The shaded letters and dots indicates substitution with different amino acids.

symptoms on the trees from which they were isolated or the plants used for biocharacterization. Two to three clones of each isolate were sequenced for comparison. When compared to standard CTV strain T36, the CPG nucleic acid sequences of the four Colombian isolates ranged from 90.9 to 92.9% similar (data not shown), and the deduced amino acid sequences ranged from 93.7 to 96.0% similar (Fig. 2), which is consistent with previous results (19). Interestingly, the three clones sequenced of the CPG of B129 all contained one additional codon, resulting in the insertion of an additional amino acid at position 48 in its deduced amino acid sequence. This is unique in the many CPG clones that we have sequenced. To enable more representative results, this codon and amino acid were not included in the sequence comparisons.

In comparison of the deduced amino acid sequences of the Colombian with T36 and other known isolates of CTV (Fig. 3), isolate B128 most closely resembled B165 and B185, both stem pitting isolates from India (13) and Japan (19), respectively. The CP sequence of isolate B128 differs by five and six amino acids from isolates B185 and B165, respectively (Fig. 2). Isolate B129 most closely resembled B249, a stem pitting isolate from Venezuela (6), from which it differs by 1 amino acid (Fig. 2). Thus the CP sequences of both B128 and B129 place them in a large group of stem pitting isolates (13, 19), which is consistent with the field observations for B128 and the biocharacterization results for both B128 and B129. The presence of the MCA13 epitope determinant in the CP sequences of both B128 and B129 (Fig. 2) confirmed their reactivity with MCA13. It is interesting to note that B128 and B129, although derived from the same tree, show more differences between them (10 amino acids) than with the other stem pitting isolates mentioned above. This is a good example of the variability that occurs within populations of CTV.

Isolates B272 and B274 resemble a group of known mild isolates, T4, T26 and T30 from Florida (19), and B32 from Spain (19) (Fig. 3), consistent with both the field observations and the biocharacterization results. The CP sequences of B274 and B272 differ by only one and four amino acids, respectively, from T30 (Fig. 2). The presence of tyrosine rather than phenylalanine at amino acid position 124 of their CP sequences corroborated the MCA13 non-reactivity of B272 and B274.

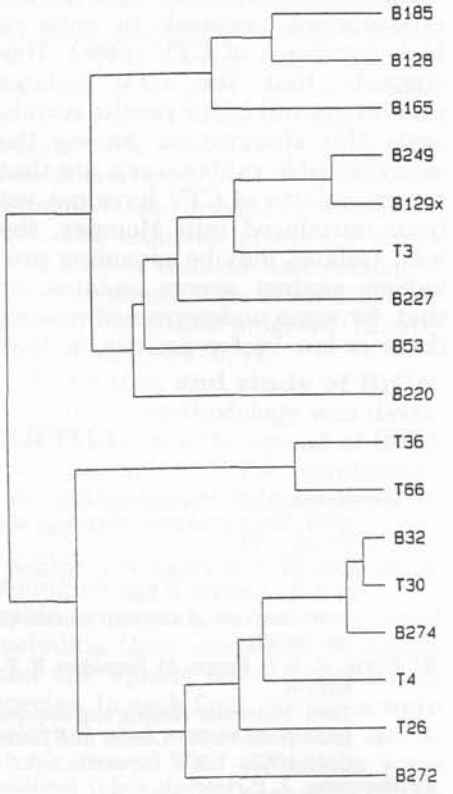

Fig. 3. A dendrogram showing the relationships of the deduced amino acid sequences of CPs of various CTV isolates. The countries of origin are indicated in the text. B129x is the B129 sequence minus the additional amino acid.

Field observations and serological evidence indicated that CTV was widespread and occurred in a very high percentage of citrus trees in Colombia. The obvious vein clearing in the leaves of limes and the pitting of stems and branches of grapefruits, limes and oranges, as well as the inability to use sour orange as a rootstock in most of Colombia suggested that severe isolates of CTV were widespread. Further evidence of this is the high percentage of samples reacting with MCA13 and by the severe reactions observed in indicator plants and in molecular characterization tests.

The situation in Mompox is especially interesting because the trees are large and productive, and sour orange continues to be used as the predominant rootstock in spite of high incidence of CTV (66%). This suggests that the CTV isolates present are mild. Our results corroborate this observation. Among the many possible explanations are that severe isolates of CTV have not yet been introduced into Mompox, the mild isolates may be providing protection against severe isolates, or that, for some undetermined reason, there is low vector activity in that region. Mompox represents an interesting research opportunity. It may provide information on the dynamics of the citrus tristeza disease in the field or be a potential source of mild isolates useful for the control of CTV in Colombia and other countries.

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LITERATURE CITED

1. Bar-Joseph, M. R. Marcus, and R. F. Lee
   1989. The continuous challenge of citrus tristeza virus control. Ann. Rev. Phytopathol. 27: 291-316.
2. Benscher, D., S. S. Pappu, C. L. Niblett, F. V. de Agudelo, F. Morales, E. Hodson, E. Alvarez, O. Acosta, and R. F. Lee
   1996. A strain of soybean mosaic virus infecting *Passiflora* spp. in Colombia. Plant Dis. 80: 258-262.
3. Cevik, B., S. S. Pappu, D. Benscher, R. F. Lee, S. M. Garnsey, S. H. Futch, P. Rucks, and C. L. Niblett
   1996. Molecular cloning and sequencing of coat protein genes of citrus tristeza virus isolated from Meyer Lemon and Homely Tangor trees in Florida, p. 47-53. *In*: Proc. 13th Conf. IOCV., IOCV, Riverside, CA.
4. Deveraux, J., P. Haerbeli, and O. Smithies
   1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Research 12: 387-395.
5. Dodds, J. A., R. J. Jordan, C. N. Roistacher, and T. Jarupat
   1987. Diversity of citrus tristeza virus isolates indicated by dsRNA analysis. Intervirology 27: 177-188.
6. Febres, V. J.
   1995. Molecular characterization of citrus tristeza virus genes and their use for plant transformation. Ph.D. Thesis. University of Florida, Gainesville, FL. 105 pp.

7. Garnsey, S. M., D. J. Gumpf, C. N. Roistacher, E. L. Civerolo, R. F. Lee, R. K. Yokomi, and M. Bar-Joseph
1987. Toward a standardized evaluation of the biological properties of citrus tristeza virus. Phytophylactica 29: 151-157.
8. Guerri, J., P. Moreno, and R. F. Lee
1990. Characterization of strains of citrus tristeza virus by peptide maps of virion coat protein. Phytopathology 80: 692-698.
9. Guzman Barney, M., K. L. Manjunath, V. J. Febres, S. S. Pappu, H. R. Pappu, R. F. Lee and C. L. Niblett
1994. Characterization of coat protein genes of citrus tristeza virus strains from Colombia. Phytopathology 83: 1403.
10. Higgins, D. G., A. G. Bleasby, and R. Fuchs
1992. Clustal V: Improved software for multiple sequence alignment. Comp. Appl. Biosci. 8: 189-191.
11. Karasev, A. V., V. P. Boyko, S. Gowda, O. V. Nikolaeva, M. E. Hilf, E. V. Koonin, C. L. Niblett, K. Cline, D. J. Gumpf, R. F. Lee, S. M. Garnsey, D. J. Lewandowski, and W. O. Dawson
1995. Complete sequence of the citrus tristeza virus RNA genome. Virology 208: 511-520.
12. Laemmli, U. K.
1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.
13. Manjunath, K. L., H. R. Pappu, R. F. Lee, C. L. Niblett, and E. L. Civerolo
1993. Studies on the coat protein gene of four isolates of citrus tristeza closterovirus from India: cloning, sequencing and expression, p. 20-27. *In*: Proc. 12th Conf. IOCV., IOCV, Riverside, CA.
14. Moreno, P., J. Guerri, J. F. Ballester-Olmos, and M. E. Martinez
1991. Segregation of citrus tristeza virus strains evidenced by double stranded RNA (dsRNA) analysis, p. 20-24. *In*: Proc. 11th Conf. IOCV., IOCV, Riverside, CA.
15. Mawassi, M., R. Gafny, and M. Bar-Joseph
1993. Nucleotide sequence of the coat protein gene of citrus tristeza virus: comparison of biologically diverse isolates collected in Israel. Virus Genes 7: 265-275.
16. Niblett, C. L.
1988. Incidence and severity of citrus tristeza virus in Colombia. (Abstr.) Phytopathology 78: 878.
17. Niblett, C. L., H. R. Pappu, S. S. Pappu, V. J. Febres, K. L. Manjunath, R. F. Lee, J. W. Grosser, and J. Schell
1993. Progress on the characterization and control of citrus tristeza virus. Proc. Fla. State. Hort. Soc. 106: 99-102.
18. Pappu, H. R., S. S. Pappu, K. L. Manjunath, R. F. Lee, and C. L. Niblett
1993. Molecular characterization of a structural epitope that is largely conserved among severe isolates of a plant virus. Proc. Natl. Acad. Sci. USA. 90: 3641-3644.
19. Pappu, H. R., S. S. Pappu, C. L. Niblett, R. F. Lee, and E. Civerolo
1993. Comparative sequence analysis of the coat proteins of biologically distinct citrus tristeza virus isolates. Virus Genes 7: 255-264.
20. Permar, T., S. M. Garnsey, D. J. Gumpf, and R. F. Lee
1990. A monoclonal antibody that discriminates strains of citrus tristeza virus. Phytopathology 80: 224-228.
21. Rhoads, D. D. and D. S. Roufa
1985. Emetine resistance of Chinese hamster cells: structure of wild type and mutant ribosomal protein S14 messenger RNA species. Mol. Cell Biol. 5: 1655-1659.
22. Rocha-Peña, M., R. F. Lee, R. Lastra, C. L. Niblett, F. Ochoa-Corona, S. M. Garnsey, and R. K. Yokomi
1995. Citrus tristeza virus and its aphid vector *Toxoptera citricida*: Serious threats to citrus production in the Caribbean countries and Central and North America. Plant Disease 79: 437-445.
23. Yokomi, R. K., R. Lastra, M. B. Stoetzel, V. D. Damsteegt, R. F. Lee, S. M. Garnsey, T. R. Gottwald, M. A. Rocha-Peña, and C. L. Niblett
1994. Establishment of the brown citrus aphid (Homoptera: Aphididae) in Central America and the Caribbean Basin and transmission of citrus tristeza virus. J. Econ. Entomol. 87: 1078-1085.