The Effects of Different Hosts and Natural Disease Pressure on Molecular Profiles of Mild Isolates of Citrus Tristeza Virus (CTV)

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ABSTRACT. All citrus trees propagated in South Africa are inoculated with a mild isolate of citrus tristeza virus (CTV) called GFMS-12. DsRNA analysis or reaction with 11 monoclonal antibodies showed only minor differences between GFMS-12-inoculated grapefruit plants maintained in the screenhouse and those exposed to natural infection in the field for 10 years. Serological differences were not detected between GFMS-12 inoculated in Marsh or Star Ruby grapefruit, Gillemberg navel, and Owari satsuma, whereas the dsRNA pattern in Star Ruby differed from that in other hosts. By a library of cDNA probes, changes in isolate composition in these four hosts were detected. Several probes that did not react with GFMS-12 or GFMS-35-infected plants kept in the screenhouse, reacted with the severe isolate GFSS-1 and with extracts from 10-yr-old field trees inoculated with GFMS-12 or GFMS-35 prior to planting. The hybridization signal with trees bearing small-sized fruits was usually stronger than that from those trees bearing normal-sized fruits. These results suggest that field trees were naturally infected by some new CTV strain(s) and that the concentration (or type) of the new strain(s) was different in trees bearing normal or small-sized fruits.

Keywords. cDNA probes, cross protection, CTV strains, double-stranded RNA analysis, ELISA, molecular hybridization, monoclonal antibodies.

Citrus tristeza closterovirus (CTV) and its aphid vector *Toxoptera citricida* (Kirkaldy) are endemic in South Africa. Stem pitting decline of grapefruit has been a major factor limiting productivity of this crop (11), but the existence of an orchard in the Western Cape containing some Marsh grapefruit trees planted in the 1920's that still produced excellent fruit despite being infected with CTV (9) raised the possibility of using these naturally occurring mild isolates to protect grapefruit trees. Two isolates GFMS-12 and GFMS-35 and several other promising isolates found during selection of budwood sources for the improvement program (24), were evaluated for mildness initially in the greenhouse (22) and then in field trees (21). Grapefruit plants inoculated with isolate GFMS-12 outperformed the others, and all citrus cultivar budwood sources (except lime) (21), rendered virus-free by shoot-tip grafting and used in the certification program, are being preinoculated with GFMS-12 (25).

The dramatic effect different environments have on CTV isolates in South Africa (5, 10) and elsewhere (4, 16) raised questions regarding the cross protecting potential of GFMS-12 and another mild isolate (GFMS-35) in grapefruit in the field. A variable performance of mild isolates has also been observed in different citrus cultivars. Both facts suggested that mild CTV isolates might contain a mixture of strains and that a different balance of strains could be established depending on the host and/or the environmental conditions.

Reaction with monoclonal antibodies (MAb) (3, 15), dsRNA analysis (6, 7, 14) and molecular hybridization with cDNA probes (1, 17) have been used to differentiate CTV isolates. An investigation was, therefore, conducted to determine by these procedures whether host and natural disease pressure could alter the strain composition of mild CTV isolates.

MATERIALS AND METHODS

Isolates and hosts. Isolate GFMS-12 was graft-inoculated on healthy Owari satsuma, Gillemberg navel, and Marsh and Star Ruby grapefruit plants, and these were kept in an insect-proof screenhouse until sampling.

GFMS-12- and GFMS-35-inoculated grapefruit plants were used in a cross protection trial after van Vuuren et al. (21). As a control, some plants had been inoculated with a severe isolate (GFSS-1). Ten years after planting some of the trees were bearing small fruits whereas others had no obvious decline symptoms and bore large fruits.

ELISA. Samples were analyzed by double antibody sandwich indirect (DASI) ELISA according to the procedure previously described (2). Microplates were coated with polyclonal antibody 879 (kindly provided by S. M. Garnsey, Hort. Res. Lab., USDA-ARS, Orlando, Florida, USA) and the following MAbs were tested as the second antibody: 3DF1, 3CA5 and 3BH6 (23), MCA 13 (15), (a gift from S. M. Garnsey), 4H6H and 3E6 (20) (a gift from H. J. Su, National Taiwan University, Taipei, Republic of China), 6 C_7F_9 and $3C_1F_{10}$ (a gift from L. Batista, Sanidad Vegetal, La Habana, Cuba) and 4F3, 4E5 and 4B1 (26) (a gift from M. Zebzami, Unité de Contrôle des Plants, DDR, Dar es Salam, Morocco).

DsRNA and hybridization analysis. Vigorous shoots were sampled from test plants, and the bark was peeled off, trimmed, freeze-dried and shipped to the laboratory at Instituto Valenciano de Investigaciones Agrarias, (IVIA), Moncada, Valencia, Spain. DsRNA was extracted with phenol-detergent, purified by CF-11 cellulose column chromatography, concentrated by ethanol precipitation, and analyzed by polyacrylamide gel electrophoresis (PAGE) as previously described (14).

For hybridization with cDNA probes, 2 µl of the dsRNA extract (containing 2 to 4 µg/ml dsRNA) were denatured with an equal volume of formamide at 90°C for 2 min, cooled on ice and immediately spotted on a PVDF membrane (Immobilon N, Millipore Corporation, Bedford, Ma). The membrane was air-dried, baked for 1 h at 80°C under vacuum, prehybridized in 6 × SSC (20 × SSC = 3 M NaCl, 0.3M sodium citrate, pH 7), 5 × Denhardt solution, 1% SDS and 100 mg/ml salmon sperm ssDNA (18), at 60°C for 3 h., and hybridized with cDNA probes (5 to 10 × 10^5 cpm/ml) in the prehybridization solution at 55°C for 16 h. The probes were ^{32}P-labeled using the Random Prime Labeling kit (Promega Inc., Madison, WI, USA). The hybridized membranes were washed three times (15 min each) in 2 × SSC containing 1% SDS at 25°C, three times in 0.1 × SSC containing 5% SDS at 55°C, and two times in 2 × SSC at 25°C, and then autoradiographed using X-Omat S film (Kodak-Pathé, France) in a cassette with X-Omatic amplifying screen (Kodak).

The cDNA probes used had between 70 and 300 bp and were selected from a library obtained from the severe CTV isolate B-2 from La Reunion (France) (1, 19).

RESULTS

The greenhouse and field isolates assayed reacted with most MAbs and only minor differences in reactivity were observed between GFMS-12 extracted from different hosts.

The dsRNA pattern obtained from Star Ruby grapefruit was different from those obtained from the other hosts (Fig. 1). The alterations suffered by isolate GFMS-12 in different hosts were confirmed in the hybridization experiment with cDNA probes (Table 1 and Fig. 2). None of the probes assayed reacted with all the extracts and one of them (R-8, data not shown) did not react

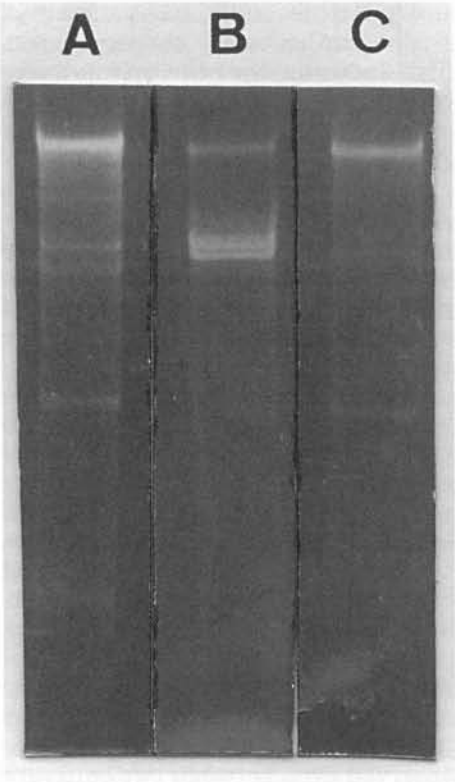

Fig. 1. DsRNA pattern obtained from (A) Gillemberg navel orange; (B) Star Ruby; (C) Marsh grapefruit inoculated with CTV isolate GFMS-12 and maintained in the screenhouse. Extracts from grapefruit plants preinoculated with GFMS-12 or GFMS-35, and then exposed for 10 years to natural disease pressure also showed dsRNA pattern C.

with any of them. The extracts from each host could be discriminated from the others by their hybridization profile (i.e: Owari Satsuma was the only source that did not react with probe T-22b; extracts from Marsh and Star Ruby grapefruit failed to react with R6; and these two sources could be differentiated by their reaction with probe I-95).

Variations in dsRNA pattern were not observed when grapefruit plants preinoculated with mild isolates GFMS-12 or GFMS-35 were exposed to natural disease pressure in the field for 10 years but their hybridization pattern did show significant alterations (Table 2 and Fig. 3). Some probes that did not react with the mild isolates kept in the screenhouse reacted with extracts from the field trees. For example, R-6 and R-8 did not hybridize with GFMS-12, but reacted with some field trees preinoculated with this isolate prior to field exposure. Similarly, R-6, I-54 or I-95, did not react with GFMS-35 in the screenhouse, but reacted with some field trees preinoculated with this isolate. In most cases, the hybridization signal obtained with plants bearing small fruits was more intense than that obtained with similar trees bearing large fruits. Only the probe T-22B reacted more strongly with GFMS-12 kept in the screenhouse than with field trees preinoculated with this isolate. Probes R-6 and T-22A reacted strongly with GFSS-1, R-8 reacted moderately, and probes T-

TABLE 1
DIFFERENTIAL HYBRIDIZATION OF CDNA PROBES WITH DSRNA EXTRACTS FROM OWARI SATSUMA (OS), GILLEMBERG NAVEL (GN), MARSH GRAPEFRUIT (MS) AND STAR RUBY GRAPEFRUIT (SRG) PLANTS INOCULATED WITH THE CTV ISOLATE GFMS-12.

GMS-12 Sub-isolate	cDNA probes				
	T-22B	T-22A	I-95	I-54	R-6
OS	-	+/-	-	++	++
GN	++	++	++	-	++
MGF	++	+/-	++	+	-
SRG	++	-	+/-	+/-	-

The intensity of the hybridization signal was evaluated as: strong (+++), medium (++), mild (+), and (-) no reaction.

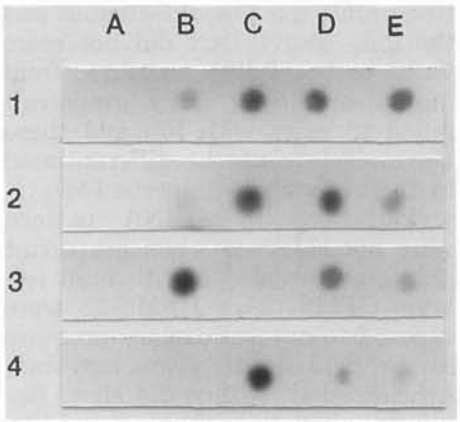

Fig. 2. Hybridization of the CTV cDNA probes, T-22B (line 1), I-95 (line 2), I-54 (line 3) and T-22A (line 4), with dsRNA extracts obtained from B. Owari satsuma, C. Gillemberg navel, D. Marsh grapefruit, and E. Star Ruby grapefruit plants inoculated with the CTV isolate GFMS-12. A. dsRNA from CTV-free plants. The hybridization was conducted as indicated in Material and Methods.

22B and I-54 gave a weak and a negative reaction, respectively.

DISCUSSION

The South African isolates assayed reacted with most MAbs used in this study independent of their pathogenic characteristics. This indicates that the epitopes recognized by these MAbs may be widespread in CTV-infected South African trees. Since CTV and *T. citricida* have been endemic in South Africa for many years, the multiple opportunities for mixed infections with different virus strains have probably caused a population of serotypes more homogeneous than in other citrus areas (3).

Variations in the dsRNA profile were observed when the mild isolate GFMS-12 was inoculated to several citrus hosts. Particularly striking were the differences observed between the profiles obtained from the grapefruit cultivars Marsh and Star Ruby. Host-dependent alterations of the dsRNA profiles were previously observed when CTV isolates were inoculated in different citrus species (8, 12, 13, 14). In the present study, the variations were observed even between two cultivars of the same species.

These variations in dsRNA profile have been attributed to the presence of several strains within some CTV isolates. It has been suggested

TABLE 2
DIFFERENTIAL HYBRIDIZATION OF CDNA PROBES WITH DSRNA EXTRACTED FROM MARSH GRAPEFRUIT PLANTS INOCULATED WITH CTV ISOLATES GFMS-12 OR GFMS-35 AND KEPT IN THE SCREENHOUSE[z], OR EXPOSED TO NATURAL CTV SUPERINFECTION IN THE FIELD FOR 10 YEARS

	cDNA Probes[y]					
CTV Isolate	T-22B	T-22A	I-95	I-54	R-6	R-8
GFMS-12[z]	++	+/-	++	+	-	-
GFMS-12[x]	+/-	+	++	+/-	+	+/-
GFMS-12[w]	+/-	+	++	+/-	+++	++
GFMS-35[z]	-	N	-	-	-	N
GFMS-35[x]	+	++	+	+/-	+++	+
GFMS-35[w]	+	+++	+++	++	+++	+++
GFSS-1[v]	+/-	+++	N	-	+++	++

[z]Plants inoculated with the mild isolate and kept in the screenhouse.
[y]The intensity of the hybridization signal was evaluated as: strong (+++); medium (++); mild (+); and (-) no reaction. N: Not done
[x]Trees bearing large-size fruit after exposure for 10 years to natural field challenge.
[w]Trees bearing small-size fruit after exposure for 10 years to natural field challenge.
[v]Plants inoculated with severe isolate.

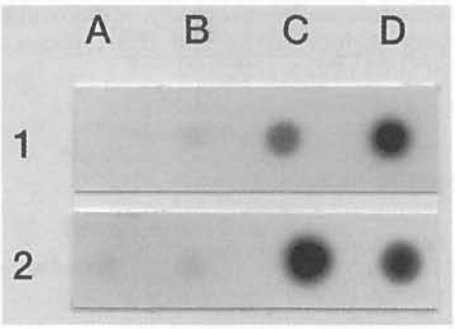

Fig. 3. Hybridization of the CTV cDNA probe R-6 with dsRNA extracts obtained from grapefruit plants. A-1 and A-2 were healthy plants; B1, C1 and D1 were inoculated with the mild CTV isolate GFMS-12, and B2, C2 and D2 with the mild isolate GFMS-35; B. Plants kept in the screenhouse. C and D. Plants preinoculated with these mild isolates and exposed for 10 years to natural disease pressure. C. Plants yielding large fruits. D. Plants yielding small fruits.

that some of the components of this mixture would be preferentially multiplied by certain citrus species whereas other species would favor multiplication of different components (12, 13). Hybridization results with cDNA probes from different parts of the genome seems to support this hypothesis. With the exception of probe R-8, that did not react with any extract, the reaction pattern of the other five probes was different. Failure of dsRNA extracts from a certain host to react with some probes would indicate absence of detectable concentrations of the homologous sequence in this host, while this sequence is present in other hosts. From this standpoint, Gillemberg navel appears to be the less selective host, as it only excluded the component homologous to probe I-54. The strain selection effected by different host species on CTV isolates may explain, in part, the host specificity observed in most mild isolates used in cross protection.

When mild isolates GFMS-12 and GFMS-35 were exposed to natural disease pressure, their hybridization pattern with several cDNA probes were altered. In one case, the field plants stopped reacting with a probe (T-22B) which recognized the mild isolate. This suggests reduced concentration of the homologous sequence due to a new balance of strains. In other cases extracts from field plants hybridized with some probes that were unable to recognize the original mild isolate kept in the screenhouse. The new sequences detected in the field plants might belong to new CTV strains introduced by natural superinfection, or they might correspond to increased replication of some of the old components when new strains were introduced into the trees. It was interesting that reaction with extracts from plants bearing small fruits was usually stronger than from plants bearing large fruits. In most cases, the reaction pattern of the small fruited trees resembled that of the severe isolate GFSS-1 used as control. Association between decline symptoms and intensity of the hybridization signal suggests that the new sequences detected belong to some severe strain(s) introduced by aphids in the preinoculated plants and their high concentration in the small-fruited trees is indicative of a cross-protection breakdown.

Hybridization with a selected panel of cDNA probes can be a sensitive and useful procedure for quick selection of the mild isolates used to preinoculate budwood sources and to monitor cross protection. Under greenhouse or screenhouse conditions the hybridization pattern can be host-dependent and in the open field it can also depend on the CTV isolates predominant in the area where the cross protection experiment is to be performed.

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