Strain Prevalence of *Citrus tristeza virus* Cross-Protecting Isolates Altered by Red Grapefruit Hosts

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ABSTRACT. In the South African citrus industry, pre-immunization of grapefruit with mild isolates is essential to reduce the effect of the endemic *Citrus tristeza virus* (CTV) on this sensitive species. Several strains of the virus occur in trees in the field and each of the two isolates used for pre-immunization, which were collected from commercial trees, contain several strains. Isolate GFMS 12 includes a severe strain while those in GFMS 35 are all mild. The influence of four red grapefruit selections (Henderson, Rio Red, Flame and Star Ruby) on the prevalence of genomic RNA variants of these two isolates, and a known severe isolate (GFSS 5), was investigated under greenhouse conditions. Individual transfers of each isolate from their greenhouse sources were made to the four grapefruit selections and two sub-transfers were made from each of these plants. Biological indexing, ELISA and single strand conformation polymorphism (SSCP) of the p27B (p27 gene fragment) and p25 CTV genes, were performed. SSCP band profiles from the four selections revealed segregation and a selective multiplication of strains within the different selections. Rio Red only supported the master sequence variant of both GFMS 12 and GFMS 35. Minor differences occurred with GFMS 12 and GFMS 35 inoculated into Star Ruby trees. No differences occurred in GFMS 35 propagated in Flame and Henderson. However, major differences were found when GFMS 12 was propagated in these two hosts. These findings were supported by the biological indexing data.

Many strains of *Citrus tristeza virus* (CTV) exist in southern Africa and they usually occur as mixed populations in citrus plants due to continuous introductions by the aphid vector *Toxoptera citricida* (Kirkaldy) (13). Although sweet oranges are also affected, grapefruit and lime are the most sensitive to CTV, and in countries where the disease is endemic, cross-protection by mild strains is the only practical solution to reduce the effect of the disease (12).

Grapefruit production forms an important part of the southern African citrus industry and comprises approximately 20% of citrus exports. Marsh and Star Ruby grapefruit selections are by far the most popular of the 16 grapefruit selections in the southern African Citrus Improvement Program (CIP). In the CIP, three major cross-protecting isolates are currently applied to the main citrus cultivars. Two isolates, GFMS 12 and GFMS 35 are applied to grapefruit. Each of these isolates is composed of more than one strain (9, 20). Passage of CTV isolates from one host to another can cause changes in the genomic RNA population composition within a host (8, 10, 11, 16). With passage, hosts can select a strain and permanently or temporarily change its prevalence in the viral population. This can lead to differences in disease expression. Moreno et al. (10) showed that, with complex isolates, strain separation could readily occur if the plant is not homogeneously invaded. One study concluded that it takes at least four years for a CTV isolate to stabilize in the host (3). Fluctuations in environmental conditions also can trigger changes in the populations in complex cross-protecting and non-protecting CTV isolates (2, 4).

Single-strand conformational polymorphism (SSCP), as described by Orita et al. (14), has proved to be useful in identifying differences in gene sequences of CTV strains. This technique has been successfully used to discriminate between genomic variants in CTV isolates using various parts of the CTV genome (6, 15, 16, 17, 18). Band intensity of cDNA on a SSCP gel is proportional to the viral RNA represented in an isolate mixture (3, 16). This technique is useful in characterizing the main genetic variant of the CTV isolate (8, 16).

Variable disease expressions under different environmental conditions and in different grapefruit selections have raised questions about the cross-protection ability of GFMS 12. The stability of this isolate as well as that of GFMS 35 was investigated in different red grapefruit selections.

MATERIALS AND METHODS

Isolates. The original isolates used for inoculations, GFMS 12, GFMS 35 and GFSS 5 (a known severe isolate), were maintained in Marsh grapefruit under aphid-free conditions in a greenhouse.

Plants. Four red grapefruit selections were used for host passage: Star Ruby, Rio Red, Flame and Henderson. Five plants of each cultivar on Swingle citrumelo rootstock were pre-immunized in the scion, using two buds per plant. After 12 mo, positive transmissions were confirmed by double antibody sandwich enzyme-linked immunosorbent assay (ELISA) using polyclonal antiserum. Readings three times that of the negative control were regarded as positive. Three plants of each selection, with variable ELISA readings (highest, intermediate, lowest), were selected. From these source plants (1, 2, 3) for each cultivar/isolate combination, two sub-source plants (A and B) were established on Troyer citrange rootstock using buds taken from different branches on the same plant. All plants were kept in an aphid-free greenhouse at a temperature regime of 24-28°C. The composition of CTV genomic RNAs in these plants was analyzed by SSCP 19 mo later.

Biological indexing. Virus-free Mexican lime and Duncan grapefruit seedlings were inoculated with buds from the source plants (1, 2, 3) and the sub-source plants (A and B) for each grapefruit selection and replicated three times. The trial was laid out according to a randomized block design in a greenhouse at a 20-24°C temperature regime. One shoot was allowed to develop per plant. Growth was measured and stem-pitting assessed 6 mo after inoculation and repeated 6 mo later. Hereafter, plants were moved to 28-32°C for another 6 mo and growth and stem-pitting assessed again. Stem pits were counted at 6× magnification using a stereo microscope. The area on which pits were counted was calculated, and the number of pits per square centimeter determined. According to these counts, isolates and sub-isolates were rated as mild (less that 20 pits per cm^2), moderate (20-50 pits per cm^2) and severe (more than 50 pits cm^2) (19).

Nucleic acid purification. Four grams of bark and leaf midrib tissue sampled from the sub-source plants were pulverized in liquid nitrogen. Double stranded RNA was isolated by CF-11 cellulose chromatography as described (5) with minor modifications (9).

Reverse transcription polymerase chain reaction (RT-PCR). Oligonucleotides corresponding to the 3' and 5' ends of genes p25 and p27B were synthesized (MWG Biotech, Munich, Germany]) based on the sequence of CTV isolate T36 (Florida) (6, 7). These genes were selected since they showed a high rate of sequence variation in a previous study (9). Two microliters of the dsRNA preparation were mixed with primers in 8 µl dH_2O, dena-

tured for 5 min at 100°C, chilled for 5 min on ice and annealed at room temperature for 30 min. RT-PCR was performed in 25 µl reactions using a one-tube RT-PCR reaction kit (Titan One Tube RT-PCR System, Roche Molecular Biochemicals, Germany). Reactions were done as recommended by the manufacturer with final concentrations of 0.4 µM of each primer, 0.2 mM of each of the four nucleotides, 5 mM DTT and 1.5 mM $MgCl_2$. Reverse transcription and amplification was done in a thermal cycler (Eppendorf Mastercycler Personal), with the following conditions: a reverse transcription step at 50°C for 30 min, followed by one cycle of denaturation at 94°C for 2 min, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 40 s at 50°C, and extension for 1 min at 68°C, with a final extension cycle of 5 min at 68°C. Resulting DNA products were examined in a 1% agarose gel stained with ethidium bromide.

Single-strand conformation polymorphism (SSCP) analysis. SSCP was performed directly on the total RT-PCR products for the different genes. A modified procedure as described by Yap and McGee (21) was followed: One microliter of RT-PCR product was mixed with 9 µl dH_2O and 1 µl 10× denaturing solution (500 mM NaOH, 10 mM EDTA pH 8.0). The mixture was heated for 10 min at 42°C and 10× loading dye added (0.5% xylene-cyanol [w/v] and 0.5% bromophenol blue [w/v] in deionized formamide). DNA strand separation was by electrophoresis in a 6% non-denaturing polyacrylamide minigel (80 mm × 70 mm × 1.50 mm), without glycerol. The gel was run in 0.5 × TBE (44.5 mM Tris-Borate, 1 mM EDTA, pH 8.0) buffer for 1.75 h, at 300 V at 8°C. For products amplified with primers for p27B, a 9% polyacrylamide minigel was used at 200 V for 3h. Gels were stained with silver nitrate as described by Beidler et al. (1) and examined on a light box.

RESULTS

ELISA. ELISA readings of the sub-source plants after 6 mo varied and bore no relation to those of the source plants, indicating that the virus is patchily distributed in the trees (data not shown). Overall, in Star Ruby, Rio Red and Henderson, GFMS 12 had the lowest titer, GFMS 35 was significantly higher and GFSS 5 was slightly higher again. For Flame, GFMS 12 and GFMS 35 had a similar titer and GFSS 5 was much higher.

Biological indexing. On Mexican lime at 20-24°C, significant variation in stem pitting counts occurred between sources [1, 2, 3] and within a plant on different branches (sub-sources A and B). A summary of the classification of stem-pitting according to visual counts of the second 6-mo period is shown in Table 1. Overall, counts were lower in the second 6-mo period, possibly because of an initial shock reaction of the seedlings to the virus. In the third 6-mo period at 28-32°C, no stem-pitting occurred with the mild isolates possibly due to suppression of CTV titers by the higher temperatures.

Mild isolates caused very few pits on Duncan grapefruit. Although counts were lower, results were similar to those on Mexican lime.

For GFMS 12, great variation in stem-pitting counts occurred not only among the three source plants, but also within a plant (sub-sources). This was more evident for the Flame and Henderson selections. Growth lengths were not as good of a criterion as stem pitting counts to distinguish between isolates. However, growth lengths did correlate positively with the stem pitting data (data not shown).

For GFMS 35 in all three sources, the stem pitting counts for the first and second 6-mo periods grouped within the mild category. A slight variation was observed within certain sources of Star Ruby,

TABLE 1
STEM-PITTING RATINGS OF MEXICAN LIME SEEDLINGS INOCULATED WITH GFMS 12 AND GFMS 35 (SOURCE PLANTS 1, 2, AND 3) AND FROM SUB-SOURCE PLANTS A AND B OF GRAPEFRUIT VARIETIES

	Stem pitting (pits/cm²)*					
	Mild		Moderate		Severe	
Selection	Source	Sub-source	Source	Sub-source	Source	Sub-source
			GFMS 12			
Rio Red			1	1A		1B
			2	2A, 2B		
		3B	3	3A		
Star Ruby			1	1A, 1B		
	2	2A		2B		
			3	3A, 3B		
Henderson	1	1A, 1B				
	2	2A		2B		
					3	3A, 3B
Flame					1	1A, 1B
			2	2A		2B
				3A	3	3B
			GFMS 35			
Rio Red	1	1A, 1B				
	2	2A, 2B				
	3	3A, 3B				
Star Ruby	1	1A, 1B				
	2	2A		2B		
	3	3B		3A		
Henderson	1	1A, 1B				
	2	2A,		2B		
	3	3A, 3B				
Flame	1	1A		1B		
	2	2A, 2B				
	3	3A, 3B				

*Mild = less than 20 pits per cm². Moderate = 20 to 50 pits per cm². Severe = more than 50 pits per cm².

Henderson and Flame after the first 6 mo, but not after the second 6 mo at the low temperature. Growth lengths did not always correlate with stem pitting results (data not shown).

For GFSS 5, all indicators inoculated with GFSS 5 showed severe stem pitting, therefore, no counts were made. Overall, growth of Mexican lime was not significantly different among or within sources except with Rio Red where significant differences occurred between sub-sources of sources 1 and 3 (6.6 and 9.2 cm respectively). At 28-32°C, growth was more vigorous because of the suppression of CTV and significant differences occurred between sources of Rio Red and Star Ruby as well as between their sub-sources. However it was still not possible to count the numerous stem pits.

SSCP analysis. In all instances, fragments of the correct size for a given gene were obtained by RT-PCR.

GFMS 12: SSCP patterns of p25

Star Ruby. No differences from the original were detected among sources and sub-sources (Fig. 1, lane 5).

Rio Red. No differences were detected between products amplified from the original sources and the sub-sources (Fig. 1, lanes 2, 3, 4).

Flame. Sources from this variety displayed patterns different from the original. Bands from sources

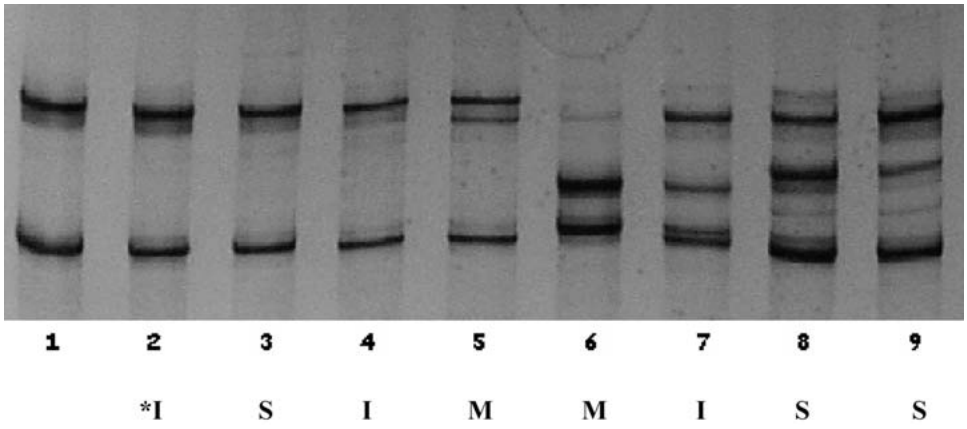

Fig. 1. SSCP profiles of gene p25 of GFMS 12 in four grapefruit selections showing the effect of the selection on strain prevalence. Lane 1: Original isolate in Marsh. Lanes 2-4: Rio Red, sources 1, 2, 3. Lane 5: Star Ruby. Lanes 6-8: Henderson, source 2 and sub-sources 2A an 2B. Lane 9: Flame. *Biological indexing in Mexican lime: M = mild, I = intermediate, S = severe.

and sub-sources were similar (Fig. 1, lane 9).

Henderson. Source 2 differed from the original and its sub-sources differed from the source as well as from each other (Fig. 1, lanes 6, 7, 8).

GFMS 12: SSCP patterns of p27B

Star Ruby. The SSCP patterns from the source plants did not differ from the pattern from the original Marsh grapefruit. Both sub-sources A and B of source plant 2 had an additional band (sub-source A of source plant 2: Fig. 2, lane 3).

Rio Red. The source plants did not differ from the original. Sub-sources A of both, sources 1 and 2, had each an additional band (sub-source A of source plant 1, Fig. 2, lane 2).

Flame. Bands of the source plants differed from the original. Sub-sources did not differ except for A and B from source 2 (Fig. 2, lanes 7, 8). B had an additional band similar to the other sub-sources.

Henderson. All three the source plants differed from the original (Fig. 2, lanes 4, 5, 6). Bands of the sub-sources corresponded with their sources.

GFMS 35

Similar to GFMS 12, no differences occurred when GFMS 35 in Star Ruby was analyzed using gene p25 and it was decided not to analyze the other selections using this gene (data not shown). Gene 27B showed an additional band in Star Ruby sub-source plant B of source plant 2. There were no differences from the original plant in any of the other three grapefruit selections with 27B.

GFSS 5

Only gene p27B was analyzed for this severe isolate. This isolate contains numerous genomic RNA variants and differences were difficult to observe (data not shown). Generally there were no differences between sources of each grapefruit selection. Only with source plant number 3 of Flame, did both sub-source plants lack some bands, suggesting suppression or total filtering out of some strains. This difference was confirmed biologically by a slightly more vigorous growth of the Mexican lime and Duncan indicator plants at the higher temperature.

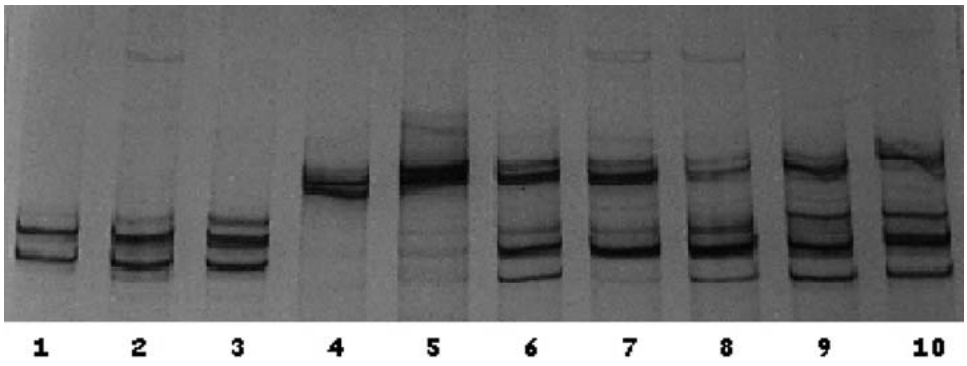

Fig. 2. SSCP profiles of gene p27B of GFMS 12 in four grapefruit selections showing the effect of the selection on strain prevalence. Lane 1: Original in Marsh. Lane 2: Rio Red, sub-source A of source 1. Lane 3: Star Ruby, sub-source A of source 2. Lanes 4-6: Henderson, sources 1, 2, 3. Lanes 7-8: Flame, sub-sources A and B of source 2. Lanes 9-10: Flame, sources 1, 3.

DISCUSSION

Single-strand conformation polymorphism (SSCP) profiles have been used successfully in the past (3, 16), to identify genomic RNA changes in CTV populations. Sambade et al. (17) observed a trend of mild isolates generally having a SSCP profile with a single sequence variant, whereas the severe isolates usually show more complex patterns. Data in this study supported this (Figs. 1 and 2). GFSS 5, a known severe isolate had complex SSCP profiles. The erratic presence or absence (suppression) of GFMS 12 genomic RNA variants within a grapefruit selection was reflected by the biological data. GFMS 12 in Flame had complex SSCP profiles and this isolate grouped into the moderate and severe stem pitting categories upon biological indexing. Simple SSCP bands in Henderson correlated with mild biological results while complex SSCP profiles indexed severe. There is enough evidence to conclude that the GFMS 12 isolate can change within a plant of a given selection, especially in the cases of Flame and Henderson. Groupings of individual plants into the severe category for GFMS 12 may be because of the severe strain within this isolate (20) becoming dominant. The results also showed that segregation within a single plant can occur, since differences between A and B locations (different branches on the same plant) were found biologically as well as with SSCPs. ELISA results also suggest that the selection has an influence on the replication of the virus in the plant, since titers varied significantly in the case of GFMS 12.

Passaging was simple, from one plant to another, of the same selection. As the source plants were only 18 mo old, it may be that strains of the GFMS 12 isolate were not fully stabilized when buds were cut for the sub-source plants as suggested by d'Urso et al. (3). SSCP was performed on the sub-source plants at 19 mo, and subsequent SSCP analyses should be done to confirm the stability of populations. The fact remains that different sequence variants in the GFMS 12 isolate seemed to be supported by different host selections. In South Africa pre-immunized plants are tested regularly by ELISA after application of the protecting CTV isolate and are distributed for multiplication to the industry as soon as the presence of CTV is detected. These pre-immunized plants are seldom older than 12 mo. This period presents the opportunity for a shift in strain

prevalence different from that of the original source plant.

GFMS 35 and GFSS 5 were much more stable within a plant, as revealed by SSCP, biological indexing and ELISA results. In the case of GFMS 35, slight differences in severity occurred with Star Ruby, Henderson and Flame upon indexing, but none fell into the severe category, confirming the results that GFMS 35 isolate does not contain any severe strains (9).

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