Diversity Among Sub-Isolates of Cross-Protecting Citrus Tristeza Virus Isolates in South Africa

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ABSTRACT. Citrus tristeza virus (CTV) disease is endemic in Southern Africa and its principle natural vector is the aphid, *Toxoptera citricida*. Aphid transmission occurs in a semi-persistent manner. The effect of the disease is minimized by deliberate infection of virus-free citrus cultivars with approved mild CTV isolates before release to the industry. Presently, three CTV isolates have been approved as pre-immunizing agents on several hundred cultivars and selections. These isolates were derived from field trees, and it is suspected that each of them is constituted of different strains. Each strain may have different effects depending on host and environment. This creates problems in South Africa due to the large variety of cultivars as well as the variation in climatic conditions of the citrus producing areas of the country. Single aphid transmissions of CTV were performed from two cross-protecting isolates, GFMS 12, without the seedling yellows (SY) component, and LMS 6, with the SY component, in attempts to identify different strains in each isolate. The percentage positive transmissions were 8% for GFMS 12 and 16% for LMS 6. Evaluation of the sub-isolates on two CTV sensitive hosts (Mexican lime and Marsh grapefruit) revealed that some of them were significantly milder or more severe than the original isolate regarding growth and stem pitting development. Significant differences occurred between sub-isolates as well. The SY component was present in 38% of the LMS 6 sub-isolates. Sub-isolates were also differentiated by their restriction fragment length polymorphism (RFLP) patterns and single strand conformation polymorphism (SSCP) of the coat protein gene. It is possible that some sub-isolates contain more than one CTV strain. The results confirm the presence of different CTV strains in the pre-immunizing isolates.

Citrus tristeza virus (CTV) causes stem pitting and decline diseases of citrus and is one of the most important pathogens world-wide (2). The disease is endemic in Southern Africa due to the occurrence of the most efficient insect vector, the brown citrus aphid, *Toxoptera citricida*. In South Africa many strains of CTV exist, and they usually occur as mixtures in a host (11). The use of decline tolerant rootstock/scion combinations has limited economical losses. However, citrus cultivars such as grapefruit and lime do not produce satisfactorily in the presence of CTV even if propagated on resistant rootstocks. To reduce the effects of the virus on these sensitive cultivars, cross protection by mild CTV isolates is used (14, 16). The development of severe stem pitting on grapefruit trees propagated from pre-immunized budwood indicated a breakdown of protection or a segregation of strains that may be present in the pre-immunizing isolates (8). When severe strains occur within an isolate, serious repercussions may be encountered in different climatic conditions, since the severe strains within the pre-immunizing isolate may become dominant (5).

In this study, we attempted to discriminate between strains that may occur within two pre-immunizing isolates in South Africa by subculturing using single aphid transmissions. The sub-isolates were evaluated for symptom expression on sensitive biological hosts and by molecular biology techniques.

MATERIALS AND METHODS

Virus isolates. Two CTV isolates, GFMS 12 and LMS 6, which are approved as pre-immunizing isolates in the Southern Africa Citrus Improvement Program were sub-cultured by single aphid transmissions. GFMS 12 was derived from grapefruit and contains only

the stem pitting component of CTV while LMS 6 which was derived from lime, contains stem pitting as well as a mild form of SY. The isolates were maintained on Mexican lime plants in an insect-free screenhouse at 24° to 28°C. The plants were pruned lightly to force new growth which is favored by the aphids prior to conducting vector tests. The aphid-transmitted isolates are referred to as sub-isolates.

Aphid transmissions. Apterous brown citrus aphids were collected in a Eureka lemon orchard and transferred to virus-free actively growing Mexican lime plants in an insect cage in the laboratory at 20° to 24°C. The aphids were transferred three times, with 24 h intervals, to new virus-free plants in order to obtain non-viruliferous insects.

In the transmission studies, groups of 50 to 80 aphids were transferred to each of the two Mexican lime plants containing the CTV isolates for a virus acquisition period of 24 h. Aphids were then placed singly on virus-free Mexican lime plants. A total of 111 aphids were transferred from GFMS 12 and 50 from LMS 6. After an inoculation access period of 24 h, the aphids were killed by spraying with a suitable insecticide. Plants were then kept in a screenhouse at 20° to 24ºC. Transmission efficiency was determined as the ratio between the number of infected plants and the total number of inoculated plants.

Enzyme-linked immunosorbent assay (ELISA). CTV infection from aphid transmissions was determined after 3 mo by double antibody sandwich ELISA using polyclonal antiserum (1). Reactions were read on a Titertek ELISA plate reader at 405 nm. A positive reaction was defined as an optical density reading of more than three times that of the negative control.

Biological indexing. Mexican lime which is sensitive to stem pitting and Marsh grapefruit which is sensitive to SY were used to assess the severity of the sub-isolates. Three seedlings of each of the two indicators were inoculated by budding and then maintained in a screenhouse at 24° to 28ºC. Plants were trained to develop a single shoot. After 6 mo the grapefruit plants were inspected for seedling yellows symptoms, the growth of both indicators was measured and the bark peeled to assess stem pitting. Stem pits were counted under a stereo microscope at 6x magnification and calculated as number of pits per cm^2.

Molecular characterization. Four grams of bark and leaf midrib tissue of Mexican lime plants was frozen in liquid nitrogen and pulverized to powder with a mortar and pestle. Double-stranded (ds) RNA was isolated using the CF-11 cellulose chromatography procedure (10). The coat protein (CP) gene was amplified in a one-tube reverse transcription polymerase chain reaction (RT-PCR) in a thermal cycler (6). The resulting amplified products were analyzed on 4% agarose gels after digestion with the restriction enzyme *Hin*f 1 to obtain restriction fragment length polymorphism (RFLP) patterns (12). Single-strand conformation polymorphism (SSCP) analysis was performed directly on the denatured PCR products of the CP gene of the different CTV sub-isolates (13).

RESULTS

Aphid transmissions. The transmission efficiency for CTV isolates GFMS 12 and LMS 6 was 8% and 16% respectively.

ELISA. OD_{405} readings of some sub-isolates differed significantly from that of the original isolate, some higher and some lower. Results of the two hosts did not complement each other, and it appears that the host may favor specific iso-

lates since an isolate may have a high reading in Mexican lime and a low reading in Marsh grapefruit (Tables 1 and 2).

Biocharacterization. *GFMS 12 sub-isolates.* In the lime host, sub-isolates GFMS 12-2 and 12-5 had significantly less virulent reactions (12-2, growth and stem pitting; 12-5, stem pitting) than the original isolate while GFMS 12-3 had significantly more stem pitting. No differences occurred between sub-isolates and the original isolate in the growth of the grapefruit host. However, stem pitting of the grapefruit was significantly less in plants inoculated with sub-isolates 12-2, 12-5 and 12-8, while plants with sub-isolate 12-3 were significantly more severely pitted than the original isolate (Table 1).

LMS 6 sub-isolates. Two sub-isolates of LMS 6, 6-1 and 6-7 induced less pitting in Mexican lime plants. None of the sub-isolates was more virulent than the original isolate. With the grapefruit plants two sub-isolates differed from each other regarding growth, but they did not differ from the original isolate. None of the sub-isolates induced any pitting in the grapefruit host. The SY component of CTV in the LMS 6 isolate was transmitted to 38% of the sub-isolates (Table 2).

Molecular characterization: *GFMS 12 sub-isolates.* RT-PCR of the CP genes was successfully performed on dsRNA extracted from all the plants infected with GFMS 12 and nine sub-isolates. When amplified CP genes were digested with *Hin*f 1, and separated by electrophoresis on a 4% agarose gel, five bands were visible which range in length from 80 to 400 bp (Fig. 1). According to the number and size of the bands when measured, the sub-isolates could be placed into three categories. One sub-isolate (12-7) gave bands similar to the original isolate while all the others differed. These sub-isolates contained one or two bands which were absent in the original. A simplified presentation of the bands is given in Table 1.

The SSCP technique revealed no differences between the CP genes of the original isolate and the sub-isolates (Fig. 2; Table 1).

LMS 6 sub-isolates. RT-PCR of the CP genes was successfully performed on dsRNA prepared from all the plants infected with LMS 6 and eight sub-isolates. When amplified CP genes were digested with *Hin*f 1, and separated by electrophoresis on a 4% agarose gel, five bands were visible ranging in length from 75 to 500 bp (Fig. 1). According to the number and size of the bands, the sub-isolates could be placed into five categories. One sub-isolate (6-6) gave bands similar to the original isolate while all the others differed. Three sub-isolates, 6-1, 6-4 and 6-7, contained bands which were absent in the original isolate. A simplified presentation is given in Table 2.

The SSCP technique revealed differences between the CP genes of the original isolate and the sub-isolates (Fig. 2; Table 2).

DISCUSSION

Two sub-isolates of GFMS 12 (12-2 and 12-5) were less virulent in the Mexican lime and grapefruit hosts. Molecular analysis showed that they are different and may still be mixtures of strains. It was disturbing to find a sub-isolate (12-3) which was more virulent than the original isolate. Growth of both hosts was not affected but the isolate induced severe stem pitting. It is possible that the severe strain in this isolate may become dominant under specific environmental conditions and may cause severe stem pitting resulting in decline and small fruit (5). This may be the reason for the severe stem pitting reported by Marais et al. (8). The molecular characterization of GFMS 12 was not well defined by the RFLP and

TABLE 1
THE EFFECT OF SUB-ISOLATES OF GFMS 12 ON THE GROWTH, STEM PITTING AND ELISA TITER READINGS IN MEXICAN LIME AND MARSH GRAPEFRUIT AND A COMPARISON OF THEIR MOLECULAR CHARACTERISTICS[z]

	Host response						Molecular characterization[y]	
	Mexican lime			Marsh grapefruit				
Isolate or sub-isolate	Growth (mm)	Pitting (pits/cm^2)	ELISA (OD_{405})	Growth (mm)	Pitting (pits/cm^2)	ELISA (OD_{405})	RFLP	SSCP
GFMS 12	432 b	26.4 bcd	1.098 cd	478 NS	20.1 b	1.298 ab	- - 3 4 5	1 2 - - -
12-1	398 b	36.2 de	1.213 b	512	7.6 ab	1.370 a	- - - 4 5	1 2 - - -
12-2	693 a	4.8 a	1.118 bcd	428	0.1 a	1.389 a	- 2 - 4 5	1 2 - -
12-3	393 b	51.7 e	1.190 bcd	408	57.1 c	1.173 c	1 - - 4 5	1 2 - - -
12-4	550 ab	17.6 abc	1.215 b	408	4.2 ab	1.291 ab	- 2 - 4 5	1 2 - - -
12-5	500 ab	5.1 a	1.197 bc	513	0.0 a	1.331 a	1 - - 4 5	1 2 - - -
12-6	500 ab	31.6 cd	1.342 a	487	4.2 ab	1.292 ab	- 2 - 4 5	1 2 - - -
12-7	530 ab	8.8 ab	1.088 d	493	18.1 b	1.395 a	- - 3 4 5	1 2 - - -
12-8	567 ab	17.3 abc	1.101 cd	453	0.4 a	1.226 bc	- 2 - 4 5	1 2 - - -
12-9	458 b	10.2 ab	0.851 d	462	5.4 ab	1.357 a	- 2 - 4 5	1 2 - - -

[z]Figures in each column followed by the same letter do not differ significantly at the 5% level (LSD). NS = not significant.
[y]Similar RFLP (Fig. 1) and SSCP (Fig. 2) bands for each isolate are indicated by the same number. - = no product.

TABLE 2
THE EFFECT OF SUB-ISOLATES OF LMS 6 ON THE GROWTH, STEM PITTING AND ELISA TITER READINGS IN MEXICAN LIME AND MARSH GRAPEFRUIT AND A COMPARISON OF THEIR MOLECULAR CHARACTERISTICS[z]

	Host response						Molecular characterization[y]	
	Mexican lime			Marsh grapefruit				
Isolate or sub-isolate	Growth (mm)	Pitting (pits/cm^2)	ELISA (OD_{405})	Growth (mm)	Pitting (pits/cm^2)	ELISA (OD_{405})	RFLP	SSCP
LMS 6	402 NS	22.5 b	1.776 ab	323[x] ab	0 NS	1.135 a	- - 3 4 5 6 7	- 2 3 4 -
6-1	525	0.0 a	1.738 abc	437 ab	0	0.323 d	1 - - - 5 - 7	- - 3 4 -
6-2	448	19.8 b	1.749 abc	373[x] ab	0	0.580 c	- - 3 4 - - 7	- - 3 4 -
6-3	455	12.1 ab	1.822 a	210[x] b	0	0.761 bc	- - 3 4 - 6 7	- 2 3 4 -
6-4	368	19.6 b	1.668 bcd	402 ab	0	0.952 ab	- 2 - 4 - - 7	- - 3 4 -
6-5	603	8.6 ab	1.664 cd	440 ab	0	0.884 b	- - 3 4 - - 7	- - 3 4 -
6-6	502	7.2 ab	1.722 abc	437 ab	0	0.842 b	- - 3 4 5 6 7	1 2 3 4 -
6-7	550	0.1 a	1.579 de	507 a	0	0.320 d	1 - - - 5 - 7	- - 3 4 -
6-8	508	3.9 ab	1.545 e	395[x] ab	0	0.829 b	- - 3 4 - - 7	- - 3 4 5

[z]Figures in each column followed by the same letter do not differ significantly at the 5% level (LSD). NS = not significant.
[y]Similar RFLP (Fig. 1) and SSCP (Fig. 2) bands for each isolate are indicated by the same figure. - = no product.
[x]Positive reaction for seedling yellows component of CTV.

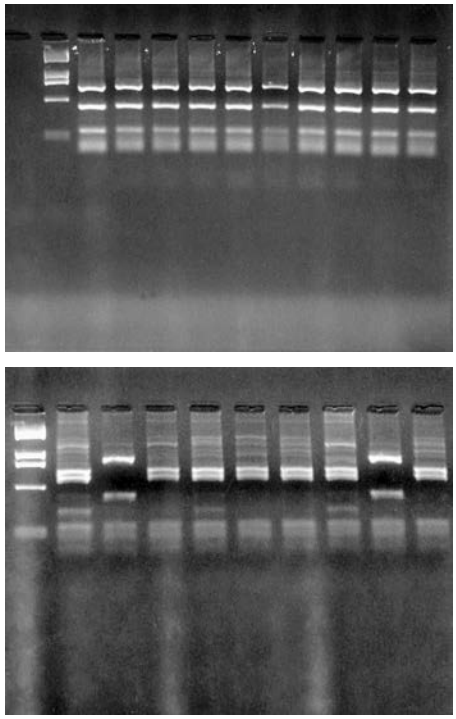

Fig. 1. RFLP patterns of single aphid transmissions of the coat protein genes of GFMS 12 (A) and LMS 6 (B) CTV isolates defined by *Hin*f 1 digestion. Restriction digests were separated on 4% agarose gels. A. GFMS 12: lane 1: pUC19 (*Hin*f 1) marker; lane 2: original; lane 3: 12-1; lane 4: 12-2; lane 5: 12-3; lane 6: 12-4; lane 7: 12-5; lane 8: 12-6; lane 9: 12-7; lane 10: 12-8; lane 11: 12-9. B. LMS 6: lane 1: pUC19 (*Hin*f 1) marker; lane 2: original; lane 3: 6-1; lane 4: 6-2; lane 5: 6-3; lane 6: 6-4; lane 7: 6-5; lane 8: 6-6; lane 9: 6-7; lane 10: 6-8. Marker bands = 1419, 517, 396, 214, 75 and 65 base pairs.

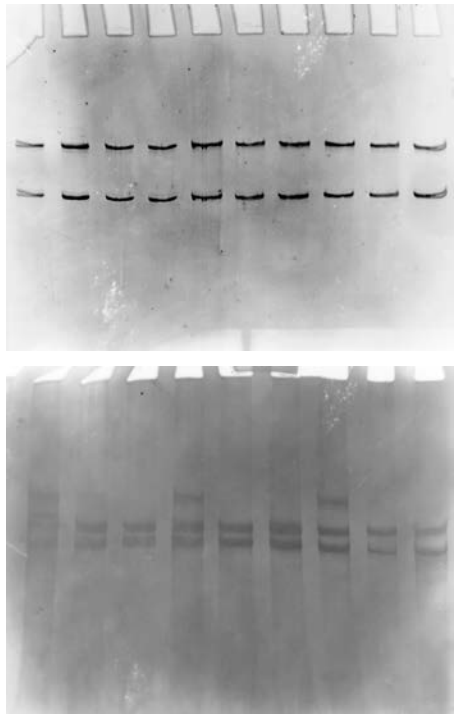

Fig. 2. SSCP patterns of single aphid transmissions of GFMS 12 (A) and LMS 6 (B) of their coat protein genes. A. GFMS 12 = lane 1: original; lane 2: 12-1; lane 3: 12-2; lane 4: 12-3; lane 5: 12-4; lane 6: 12-5; lane 7: 12-6; lane 8: 12-7; lane 9: 12-8; lane 10: 12-9. B. LMS 6 = lane 1: original; lane 2: 6-1; lane 3: 6-2; lane 4: 6-3; lane 5: 6-4; lane 6: 6-5; lane 7: 6-6; lane 8: 6-7; lane 9: 6-8. Electrophoresis was performed under non-denaturing conditions at room temperature at 300 volts for 2h in 8% acrylamide gels with 5% glycerol. Gels were stained with silver nitrate.

SSCP techniques. Digestion of the CP gene by the restriction enzyme *Rsa* 1 as well as the use of the P20 gene did not give better discrimination (data not shown). None of the RFLP profiles of the GFMS 12 sub-isolates correspond with the RFLP groups described by Gillings et al. (6) and therefore it appears that they all still may contain more than one strain. Additional single aphid transmissions will have to be done from the sub-isolates to try to separate individual strains. More sensitive molecular differentiation techniques have to be applied to differentiate between GFMS 12 sub-isolates.

Sub-isolates LMS 6-1 and LMS 6-7 were less virulent on the Mexican lime host than the original isolate. They had similar RFLP profiles and appear to be in the RFLP group 5 of Gillings et al. (6). The other sub-isolates appear to contain mixed strains and further sub- culturing will be necessary. It appears that the LMS 6 isolate does not contain strains that induce severe stem pitting. The SY component of CTV that is present in the original LMS 6 iso-

late was only transmitted to three sub-isolates. They could not be distinguished by RFLP and SSCP techniques when using the CP gene.

The results of this investigation clearly indicate that the South African pre-immunizing CTV isolates GFMS 12 and LMS 6 are constituted of more than one strain. Climatic variation of the citrus producing areas in South Africa (3), together with the host, will contribute to segregation of strains within these isolates (4, 5, 9). When an isolate contains a severe strain, like GFMS 12, the severe strain may become dominant and severe symptoms of CTV may develop at an early stage as reported by Marais et al. (8) on 11-yr-old trees. In contrast, it was reported that trees pre-immunized in Australia with an Australian isolate, which appears to contain only one strain, were producing well at 27 yr (7).

The poor performance of Star Ruby grapefruit that were pre-immunized by GFMS 12 necessitated the introduction of an additional isolate (GFMS 35) for pre-immunization of red grapefruit in South Africa. However, this isolate may also contain severe strains as was indicated by the Nel Ruby grapefruit selection (15). Field evaluations of potential mild protective strains are long-term operations, and it may be beneficial for the interim to eliminate the severe strain from GFMS 12 by recombining sub-isolates that do not carry the severe strain.

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