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ORIGINAL ARTICLE

Genotype composition of populations of grapefruit-cross-protecting citrus tristeza virus strain GFMS12 in different host plants and aphid-transmitted sub-isolates

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Abstract Citrus tristeza virus (CTV) causes severe losses in grapefruit production in South Africa and requires mild-strain cross-protection to maintain production. Unfortunately, cross-protection breakdown of the pre-immunizing CTV grapefruit mild source GFMS12 is prevalent in grapefruit in South Africa. The CTV genotype composition of the GFMS12 population inoculated onto different hosts was determined by sequencing part of ORF1a and the p23 gene of multiple clones from each plant. Analysis of the GFMS12 population in Mexican lime and Marsh and Star Ruby grapefruit varieties revealed that at least four genotypes occur in the GFMS12 population and that genotype compositions differed amongst the populations in different host plants. Single-aphid-transmitted sub-isolates derived from the GFMS12 mother population on Mexican lime appeared to contain three populations of a mixture of VT-like and recombinant B165/VT-like genotypes; a mixture of recombinant RB/VT- and B165/VT-like genotypes; and a single recombinant B165/VT-like genotype. This study underlines the importance of determining the genotype composition of a potential CTV pre-immunizing source on a range of inoculated host species before utilization.

Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers JQ819902 to JG820013.

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Introduction

Citrus tristeza virus (CTV) is an aphid-borne closterovirus that is endemic in South Africa. Stem pitting and decline symptoms of CTV have been major factors limiting productivity of grapefruit locally. Without CTV cross-protection, it has been suggested that grapefruit production would be uneconomical in South Africa [19]. Marsh grapefruit trees planted in the 1920s and infected with CTV but still producing excellent fruit in the 1970s served as mild sources to potentially protect grapefruit trees. These sources, grapefruit mild strain GFMS 12, GFMS 35, and others were evaluated for mildness, initially in the greenhouse [21], and then in field trials [19]. In 1973, the Southern African Citrus Improvement Scheme (SACIS) was initiated, and until 2007, all grapefruit selections were pre-immunized with the GFMS12 source. Star Ruby grapefruit, pre-immunized with the GFMS12 source, however, later showed varying degrees of stem-pitting and variable fruit size in field trials and on trees for budwood production [20, 22]. The heterogeneity of the mother GFMS12 source was demonstrated, and based on biological indexing, a severe CTV strain with severe stem-pitting was found in it [23]. CTV strains within GFMS12 were separated by single *Toxoptera citricida* transmissions and sub-isolates GFMS12-1 to GFMS12-9 were established [23]. Two sub-isolates were shown to be less virulent, while one sub-isolate was more virulent than the original GFMS12

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source [23]. Additionally, the presence of seedling yellows (CTV-SY) symptoms, which were not found in GFMS12 plants maintained in greenhouses but were found in some plants in the field, suggested that at least some plants that were pre-immunized with GFMS12 had become superinfected with additional CTV strains, probably introduced by aphids [18]. Altered single-strand conformational polymorphic (SSCP) patterns that did not correspond to the SSCP profile of the original GFMS12 source supported this [18]. Recent studies [5] have shown that hyperinfection exclusion in CTV is genotype specific, suggesting that cross-protecting sources should contain mild variants of all CTV genotypes circulating within a given area in order to remain effective.

The purpose of this study was to characterise the genotype composition of the greenhouse-maintained GFMS12 mother source population on various citrus hosts as well as that of single-aphid-transmitted sub-isolates derived from it [23] in order to better investigate the role of various CTV genotypes in cross-protection breakdown of this mild source. The genotype composition in each population was determined by sequencing multiple clones (between 22 and 64) of two genomic regions.

Materials and methods

Virus isolates and sub-isolates

The original field-collected GFMS12 isolate was maintained on Marsh grapefruit on rough lemon rootstock in a glasshouse. This source was used to pre-immunize grapefruit plants within the Citrus Improvement Scheme (S.P. van Vuuren, personal communication). The Star Ruby and Marsh grapefruit varieties (*C. paradisi* Macf.) on rough lemon rootstocks and Mexican lime (*Citrus aurantifolia*) (L.) Swingle) plants were inoculated from the original GFMS12 isolate (S.P. van Vuuren, personal communication) and maintained in an insect-free greenhouse in Nelspruit for eight years prior to this study. GFMS12 sub-isolates (12-1; 12-4; 12-5; 12-6; 12-7; 12-8; and 12-9) on Mexican lime were obtained by single-aphid transmissions (*Toxoptera citricida* (Kirkaldy)) from the GFMS12-inoculated Mexican lime plant obtained in a previous study [23]. Sub-isolates were sampled, and their A-region and p23 gene DNA products were sequenced at two different time points: initially when plants were introduced into the glasshouse in Pretoria and then again between 2 and 3 years later.

RNA extraction, cDNA synthesis, and PCR amplification

Bark from different parts of the CTV-infected plant was pulverized with liquid nitrogen, and total RNA was extracted using an SV Total RNA Isolation System (Promega) according to the manufacturer's protocol. A two-step RT-PCR protocol was utilized to detect and amplify CTV RNA. Two pairs of primers targeting the A region (ORF1a) [14] and the p23 gene [15] (Table 1) were used. Reverse transcription was performed according to Herron [9] using either the PM51 or A-R reverse primers (Table 1). PCR reactions of 50 µl contained 10 µl of the primed cDNA and a mixture containing 100 pmoles of each primer (PM50/PM51 or A-F/A-R); 2.5 mM $MgCl_2$; 1x Bioline Buffer; 2.5 U BioTaq (Bioline); 0.14 mM each of dATP, dCTP, dGTP and dTTP; and 0.2 µg/µl BSA. The thermal cycle conditions were as follows: 92 °C for 2 min, 40 cycles of 92 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min, with extension for 10 min at 72 °C. The resulting PCR products were separated by 1 % agarose gel electrophoresis. The PCR products were purified using a Wizard® SV Gel and PCR Clean-Up Kit (Promega) according to the manufacturer's instructions.

Cloning and sequence analysis of GFMS12 sources

Characterization of the genotype composition of GFMS12 populations on Mexican lime, Star Ruby and Marsh grapefruit plants was performed by cloning the PCR product of the A region and the p23 gene from each source and sequencing multiple clones. Cloning was done using the pGEM-T Easy Vector cloning system (Promega) according to the manufacturer's instructions, followed by transformation of *Escherichia coli* JM109 competent cells (Promega). Recombinant colonies were selected by blue/white selection and grown overnight in Luria broth with 100 ug/ml ampicillin (USB). Plasmids were isolated using the alkaline lysis method [16] and screened by PCR using pGEM-T vector-specific primers T7 and Sp6 (Integrated DNA Technologies; IDT) (Table 1), and PCR products were purified [25]. Sequences of cloned plasmids were determined using primers SP6 or T7.

The sequences that were obtained were trimmed using the BioEdit sequence alignment editor (v7.0, Tom Hall, Isis Pharmaceuticals, Inc 1997-2004), and alignments were carried out using the ClustalW alignment software (EBI) incorporated in the BioEdit software [17]. Sequences were aligned with the cognate regions of 18 full-genome reference sequences retrieved from GenBank. Cognate regions from the full-genome sequences of CTV isolates T30 (AF260651) and T36 (U16304) from Florida; T318A (DQ151548) and T385 (Y18420) from Spain; VT from Israel (U56902); SY568 (AF001623) from California; Mexican CTV isolate (DQ272579); NUagA (AB046398) from Japan; B165 (EU076703) and Kpg3 (HM573451) from India; Qaha (AY340974) from Egypt; HA16-5 (GQ454870) and HA18-9 (GQ454869) from Hawaii; and

Table 1 Primers used for the RT-PCR amplification of GFMS 12 sources and sub-isolates

Primer	Sequence (5′-3′)	Region	Position[1]	Product size	Author
A-F forward	ACGTGTTCGTGAAACGCGG	ORF1a	2021-39	528 bp	Rubio *et al.* [14]
A-R Reverse	GTCGATAACTCGACAAACGAGC		2527-48		
PM 50 Forward	ACTAACTTTAATTCGAACA	p23	18347-65	697 bp	Sambade *et al.* [15]
PM51 Reverse	AACTTATTCCGTCCACTTC		19026-44		
T7	TAATACGACTCACTATAGGG	pGEM-T vector	N/A	N/A	Promega pGEM-T Easy vector system (Promega, USA)
SP6	ATTTAGGTGACACTATA GAA				

[1] Nucleotide positions of primers relative to the T36 isolate (GenBank accession U16304)

New Zealand resistance-breaking isolates NZRB-M12 NZRB-G90, NZRB-TH28, NZRB-TH30 and NZRB-M17 (FJ525431-35) were retrieved from GenBank and included in the analysis to provide phylogenetic references. The MEGA 3.1 package was used to perform the phylogenetic and distance analyses [11]. Unrooted dendrograms were constructed after bootstrapping to 1000 replicates by the neighbour-joining (NJ) method using the Jukes-Cantor base substitution model. Dendrograms with bootstrap values of more than 70 % were generally regarded as providing evidence for a phylogenetic grouping. The graphical output for the 50 % majority rule consensus trees was obtained by using MEGA 3 Tree Explorer version 3.1 [11]. Recombination was analyzed using the GENCOV function of RDP-V2 Beta 08.

Direct sequencing without DNA cloning was done on GFMS12 single-aphid-transmission sub-isolates 12-1; 12-4; 12-5; 12-6; 12-7; 12-8 and 12-9 [23] to obtain the consensus sequence of the population in order to select a representative of each potentially isolated genotype. Sequencing was performed a) initially when plants were obtained from Citrus Research International, Nelspruit, and b) repeated after a 2- to 3-year period of maintenance under glasshouse conditions in Pretoria. Direct nucleotide sequence determination was done in both directions using an ABI Prism DNA sequencer 377 (Perkin-Elmer) with A-F, A-R, PM50 and PM51 primers. The DNA was purified by the EDTA/NaOAc/EtOH method [16]. The genotype composition of the population for representative sub-isolates was determined by sequencing multiple clones of the A-region and p23 gene amplicons as described above for the GFMS12 mother sources.

Biological indexing

The pathogenicity of GFMS12 sub-isolates was determined by inoculating the virus source on greenhouse-maintained, container-grown plants of five recognized [7] citrus indicators: Mexican lime (ML); sour orange (SO) (*C. aurantium*); Duncan grapefruit (DG) (*C. paradisi*); Madam Vinous (MV) sweet orange (*C. sinensis*); and sweet orange grafted on sour orange rootstock (SW/SO). Three plants of each indicator/virus source were graft-inoculated with bark pieces from CTV-infected donors (GFMS12 source on Mexican lime; sub-isolates 12-7, 12-8, and 12-9) with uninoculated plants kept as controls. Symptoms were monitored periodically over a six-month period for SO, SW/SO, ML plants while DG and MV plants were monitored for a further four months [7]. Intensity of stem-pitting was scored as follows: 0-20 pits/cm^2 was considered mild; 21-50 pits/cm^2, moderate; and >51 pits/cm^2, severe [23].

Results

The CTV genotypes present within the GFMS12 sources maintained on grapefruit varieties Marsh and Star Ruby and on Mexican lime were identified from sequences of multiple clones of amplicons of the A-region (ORF1a) and the p23 gene and used to prepare dendrograms vs. cognate regions of known CTV genotypes (Figs. 1 and 2). The number of clones of each identified genotype in the GFMS12 sources gives an indication of the genotype composition present (Table 2). From this it is evident that GFMS12 consists of multiple genotypes and that the populations in different plants appear to have differing genotype compositions. Incongruences in genotype composition using the A-region and the p23 gene within the same population were also observed. The genotypes present in the GFMS12 populations in the two grapefruit cultivars showed similar incongruences. Both populations were composed of CTV genotypes with only VT-like p23 genes, and both contained CTV genotypes with B165-like and T30-like A-regions (Table 2). In the GFMS12 population on Star Ruby, an additional genotype with a VT-like A-region was observed (Table 2). No clones containing RB-, HA16-5-, Kpg-3-, or T36-like sequences in the A-region or p23 gene were obtained in the grapefruit cultivars. The relative proportion of cloned plasmids with specific genotype sequences in the two grapefruit

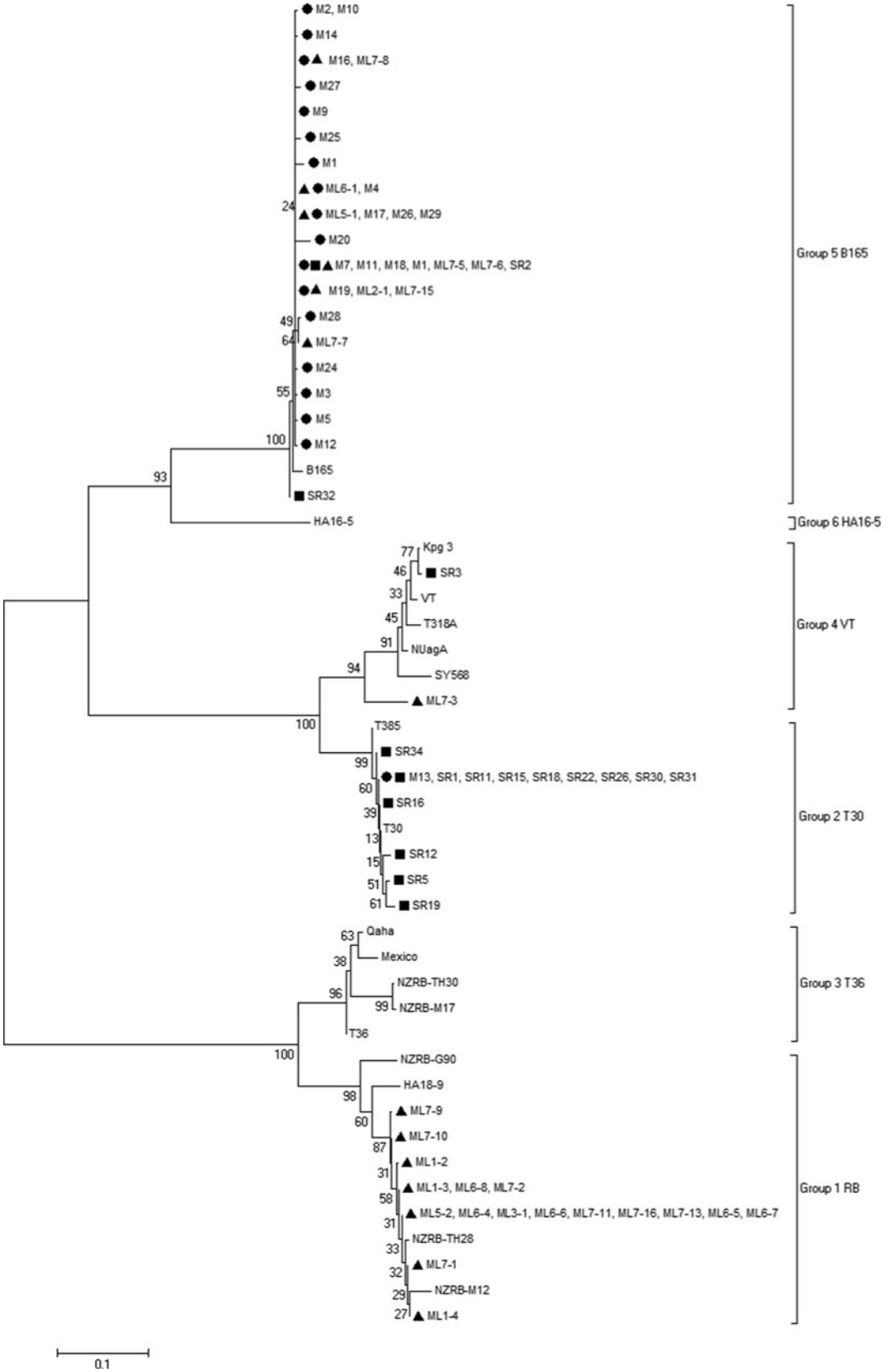

Fig. 1 Neighbour-joining dendrogram depicting phylogenetic relationships between clones of A-region nucleotide sequences of CTV GFMS12 on Marsh (M) (circles), Star Ruby (SR) (squares), Mexican lime (ML) (triangles), and cognate reference CTV sequences. Bootstrap values (1000 replicates) supporting a particular phylogenetic grouping is are shown at the branch points

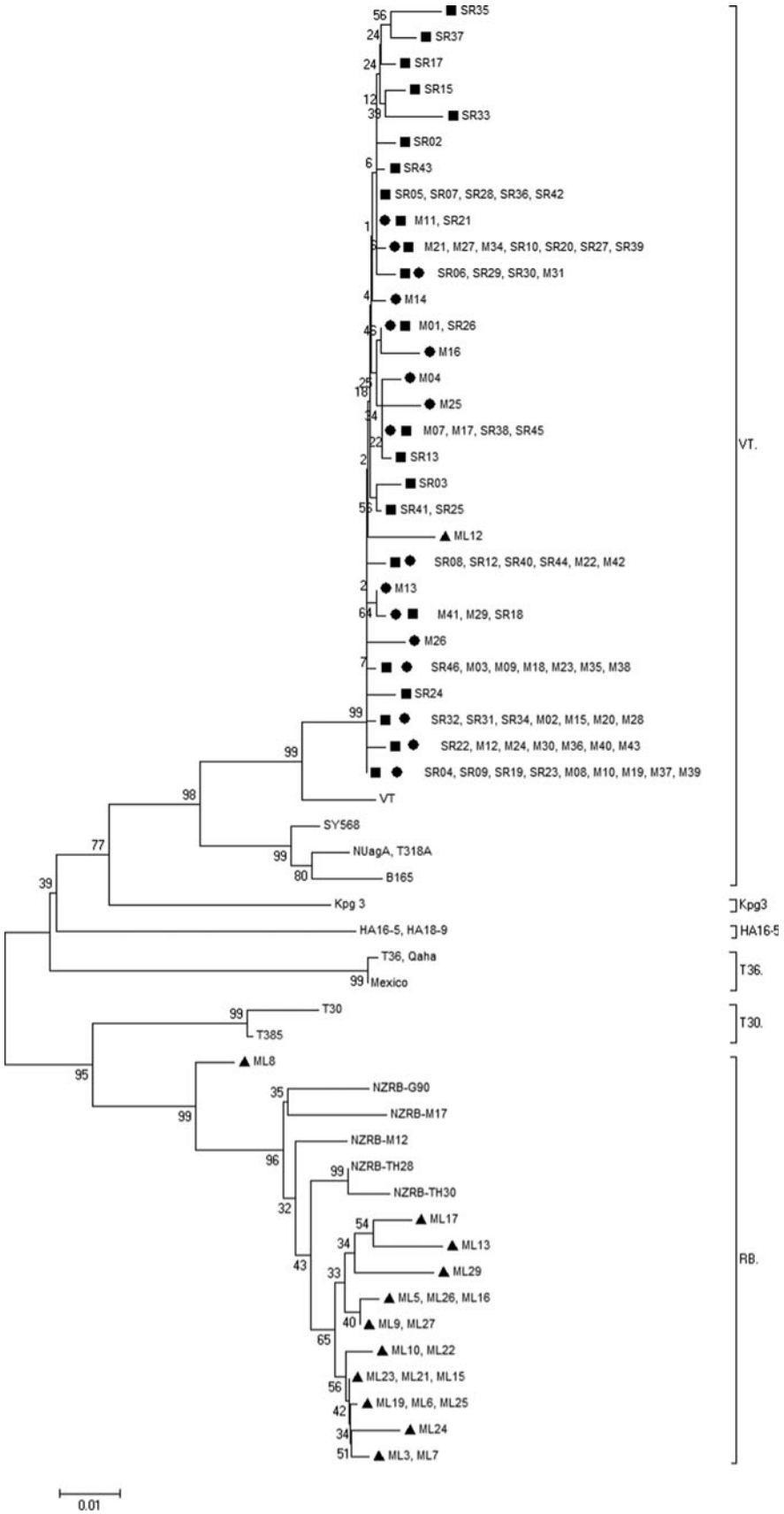

Fig. 2 Neighbour-joining dendrogram depicting phylogenetic relationships between clones of p23 gene nucleotide sequences of CTV GFMS12 on Marsh (M) (circles), Star Ruby (SR) (squares), Mexican lime (ML) (triangles), and cognate reference CTV sequences. Bootstrap values (1000 replicates) supporting a particular phylogenetic grouping are shown at the branch points

Table 2 The population diversity in the A-region (ORF1a) and p23 gene of GFMS 12 sources on Mexican lime, Marsh and Star Ruby grapefruits

Source	Sequence	No. of clones	Percentage CTV genotype						
			B165	HA16-5	Kpg3	RB	T30	T36	VT
GFMS12 Mexican Lime	A-region	27	30*	0	n/a	66.0	0	0	4.0
	p23 gene	22	n/a	0	0	95.0	0	0	5.0
GFMS12 Marsh	A-region	24	96.0	0	n/a	0	4.0	0	0
	p23 gene	40	n/a	0	0	0	0	0	100.0
GFMS12 Star Ruby	A-region	16	13.0	0	n/a	0	81.0	0	6.0
	p23 gene	42	n/a	0	0	0	0	0	100.0

The percentage of cloned sequences from each source that grouped with reference genotypes (B165, HA16-5, Kpg3, RB, T30, T36 and VT) are shown

n/a not applicable, no differentiation of such a genotype based on this region

* Percentage of clones sequenced

Table 3 Intra-group nucleotide diversity values from sequences derived from the A-region (ORF1a) and p23 gene that were estimated for the B165-, HA16-5-, Kpg3-, RB-, T30-, T36- and VT-like genotypes of CTV from each GFMS12 source (Marsh and Star Ruby grapefruits and Mexican lime)

Source	Sequence	CTV genotype						
		B165	HA16-5	Kpg3	RB	T30	T36	VT
GFMS12 Marsh	A-region	0.008 (n = 23)	–	n/a	–	n/c	–	–
GFMS12 Star Ruby	A-region	0.065 (n = 2)	–	n/a	–	0.01 (n = 13)	–	n/c
GFMS12 Mexican Lime	A-region	0.001 (n = 8)	–	n/a	0.06 (n = 17)	–	–	n/c
GFMS12 Marsh	p23 gene	n/a	–	–	–	–	–	0.009 (n = 39)
GFMS12 Star Ruby	p23 gene	n/a	–	–	–	–	–	0.009 (n = 42)
GFMS12 Mexican Lime	p23 gene	n/a	–	–	0.018 (n = 20)	–	–	n/c

The number of sequences (n) used to estimate nucleotide diversity is shown

n/a not applicable, differentiation of genotype based on this region not generally accepted

n/c no comparison possible, single sequence observed

– none such sequences obtained

populations differed, however. Most cloned A-region amplicons had sequences that were B165-like in Marsh while being T30-like in Star Ruby. The Mexican lime population differed considerably from the two grapefruit populations, with most sequenced clones of both the A-region and the p23 gene being RB-like, a genotype sequence not observed in clones of either sequenced region from either of the grapefruit populations. The GFMS12 population in Mexican lime also contained B165- and VT-like genotypes, as was obtained in the two grapefruit populations.

Identical nucleotide sequences within cloned amplicons from different GFMS12 populations were often obtained. These were collapsed into single branches on the dendrogram (branches with multiple symbols in Figs. 1 and 2). Commonly, however, some variation in sequence within any given genotype, even within a single GFMS12 population, was observed. The following example illustrates these two occurrences: eight clones of A-region amplicons from GFMS12 on Star Ruby and one from GFMS12 on Marsh yielded identical sequences clustering within the T30-like group (Fig. 1); however, clones of this sequence, along with five other clones, all from the GFMS12 population in Star Ruby, within the T30 clade had 1 % nucleotide diversity (Table 3). The average intra-genotype nucleotide diversity for the A-region and p23 gene of cloned amplicons from GFMS12 Mexican lime, Marsh and Star Ruby grapefruit populations are shown in Table 3. Higher nucleotide diversity exists amongst strains in the variable A-region than in the more conserved p23 gene. Recombination analysis was performed using the GENCOV function of RDP-V2 Beta 08, with no genotype recombination events being found within any of the A-region sequences of any of the GFMS12 populations. Recombination with an unknown parental sequence was detected in the p23 gene of the RB-like genotype identified within the GFMS12 Mexican lime population (data not presented).

Direct sequencing of the A-region and the p23 gene of single-aphid-transmission sub-isolates of GFMS12 on Mexican lime yielded the population consensus sequence, effectively that of the dominant genotype (Fig. 3). Based on direct sequences of the A region when sources were initially moved from greenhouses in Nelspruit to those in Pretoria, a population consensus of a VT-like dominant genotype was observed in GFMS12 sub-isolates 12-1, 12-4, 12-6, and 12-9; a B165-like genotype in sub-isolate 12-8; and a RB-like genotype in sub-isolates 12-5 and 12-7, while after a 2- to 3-year period in the Pretoria greenhouse, the population consensus sequences shifted to that of a dominant B165-like genotype for all sub-isolates (Fig. 3A). Analysis of the p23 gene of the sub-isolates over the three-year time period suggested that they all contained VT-like dominant genotypes, with the consensus sequence obtained from sub-isolates 12-5 and 12-7 grouping more closely to the T318A and NUagA variants of the VT-genotype, and sub-isolates, 12-1, 12-4, 12-6, 12-8 and 12-9 being more related to the type VT isolate (Fig. 3B).

The combined A-region and p23 gene analyses of the GFMS12 sub-isolates suggest that three populations with apparently different CTV genotypes were present. Sub-isolates 12-1, 12-4, 12-6 and 12-9 consisted of a B165-like and VT-like sequence type in the A-region but a VT-like sequence type in the p23 gene, representing a mixture of a possible recombinant B165/VT-like strain and a VT-like strain. Sub-isolates 12-5 and 12-7 had an RB-like and B165-like sequence type in the A-region and a VT-like sequence in the p23 gene, representing a mixture of possible recombinant RB/VT-like and B165/VT-like strains. Sub-isolate 12-8 had a B165-like sequence type in the A-region but a VT-like sequence type in the p23 gene, representing a single possible recombinant B165/VT-like strain that was maintained over time. Based on the direct sequencing information, sub-isolates 12-7, 12-8 and 12-9 were chosen as representatives of the three populations found. Dendrograms derived from comparison of sequences of 20-30 clones of the A-region and p23 gene of the representative sub-isolates with those of the cognate region of known CTV genotypes are not presented, as these clones all contained sequences that had already been identified by direct sequencing. Based only on the second time point after direct sequencing, sub-isolates 12-7 and 12-8 were B165-like, sub-isolate 12-9 was a mixture of VT-like and B165-like in the A-region, while all of them still had VT-like p23 gene sequences. These three sub-isolates were indexed in triplicate in each of five citrus indicator hosts, and their symptoms were compared to those observed in the GFMS12 pre-immunizing source (Table 4). Sub-isolate 12-7 yielded mild symptoms, whereas symptoms of sub-isolates 12-8, 12-9 and the GFMS12 source were moderate under the experimental conditions used. The GFMS12 source had moderate vein clearing and SP in Mexican lime, and mild stunting and moderate SP in Duncan grapefruit. Sub-isolate 12-7 was milder than the GFMS12 source for Mexican lime and Duncan grapefruit, and in addition displayed decline in SW/SO. Sub-isolate 12-8 (B165-like) did not display any stem-pitting symptoms in grapefruit. Sub-isolate 12-9 produced symptoms on all five indicators with mild SP and stunting occurring. The symptom profile induced by sub-isolates 12-9 and 12-8 differed from that of the GFMS12 source.

Discussion

In a recent study by Folimonova *et al*. [5], it was shown that superinfection exclusion by CTV sources is genotype specific, with a given CTV genotype only providing superinfection exclusion against other sources of the same T36 genotype. These results have direct bearing on the strategy of mild-strain cross-protection of citrus, where it is now evident that mild forms of all CTV genotypes circulating in a given citrus production area are required as pre-immunizing sources to achieve sustainable mild-strain cross-protection. It is therefore critical to fully characterise, at least to the genotype level, the CTV populations used for pre-immunization, as well as to identify and characterise those able to overcome the cross-protecting infections. In this study, we therefore determined the genotype composition of the GFMS12 pre-immunizing source, which, while initially showing great promise as a cross-protection source, fell into disuse due to cross-protection breakdown in the field [22]. We also determined the genotype composition of populations obtained from the mother source by single-aphid transmission to determine their usefulness as pre-immunizing sources.

We have shown that the GFMS12 population contains at least RB-, T30-, VT-, and B165-like genotype sequences in the Mexican lime, Marsh and Star Ruby GFMS12 populations tested. We suspect that all four genotypes are in the GFMS12 population even though the full complement was not found in any of the individual populations, and that the populations in the different plants represent various combinations and differing ratios of the components present in the original GFMS12 population. We conclude this because the possibility of introduction of additional CTV genotypes by aphid transmission was negligible, since populations were maintained in insect-free greenhouses throughout their lifetime. The study therefore also confirms the mixed-"strain" nature of GFMS12 suggested previously by differing dsRNA and SSCP patterns [2, 23]. In all three GFMS12 populations, a predominant genotype with additional minor genotypes was observed, similar to what has been reported previously for CTV populations [10, 12].

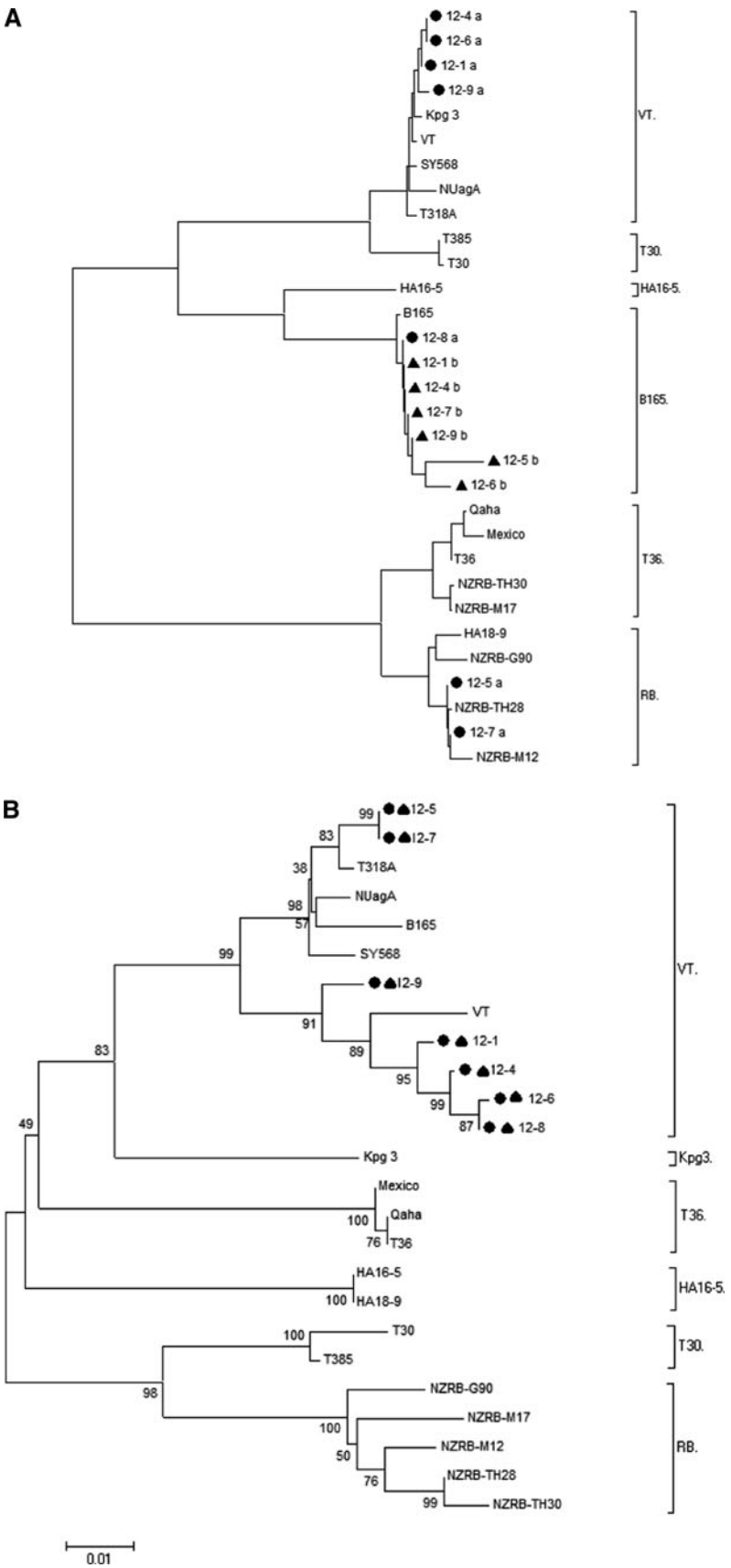

Fig. 3 Neighbour-joining dendrogram of the (**A**) A-region (nt 2021-2548) and (**B**) p23 gene, depicting the phylogenetic relationships of nucleotide sequences of GFMS12 sub-isolates sequenced (a) initially upon introduction in the greenhouse (dots) and then (b) after 2-3 years of maintenance under greenhouse conditions (triangles) and references using the Jukes-Cantor parameter. Bootstrap values (1000 replicates) supporting a particular phylogenetic grouping are shown at the branch points

Table 4 Biological indexing of GFMS12 source and sub-isolates on different host responses

Isolate	Host response[a]							
	Mexican lime (ML)		Madam Vinous (SW)		Sour Orange (SO)	SW/SO	Duncan Grapefruit (DG)	
	Vein clearing	Stem-pitting	Stunting	Stem-pitting	Stunting/Seedling Yellows	Stunting	Stunting	Stem-pitting
Sub-isolate 12-7	+	+	-	-	-/-	+	+	+
Sub-isolate 12-8	++	++	+	+	+/+	+	+	-
Sub-isolate 12-9	++	++	+	-	+/+	+	+	+
GFMS 12	++	++	-	-	-/-	-	+	++

[a] Symptom consistently observed and mild (+), moderate (++), not observed (-)

The GFMS12 population from Mexican lime was composed of 66 % RB-, 30 % B165-, and 4 % VT-like genotypes when the analysis was based on the A-region but was composed of 95 % RB- and 5 % VT-like genotypes when it was based on the p23 gene. Such incongruent relationships have been found in the past for CTV [14] and may represent recombination events between diverged sequence variants. This may suggest that about 30 % of the genotypes present in the GFMS12 population on Mexican lime are probably a B165 (A-region)/RB (p23 gene) recombinant. To determine the actual recombination sites, further sequence determination of the region between the A-region and the p23 gene, but preferably the whole genome, is envisaged – a goal made possible with next-generation sequencing technology. It will be important to determine the extent of genotype-specific hyper-exclusion [5] that occurs amongst a range of variants and recombinants within a specific genotype. Full-genome sequencing will also help to distinguish if any sequence variants are dRNAs or recombinant full-length genomes.

Interestingly, the GFMS12 populations differed between varieties of the same host, with the genotype composition on grapefruit cv. Marsh being 96 % B165- and 4 % T30-like; in contrast, in grapefruit cv. Star Ruby, it was 81 % T30-, 13 % B165- and 6 % VT-like based on the A-region. Only VT-like sequences were obtained from multiple clones of the p23 gene on both grapefruit cultivars. This suggests that along with some VT-like genotypes within the Star Ruby GFMS12 plant, some are likely to be recombinants between the B165- or T30-like genotypes in the 5′ end and VT-like genotypes in the 3′ end. A previous study showed that a large number of genetic variants were generated by recombination between major genotypes [24]. Potentially, cv. Marsh may be selecting for the B165/VT recombinant, and cv. Star Ruby may be selecting for the T30/VT recombinants. Analysis of GFMS12 populations in replicated individuals of these two hosts is required to confirm this.

Three of the four genotypes observed in the parental GFMS12 populations were detected amongst seven single-aphid-transmission sub-isolates from Mexican lime [23] that were tested by direct sequencing and hence could represent the most dominant genotypes or the most efficiently transmitted. These included VT-like, possible RB/VT-like and B165/VT-like recombinant genotypes. None of the sub-isolates contained the RB-like or recombinant B165/RB sequences that are found in the GFMS12 parental population. Direct sequencing of the A-region of GFMS12 sub-isolates showed the detection of different genotypes over time. Initially, four sub-isolates had a VT-like genotype, and two sub-isolates had a RB-like sequence type, but two to three years later, they had a B165-like sequence type. The change in the population of sub-isolates 12-7, 12-8 and 12-9 was confirmed by cloning the A-region at the second time period. The change in genotypes present in sub-isolates over time suggests that the B165/VT genotype was repressed initially or segregated with graft transmission but with time was able to replicate and dominate the other sequence types (VT-like or RB/B165-like recombinant) present under the experimental conditions in the Pretoria greenhouse. A mixture of RB/VT-like and B165/VT-like recombinant genotypes for sub-isolate 12-7, a B165/VT-like recombinant genotype for sub-isolate 12-8, and a mixture of VT-like and B165/VT recombinant genotypes for sub-isolate 12-9 were selected as representatives of the three genotype populations found within the sub-isolates. To accurately determine the pathogenicity of genotypes present in pre-immunizing sources, representatives of the genotype populations were evaluated. The symptoms observed for sub-isolate 12-7 are possibly due to the putative recombinant RB/VT genotype. RB isolates are generally mild in most indicators, with no SP or SY reactions (S. Harper, personal communication). Sub-isolate 12-8 (B165-like) had no stem-pitting in grapefruit and was very similar to the B165 isolate from India [13]. It cannot be predicted whether symptoms are associated with particular regions of the genome or if they are induced by the predominant variant, minor variants or by a combination of variants of the population. Sub-isolate 12-9 had symptoms on all five indicators, with mild SP and stunting possibly attributed to the VT-like and putative recombinant

B165/VT genotype. In previous studies, Mexican lime and grapefruit varieties Star Ruby and Marsh infected with GFMS12 displayed moderate, mild, and mild-to-moderate stem-pitting symptoms, respectively [19, 23]. These symptoms are not generally caused by RB-like or T30-like genotypes but could potentially be caused by the B165- and VT-like genotypes present as minor or major components of the population, as shown by Černi *et al.* [3]. Since a SY pathogenicity determinant was mapped to the p23 gene [1], it is possible that the symptoms observed for GFMS12 can be attributed to the RB- (Mexican lime) and VT-like strains (Marsh and Star Ruby) found in the p23 gene.

In this study, the RB-like strain of CTV is reported for the first time in South Africa, previously only being reported from New Zealand [8]. RB strains are able to infect the economically important rootstock species *Poncirus trifoliata* [4], hybrid rootstocks of which are commonly used in South Africa, which is resistant to most isolates of CTV [6]. We did not confirm the resistance-breaking nature of GFMS12 or sub-isolate 12-7 by inoculating it onto *P. trifoliata*, but this would be essential to test local RB-like isolates in the future.

This study underlines the importance of determining the genotype composition of a potential CTV pre-immunizing source on a range of inoculated host species before utilization. Even with the current study, the possibility of additional genotypes being present in sub-detectable levels in the three GFMS12 populations tested cannot be excluded, and these may only present themselves at detectable levels in additional host species and with larger numbers of cloned sequences analysed. Additionally, a clearly unbiased method of determining the genotype composition of greater numbers of CTV populations must be developed. While we have tried to minimize genotype bias by PCR by using primers to sequences that are conserved amongst genotypes that do not preferentially amplify specific genotypes and have been used in similar studies [14, 15], the occurrence of bias cannot be discounted completely. The absence of some CTV genotypes in the GFMS12 populations (e.g., HA16-5, T30 and T36) could leave trees pre-immunized with this source vulnerable to these genotypes should the genotype-specific hyper-exclusion phenomenon [5] be a general principle amongst CTV genotypes. It is possible that at least some of the instances of cross-protection breakdown observed in South Africa grapefruit orchards may be due to superinfection of these additional genotypes in plants pre-immunized with GFMS12. Studies are in progress to determine whether such additional genotypes are found in GFMS12-pre-immunized grapefruit trees in the field displaying severe CTV symptoms (Read, unpublished results).

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