

Changes in the *Citrus tristeza virus* Status of Pre-immunized Grapefruit Field Trees

J. B. van der Vyver, S. P. van Vuuren, M. Luttig, and J. V. da Graça

ABSTRACT. Certain grapefruit selections pre-immunized with South African *Citrus tristeza virus* (CTV) cross-protecting isolates GFMS 12 and GFMS 35 displayed changes in the degree of protection conferred by yielding unacceptably high percentages of small fruit, unsuitable for export. These isolates, as well as mild isolate LMS 6, from 10-yr-old Star Ruby, Nel Ruby and Marsh grapefruit field trees, were therefore biologically evaluated in CTV-sensitive plants and examined by single-strand conformation polymorphism (SSCP) analysis. CTV isolates from naturally infected trees (planted virus-free) of the same selections were similarly examined. Samples were taken for testing from each of two sectors of 1.0 m², one with large normal healthy looking leaves and the second with small, cupped leaves, on the outer canopy of each tree. The biological data indicated that the cross-protecting isolates had not retained their original status since CTV seedling yellows (CTV-SY) (only LMS 6 harbors the CTV-SY component) as well as severe stem pitting reactions were recorded in many of the trees, including those pre-immunized with GFMS 12 or GFMS 35. Furthermore, SSCP analysis of the CTV coat protein gene revealed the presence of additional CTV strains in some trees, compared to the original isolates kept in a glasshouse as controls. This can be attributed to super-infection by other CTV strains introduced by the brown citrus aphid vector. DNA bands present in the SSCP profiles from the original isolates were sometimes absent (LMS 6) or less intense (GFMS 12) in some of the grapefruit selections. Although changes in the viral RNA populations within a tree may have occurred, this did not necessarily indicate cross-protection failure and should be further investigated.

The genome of *Citrus tristeza virus* (CTV) is a positive sense, single-stranded RNA organized into 12 open reading frames, resulting in at least 17 protein products (9). The coat protein gene (CPG) is the most extensively analyzed part of the genome. The reported homology of the CPG sequence and deduced amino acid composition of biologically diverse isolates is high, regardless of geographic origin (7, 16), however, several differences in nucleotide and amino acid composition, unique for an isolate, have been identified (12, 16, 17). Many strains of CTV exist and in countries where the disease is endemic, they usually occur as mixtures in a host due to continuous introductions by the main aphid vector, *Toxoptera citricida* (Kirkaldy) (1, 21).

Factors such as cultivar, environment, and multiple infection by aphids, affect the composition or balance of strains within a host (1, 4, 13, 14, 21). A CTV isolate that displays mild symptoms when biologically indexed, may contain severe

strains that can be expressed under certain conditions. Therefore glasshouse-selected cross-protection isolates may differ in their performance under field conditions (22). Differences in symptom expression of daughter trees, derived from the same mother source, planted in different climatic regions were reported (3, 5).

When plants are inoculated with complex isolates, strain separation can readily occur during systemic invasion (13). Hosts can influence the CTV strain balance, as shown by passage through grapefruit, smooth Seville orange and Mexican lime (8, 13, 14). Permanent and non-permanent separation of CTV strains in an isolate after host passage or by stem slash-inoculation was also confirmed by Rubio et al. (19), using single strand conformation polymorphism (SSCP) analysis.

Grapefruit export is important for southern Africa but small fruit size, an effect of CTV, is a constraint. In South Africa two isolates are currently used to cross-protect grape-

fruit, GFMS 12 and GFMS 35 (both derived from grapefruit and without seedling yellows). Isolate LMS 6 (derived from lime, containing a mild form of seedling yellows) is applied in cross-protecting sweet oranges, mandarins and lime. These isolates contain multiple strains (10, 26). Results from field experiments showed that these isolates afforded good protection for several years in Marsh grapefruit (24) and lime (23). However, unacceptably high percentages of small fruit occurred where GFMS 12, GFMS 35 and LMS 6 were used to pre-immunize Star Ruby, Nel Ruby and Marsh grapefruit selections respectively (25). On the contrary, GFMS 12 afforded good protection in Nel Ruby and GFMS 35 was good in Star Ruby. Control trees that were planted virus-free and became infected naturally by aphids, showed mild CTV stem pitting symptoms, and van Vuuren and van der Vyver (25) attributed the small fruit production of some of the grapefruit selection/mild CTV isolate combinations, to possible strain shifts within the isolates, caused by the hosts.

This investigation was conducted to determine the CTV status in trees with sectors of small and normal size leaves in the canopy of 10-yr-old field trees used by van Vuuren and van der Vyver (25) in their study, in order to explain the variability observed in fruit symptom expression in different selections. The CTV status was determined by biological indexing and SSCP analysis of the CPG. An attempt was also made to detect any correlation between CTV molecular profiles and small fruit together with reduced leaf size.

MATERIALS AND METHODS

Isolates. The original cross-protection isolates used for pre-immunization were maintained in Marsh grapefruit (GFMS 12 and GFMS 35) and Mexican lime (LMS 6) in an aphid-free greenhouse. Unlike

GFMS 12 and GFMS 35, LMS 6 contains a mild form of seedling yellows (CTV-SY) (10, 26) in addition to several other strains. Although the protection capacities of the isolates have been established, the contributions of each strain towards cross-protection have not been determined. Therefore, two buds were used to pre-immunize virus-free plant material in an attempt to transfer the whole complement of each isolate.

Test Trees. Ten-year-old Star Ruby, Nel Ruby and Marsh grapefruit trees on Rough lemon rootstock pre-immunized with GFMS 12, GFMS 35 and LMS 6 respectively, and virus-free trees, were selected from a previous trial (25). The trial was planted as a randomized block design with five trees for each treatment in a grapefruit production area. Surrounding orchards included sweet oranges and mandarins. Three trees in each treatment which yielded on average the highest percentage of small fruit (diameter less than 75 mm) over the last 5 yr were selected for testing (Table 1).

On each tree, two sectors on the outer canopy (A and B), each of approximately 1.0 m², at a height of approximately 1.7 m were selected. Sector A on each tree was where small leaves (possibly as a result of CTV super-infection or segregation) were prominent, while sector B consisted of large, healthy looking leaves. The occurrence of small and large leaves varied from tree to tree and in some instances only one branch had either small or large leaves. Each sector was marked clearly with spray paint for easy location at harvest time when fruit size was measured (see below). In each sector a representative sample of shoots (approximately 15 cm long) with mature leaves, was cut.

Samples were taken in early winter and used for biological indexing on Mexican lime and Duncan grapefruit seedlings, determination of average leaf area, and molecular characterization.

TABLE 1
 AVERAGE PERCENTAGE SMALL FRUIT, LEAF AND FRUIT SIZE IN TWO SELECTED AREAS OF THE CANOPY, AND BIOLOGICAL INDEXING RESULTS OF THE SELECTED AREAS FOR THREE 10-YR-OLD GRAPEFRUIT SELECTIONS PRE-IMMUNIZED WITH DIFFERENT *CITRUS TRISTEZA VIRUS* (CTV) ISOLATES BEFORE PLANTING IN THE FIELD COMPARED WITH TREES THAT WERE PLANTED VIRUS-FREE^v

CTV isolate	Host ^v	Small fruit (%) ^x	Canopy sample area	Leaf size (mm ²)	Fruit diameter (mm)	Growth (mm)		Stem pitting ^y		Seedling yellows	
						ML	DG	ML	DG	# Positive trees/3 ^z	# Positive seedlings/9
GFMS 12	SR	9.6	A	228 a	74.8 a	173 a	204 a	2.2 a	1.8 a	1	3
			B	442 b	79.8 b	209 a	249 a	1.9 a	1.6 a	1	2
	NR	2.9	A	192 a	82.8 a	162 a	257 a	2.1 a	2.1 a	1	3
			B	350 b	86.3 b	189 a	170 b	2.3 a	2.6 b	2	5
	M	0.5	A	227 a	73.6 a	79 a	114 a	3.0 a	2.3 a	2	2
			B	386 b	83.4 b	111 b	182 b	2.5 b	2.2 a	2	2
GFMS 35	SR	1.5	A	167 a	70.2 a	191 a	163 a	2.6 a	2.6 a	1	2
			B	365 b	80.6 b	204 a	143 a	2.0 a	2.2 a	1	2
	NR	17.1	A	181 a	66.2 a	94 a	117 a	2.7 a	2.7 a	3	9
			B	334 b	77.3 b	83 a	147 a	2.9 a	2.6 a	3	7
	M	3.1	A	213 a	78.6 a	111 a	143 a	2.7 a	2.3a	1	3
			B	373 b	82.9 b	132 a	177 a	2.7 a	2.6 a	3	8
LMS 6	SR	2.5	A	220 a	79.1 a	325 a	226 a	1.9 a	2.0 a	1	1
			B	368 b	85.4 b	210 a	208 a	2.0 a	1.8 a	3	4
	NR	1.9	A	199 a	76.3 a	112 a	173 a	2.9 a	2.3 a	3	9
			B	389 b	84.2 b	86 a	178 a	2.6 a	1.8 a	3	7
	M	6.8	A	196 a	79.0 a	85 a	156 a	3.0 a	2.8 a	3	6
			B	326 b	81.6 a	108 a	201 b	2.7 a	2.2 a	2	4
Virus-free	SR	3.2	A	198 a	71.7 a	214 a	201 a	2.2 a	1.6 a	2	2
			B	380 b	86.0 b	154 a	211 a	2.6 a	2.4 b	1	3
	NR	8.5	A	213 a	75.8 a	137 a	234 a	2.6 a	2.7 a	2	6
			B	390 b	85.0 b	173 a	178 a	2.2 a	2.4 a	2	5

^vFigures of the A and B areas of each treatment followed by the same letter do not differ significantly at the 5% level (Fisher's LSD), na = not applicable.

^wGrapefruit hosts: SR = Star Ruby, NR = Nel Ruby, M = Marsh, Control = original CTV isolates kept in an aphid-free greenhouse.

^xAverage % small fruit (diameter less than 75 mm) of the total yield at production years 6 to 10 by each cultivar.

^yVisual stem pitting rating: 0 = none, 1 = mild, 2 = moderate, 3 = severe. ML = Mexican lime; DG = Duncan grapefruit.

^zWhen one indicator of either the A or B area gave a positive reaction, the tree was regarded as positive for Seedling Yellows.

TABLE 1 (CONTINUED)
 AVERAGE PERCENTAGE SMALL FRUIT, LEAF AND FRUIT SIZE IN TWO SELECTED AREAS OF THE CANOPY, AND BIOLOGICAL INDEXING RESULTS OF THE SELECTED AREAS FOR THREE 10-YR-OLD GRAPEFRUIT SELECTIONS PRE-IMMUNIZED WITH DIFFERENT *CITRUS TRISTEZA VIRUS* (CTV) ISOLATES BEFORE PLANTING IN THE FIELD COMPARED WITH TREES THAT WERE PLANTED VIRUS-FREE^v

CTV isolate	Host ^w	Small fruit (%) ^x	Canopy sample area	Leaf size (mm ²)	Fruit diameter (mm)	Growth (mm)		Stem pitting ^y		Seedling yellows	
						ML	DG	ML	DG	# Positive trees/3 ^z	# Positive seedlings/9
	M	1.3	A	217 a	77.3 a	70 a	204 a	2.8 a	2.2 a	2	6
			B	434 b	78.9 a	81 a	201 a	2.7 a	2.3 a	2	6
GFMS 12	Control	na	na	na	na	327	555	1.0	1.0	na	0/9
GFMS 35	Control	na	na	na	na	389	468	1.0	1.0	na	0/9
LMS 6	Control	na	na	na	na	314	238	1.0	0.0	na	5/9
Virus-free	Control	na	na	na	na	571	681	0.0	0.0	na	0/9

^vFigures of the A and B areas of each treatment followed by the same letter do not differ significantly at the 5% level (Fisher's LSD), na = not applicable.

^wGrapefruit hosts: SR = Star Ruby, NR = Nel Ruby, M = Marsh, Control = original CTV isolates kept in an aphid-free greenhouse.

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Biological indexing. Glasshouse-grown Mexican lime and Duncan grapefruit seedlings with a stem diameter of approximately 0.5 cm were each inoculated with two buds from different shoots in a sample and replicated three times. After inoculation, plants were cut back, kept in a glasshouse with a temperature regime of 24–28°C, and one shoot allowed to develop per plant. Six months after inoculation, growth was measured, and stem pitting severity rated visually. A scale of 0, 1, 2 and 3 (none, mild, moderate and severe stem pitting respectively) was used. CTV-SY symptoms on Duncan grapefruit seedlings were recorded as absent or present.

Leaf area. The average leaf area of each sample was determined by measuring 10 leaves at random with a digital leaf area meter (CI-202 Portable Leaf Area Meter, Ben Meadows Company, Janesville, USA).

Nucleic acid purification and RT-PCR. For each sample sector, 4 g of bark and midrib tissue were stripped and pooled, then pulverized in liquid nitrogen with a sterile mortar and pestle. Isolation of dsRNA by CF-11 cellulose chromatography was performed as previously described (6). Primers for amplification of the CPG were synthesized (MWG Biotech, München, Germany) based on the sequence of Florida isolate T36 (18). The forward and reverse primers were, 5'-ATGGACGACGAAACAAAG-3' (CTV-CPL) and 5'-TCAACGTGTGTTGAATTT-3' (CTV-CPR), respectively. Two microliters of dsRNA were mixed with both primers (0.4 µM of each primer), first denatured 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 reaction mixtures after adding 0.2 mM of each of the four dNTPs, 5 µl RT-PCR buffer (5X), 5 mM DTT, 0.5 µl enzyme mix (AMV reverse transcriptase and Expand High Fidelity enzymes; Roche Molecular Biochemicals, GmbH) and 7.75 µl

dH₂O to the RNA-primer mix. Reverse transcription and amplification were done in a thermal cycler (Eppendorf Mastercycler Personal) in a one-tube RT-PCR reaction (Titan One Tube RT-PCR System, Roche Molecular Biochemicals, GmbH). Thermocycling conditions were: A reverse transcription step at 50°C for 30 min, amplification for 2 min at 94°C, 40 s at 50°C, 1 min at 68°C (one cycle) and 30 s at 94°C, 40 s at 50°C, 1 min at 68°C (35 cycles) and 30 s at 94°C, 40 s at 50°C, 5 min at 68°C (one cycle). RT-PCR products were examined in a 1% agarose gel stained with ethidium bromide.

Single-strand conformation polymorphism (SSCP) analysis. SSCP was performed directly on the RT-PCR products. A modified procedure as described by Yap and McGee (27) was followed: One microliter RT-PCR product was mixed with 9 µl dH₂O and 1 µl 10X denaturing solution (500 mM NaOH, 10 mM EDTA, pH 8.0). The mixture was heated for 10 min at 42°C and 10X loading dye added (0.5% xylene-cyanol [w/v] and 0.5% bromophenol blue [w/v] in deionized formamide). Denatured DNA were separated by electrophoresis in a 6% non-denaturing polyacrylamide minigel (80 mm × 70 mm × 1.50 mm), without glycerol. The gel was run in 0.5X TBE (44.5 mM Tris-borate, 1 mM EDTA, pH 8.0) buffer for 1.75 h, 300 V at 8°C. Gels were stained with silver nitrate as described by Beidler et al. (2).

Fruit size. During the following harvest season, approximately 10 mo after the samples were taken, the diameters of 10 fruits in each sector, taken at random, were measured with a digital caliper.

RESULTS

Leaf and fruit size. The average leaf size of the two sectors (A = small and B = large leaves) differed significantly in all treatments as was expected (Table 1). Fruit size

corresponded with the leaf size and in all instances the fruit in sectors with small leaves were significantly smaller than fruit in large-leaf sectors. The sizes of fruit from small leaf sectors of Star Ruby and Nel Ruby, pre-immunized with GFMS 35, as well as those of the Star Ruby trees planted virus-free, were below the export standard of 73 mm.

The percentage of small fruit yielded over five years, was the highest in Nel Ruby trees pre-immunized with GFMS 35, Star Ruby trees with GFMS 12, Marsh trees with LMS 6 and Nel Ruby trees that were planted virus-free (Table 1).

Biological indexing. A drastic decrease in growth and increase in stem pitting in Mexican lime and Duncan grapefruit seedling indicators occurred where they were inoculated with the field sources in comparison with the original sources that had been kept under aphid-free conditions (Table 1). The only exception was the A sector of the Star Ruby trees that were pre-immunized by LMS 6. On Mexican lime the presence of severe CTV strains (severe stem pitting reaction) was revealed in 59% of the selected sectors, while 35% were moderate and only 6% mild. On the Duncan grapefruit, 31% of the sectors caused a severe CTV reaction, 54% moderate and 15% mild.

Overall, growth corresponded with stem pitting severity in both indicators (Table 1). Significant differences in growth and stem pitting in Mexican lime inoculated with material from sectors A and B occurred only where Marsh was pre-immunized with GFMS 12. The reaction of Duncan grapefruit indicated that the A sector (small leaves) of the Nel Ruby with GFMS 12, contained significantly milder strains than the B sector (large leaves), although fruit from the B sector was significantly larger than from the A sector. Nevertheless, the fruit size of 83 mm in the A sector was well beyond the minimum standard for export.

A number of cases occurred where good growth was accompanied by severe pitting (several deep pits) in contrast to numerous small shallow pits and severe stunting. The presence of CTV-SY generally contributed to stunted growth of Duncan grapefruit irrespective of leaf size in the sector.

All sectors of the Nel Ruby trees pre-immunized with GFMS 35 and LMS 6 were positive for the CTV-SY component (Table 1). With the other treatments the occurrence of CTV-SY varied between trees as well as between sectors, i.e., one tree of Nel Ruby with GFMS 12 indexed positive for CTV-SY in both sectors (3/3 positive indicators in sector A and 3/3 positive in sector B); a second tree was negative in both sectors; the third tree was negative in sector A and positive in sector B (2/3 positive).

RT-PCR. Samples from individual trees of all cultivars pre-immunized with GFMS 12, GFMS 35 and LMS 6 as well as those planted virus-free, yielded a DNA product of approximately 670 base pairs corresponding to the known size of the CTV-CPG. The RT-PCR products could not be obtained from Star Ruby tree 3 sector A, Nel Ruby tree 2 sector A, and both sectors of Marsh tree 3 after several attempts. No DNA bands were obtained from uninoculated glass-house plants used as controls.

SSCP analysis. SSCP profiles of the CPG for all the isolates in their various hosts are shown in Figs. 1, 2 and 3. Changes in the pre-immunized field trees are evident from all SSCP profiles when compared to the original isolates. Trees planted virus-free also contained multiple DNA bands confirming introductions of CTV by aphids (Fig. 4).

SSCP profiles for the different isolates showed DNA bands of a high intensity (pre-dominant strains) forming a unique pattern for each host. Numerous bands of low intensity occurred, which were taken into consideration when noting differences between the sectors.

Regarding GFMS 35, all the hosts contained the original DNA bands and also additional bands, in samples of both sectors from all grapefruit selections (Fig. 1). In Nel Ruby, all SSCP profiles were the same for both sectors as well as between trees. Differences between sectors A and B occurred in Star Ruby (tree 1) and Marsh (trees 1, 2, 3). For each grapefruit selection, the SSCP profile for GFMS 35 was unique and differed in complexity.

For GFMS 12, variation occurred in intensity of the top original DNA band in all hosts and especially with Nel Ruby (Fig. 2). Differences between the A and B sectors occurred in profiles for Marsh (trees 1, 2) and Nel Ruby (tree 3).

LMS 6 showed the highest variation in DNA band intensity of all three isolates (Fig. 3). The top DNA band was absent in some trees of all the hosts and the middle band was in one case displaced (Marsh, tree 3, lanes 6 and 7). It is evident that the strain composition of LMS 6 in this tree differed from that of the origi-

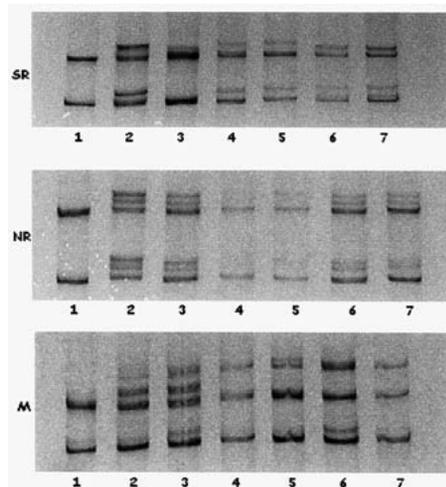


Fig. 1. SSCP profiles of 10-yr-old Star Ruby (SR), Nel Ruby (NR) and Marsh (M) grapefruit trees pre-immunized with *Citrus tristeza virus* isolate GFMS 35 before planting. SR; NR; M - Lane 1: Original GFMS 35 isolate; Lanes 2-3: Tree 1, sectors [A] and [B]; Lanes 4-5: Tree 2, sectors [A] and [B]; Lanes 6-7: Tree 3, sectors [A] and [B].

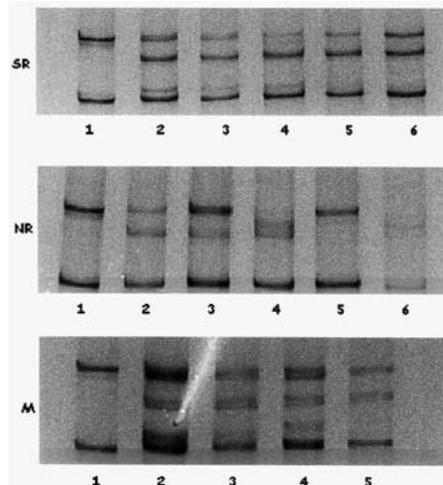


Fig. 2. SSCP profiles of 10-yr-old Star Ruby (SR), Nel Ruby (NR) and Marsh (M) grapefruit trees pre-immunized with *Citrus tristeza virus* isolate GFMS 12 before planting. SR - Lane 1: Original GFMS 12 isolate; Lanes 2-3: Tree 1, sectors [A] and [B]; Lanes 4-5: Tree 2, sectors [A] and [B]; Lane 6: Tree 3, sector [B] (No RT-PCR product obtained from tree 3, sector A). NR - Lane 1: Original GFMS 12 isolate; Lanes 2-3: Tree 1, sectors [A] and [B]; Lane 4: Tree 2, sector [B] (No RT-PCR product obtained from tree 2, sector A); Lanes 5-6: Tree 3, sectors [A] and [B]. M - Lane 1: Original GFMS 12 isolate; Lanes 2-3: Tree 1, sectors [A] and [B]; Lanes 4-5: Tree 2, sectors [A] and [B]; (No RT-PCR products obtained from tree 3, sectors A and B).

nal as well as from that of trees 1 and 2. Differences between sectors A and B were obtained in Marsh (trees 1, 2, 3) and Star Ruby (trees 1, 2, 3).

Trees that were planted virus-free and became naturally infected also displayed variation in their SSCP profiles (Fig. 4). Different patterns occurred among trees of the same selection for all three selections. Two Star Ruby trees (2 and 3) displayed the same patterns. The SSCP profiles between sectors of a tree were similar except for tree 1 of Marsh.

DISCUSSION

Biological indexing indicated a deviation from the original pre-immunizing isolates, and the SSCP

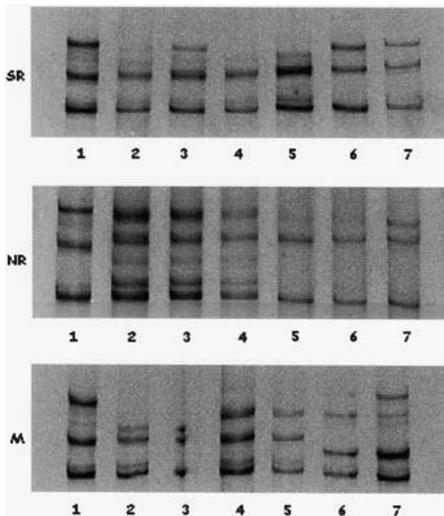


Fig. 3. SSCP profiles of 10-yr-old Star Ruby (SR), Nel Ruby (NR) and Marsh (M) grapefruit trees pre-immunized with *Citrus tristeza virus* isolate LMS 6 before planting. SR; NR; M - Lane 1: Original LMS 6 isolate; Lanes 2-3: Tree 1, sectors [A] and [B]; Lanes 4-5: Tree 2, sectors [A] and [B]; Lanes 6-7: Tree 3, sectors [A] and [B].

profiles confirmed that the CTV status of pre-immunized field trees had changed after ten years in the field. Ideally a cross-protecting isolate should be stable in the host under different environmental conditions (15).

The presence of small leaves correlated with the production of small fruit but not with severe stem pitting or stunting. Two types of severe stem pitting were observed, numerous small pits which were associated with stunting, and deep pits and grooving that were accompanied by good growth.

On a molecular level, the results revealed the presence of additional sequence variants for the CPG in the SSCP profiles since the number of bands in the profile for the original isolate did not correlate with that of the field isolate. Furthermore, LMS 6 was the only isolate that originally contained a mild CTV-SY component. Mild and severe CTV-SY was recorded by biological indexing on the Duncan grapefruit for all the

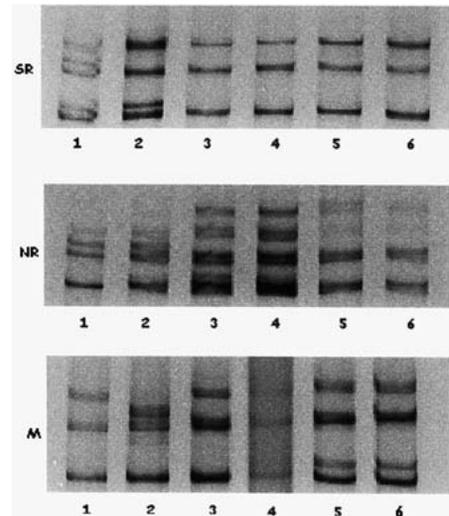


Fig. 4. SSCP profiles of 10-yr-old Star Ruby (SR), Nel Ruby (NR) and Marsh (M) grapefruit trees that were planted virus-free. SR; NR; M - Lanes 1-2: Tree 1, sectors [A] and [B]; Lanes 3-4: Tree 2, sectors [A] and [B]; Lanes 5-6: Tree 3, sectors [A] and [B].

treatments, suggesting super-infection. According to McClean (11) grapefruit affords resistance to CTV-SY, and infected trees will be free of this component within two years if not re-infected. However, orchards of sweet orange and mandarin, which naturally harbor CTV-SY, bordered this grapefruit orchard and provided an abundant and perpetual source of CTV-SY.

There is no conclusive evidence that host interference caused a change in strain prevalence in the CTV isolates originally applied, although there are a few observations that need consideration. Firstly, GFMS 35 in Nel Ruby afforded poor protection. Five of the six sectors tested caused severe stem pitting on Mexican lime and were indistinguishable by SSCP analysis. The trees were not located close to each other but still contained the same SSCP profiles. Comparing the profiles from the different selections, there appears to be a tendency for each selection to support specific strains. The more complex

SSCP profiles in the Nel Ruby samples indicate the presence of additional strains. This indicates the presence of additional strains that could be severe (20) and responsible for the high percentage of small fruit. In contrast, it was not possible to correlate the high percentage small fruit that occurred in the Star Ruby trees, pre-immunized with GFMS 12, with the SSCP profiles that contained fewer DNA bands.

Secondly, some DNA bands in the SSCP profiles for the CPG from LMS 6 and GFMS 12 were absent, or present in very low concentrations within certain trees of the different selections. Rubio et al. (19) showed that the intensity of DNA bands reflected the titer of the corresponding strain in the isolate. Multiplication and movement of these CTV strains may have been suppressed in the host and therefore not detected. Other factors such as temperature could also have played a role in altering the isolate composition or strain prevalence within and between trees of a selection (3, 5).

Thirdly, differences in SSCP profiles between A and B sectors in individual trees strongly indicate uneven distributions of the CTV strains within the tree, possibly due to aphid introductions.

In an attempt to differentiate between mild and severe strains, Sambade et al. (20) observed a trend

of mild isolates to generally have SSCP profiles of that of a pre-dominant strain, whereas severe isolates usually showed more complex DNA band patterns. SSCP analysis of GFMS 12 in Nel Ruby, where fruit and tree quality were evidence of good cross-protection, revealed only simple band patterns. Severe stem pitting and complex SSCP profiles were correlated in the majority of cases e.g., both sectors from GFMS 35 in Marsh (tree 1: Fig. 1, lanes 2 and 3), GFMS 12 in Marsh (tree 1: Fig. 2, lanes 2 and 3), LMS 6 in Nel Ruby (tree 1: Fig. 3, lanes 3 and 4) and Nel Ruby planted virus-free (tree 2: Fig. 4, lanes 3 and 4).

The results indicated that the new strains introduced by aphids appeared to be more severe than van Vuuren and van der Vyver (25) suggested. There was no evidence from SSCP analysis of segregation of strains within the original pre-immunizing isolates, in different sectors of the trees. None of the SSCP profiles corresponded with those reported previously for single aphid transferred sub-isolates from GFMS 12, GFMS 35 and LMS 6 (10, 26).

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