Comparison of the Coat Protein Gene Sequences of Citrus Tristeza Virus Isolates in New Zealand

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ABSTRACT. The citrus tristeza virus (CTV) coat protein gene (CPG) sequences from isolates in New Zealand, Florida and the South African Nartia strain were compared. The results demonstrated a high degree of homology at the nucleotide and deduced amino acid level between all isolates. The CPG amino acid sequences of three New Zealand isolates were identical, however, all other New Zealand isolates had unique amino acid residues. A cluster dendrogram showed tightly clustered phylogenetic groupings within the New Zealand isolates, but that the New Zealand isolates were dissimilar from the Florida and South African isolates.

The New Zealand citrus industry developed in the early 1800s, with unrestricted importation of citrus trees by early missionaries (5). As there were no quarantine restrictions in place, pests and diseases associated with citrus were introduced into New Zealand along with the trees. Consequently, New Zealand has citrus tristeza virus (CTV) isolates with biological characteristics ranging from mild through to quick decline and severe stem pitting, and the CTV vector, the brown citrus aphid, *Toxoptera citricida* (Kirkaldy), is endemic. As the result of an 'incompatibility problem' of sweet orange on sour orange rootstocks encountered in the 1940's (10), New Zealand citrus propagators of the day had to use alternative rootstocks, Trifolate orange, citranges, and sweet orange, which were, by chance, tolerant of CTV. Consequently, although we have the quick decline strains of CTV in New Zealand, we have not experienced the devastating losses seen in the various citrus growing areas around the world.

Most commercial citrus trees grown in New Zealand are infected with one or more CTV isolates. A form of natural mild strain cross protection has apparently evolved, as evidenced by healthy looking trees growing in the orchard, but when isolates are separated, some severe stem-pitting isolates are evident on biological indicator plants (2). The severe CTV isolates are only becoming evident when uninfected imported cultivars or plants from our breeding selections become infected in the nursery or field.

In this study, CTV isolates collected from several commercial varieties grown in the three citrus-growing regions in New Zealand were studied. Their biological characteristics were determined using biological indexing, and their coat protein genes (CPGs) were cloned and sequenced. Based upon sequence analysis the relationships were determined between the New Zealand isolates and isolates T3, T26, T30, T36 and T66 from Florida and the South African Nartia B7 isolate.

MATERIALS AND METHODS

New Zealand CTV isolates. The CTV isolates studied and their biological properties are given in Table 1. CTV isolates were collected mainly from commercial citrus orchard trees and from the germplasm collection or advanced breeding selection blocks at Kerikeri Research Centre. Isolates were propagated through graft transmission. The isolates were maintained in Madam Vinous sweet orange, Mexican lime, Duncan grapefruit and *Citrus excelsa* under glasshouse

TABLE 1
CHARACTERISTICS OF NEW ZEALAND CITRUS TRISTEZA VIRUS ISOLATES USED IN THIS STUDY

			Biological characteristics[z]				
			Seedling yellows	Stunting	Stem pitting		
Isolate	ELISA polyclonal	Quick decline	EL[y]	DG[x]	MV[w]	DG	ML[v]
NM83	1	1	1	1	1	1	1
N1	1	3	1	1	1	1	4
N3	1	3	1	1	1	1	4
N4	1	3	1	1	1	1	4
N5	1	3	1	1	1	1	4
N6	1	3	1	1	1	1	4
N7	1	3	1	1	1	1	4
N8	1	3	1	1	1	1	4
N9	1	3	1	1	1	1	4
N10	1	3	1	1	1	1	4
N11	2	5	2	4	1	1	4
N12	2	5	2	4	1	1	4
N13	2	5	2	4	1	1	4
N14	2	5	2	4	1	1	4
N20	3	3	2	2	1	2	4
N21	3	3	2	2	1	2	4
N22	3	3	2	2	1	2	4
N27	3	4	3	5	2	1	5
N37	3	5	4	1	4	1	4
N38	3	5	4	1	4	1	4
N39	3	5	4	1	4	1	4
N40	3	5	4	1	4	1	4

[z]Key - 1 = nil, 2 = mild, 3 = moderate, 4 = severe, 5 = very severe.
[y]EL = Eureka lemon; [x]DG = Duncan grapefruit; [w]MV = Madam Vinous; [v]ML = Mexican lime.

conditions with mean maximum temperatures of 28°C and mean minimum temperature of 18°C.

Extraction of CTV specific dsRNA. Double stranded (ds) RNA analysis was carried out by phenol chloroform/isoamyl alcohol extraction of nucleic acids from 5 g of fresh bark tissue, purified by CF11 cellulose chromatography, and nucleic acids separated on 6% polyacrylamide gels by electrophoresis, following the method of Gillings et al. (4). The dsRNA was visualized by staining with silver nitrate.

cDNA cloning. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out on the dsRNA. An aliquot of 4.5 µl of purified dsRNA was heat treated under three drops of paraffin oil for 5 min at 94°C, chilled on ice, and then the cDNA synthesis and amplification of the CTV CPG was performed following the procedure of Gillings et al. (4). Thermal cycling was performed in a Stratagene Robocycler 40™ temperature cycler. The PCR products were separated from the overlay of paraffin oil by sequential phenol/chloroform extractions, the cDNA was pelleted by the addition of $^1/_{10}$ volume of 3M sodium acetate and 2.5 volumes of absolute ethanol; this was chilled overnight at -20°C and the PCR product collected by centrifugation. The pellet was washed with 70% ethanol, dried under vacuum and resuspended in 20 µl sterile water. Ten percent of the PCR product was checked and visualized on 1.2% agarose/TBE gels by ethidium bromide staining. Amplified PCR products were separated on 1.5%

agarose gels and eluted using a Bresaclean gel extraction kit according to the manufacturer's protocol (Bresatec Pty Ltd., Australia).

The purified DNA was digested with EcoRI and XbaI restriction enzymes and ligated into the pGEM-5 vector digested with the same two enzymes. Competent cells of the DH5á strain of *E. coli* were transformed with the plasmid vectors. Recombinant colonies were selected and tested for the presence of the CTV CPG.

Sequence analysis. The purified CPG PCR products and/or inserts in pGEM-5 plasmid were then sequenced in a commercial laboratory using the Applied Biosystems International protocol. Sequence analysis was performed using GCG PileUp and Clustal V sequence analysis programs (3, 6). A dendrogram showing the relationship of the deduced amino acids was constructed using the Tajima and Nei (9) correction, neighbor joining and mid-point rooting.

RESULTS AND DISCUSSION

The CPGs of several biologically different isolates of New Zealand CTV have been cloned and sequenced. Comparative analysis of the nucleotide sequence and deduced amino acids show a high degree of homology among the different isolates. There was 90% direct sequence similarity between the New Zealand isolates at the nucleotide level (data not shown) and 88% similarity at the amino acid level (Fig. 1).

However, unlike the results of studies on CTV isolates from Florida, Colombia and Spain (1, 8), the association between biological characteristics and the CPG sequences of the New Zealand isolates was not as clear. Biologically mild isolates were clustered with stem pitting and quick decline isolates. CPGs of the New Zealand CTV isolates which have been sequenced all have a thymine (T) at position 371 in the nucleotide sequence (data not shown) and phenylalanine (F) at amino acid position 124 (Fig. 1). Consequently, the MCA13 epitope is present in all of the cloned New Zealand CTV isolates which included NM83, a mild isolate selected for a consistent nil reaction on all indicator plants tested, and N20, N21 and N22 which are moderate stem pitting isolates but with slight quick decline activity. These results are similar to those of Kano and his colleagues (7), who found that M15A and M23A, two mild Japanese CTV isolates, have the same residue at position 124 in the amino acid sequence. This raises a question concerning the reliability of using the presence or absence of the MCA13 epitope in the CPG to classify a CTV strain as either mild or severe.

The deduced amino acid sequences of New Zealand isolates were compared with T3, T26, T30, T36 and T66 isolates from Florida and the South African Nartia B7 strain (Fig. 2). The New Zealand isolates clustered in four distinct domains. Isolates N37, N38, N39 and N40 clustered together with the mild isolate NM83, and were phylogenetically distinct from other New Zealand isolates and the South African and Florida isolates. Clones N37, N38, N39 and N40 differed from each other by one or two amino acids and from NM83 by six, six, six and five amino acids, respectively (Fig. 1). N37, N38, N39 and N40 are biologically different from NM83, in that these isolates cause severe stem pitting in Madam Vinous and Mexican lime, severe seedling yellows in Eureka lemon, severe stunting in Mexican lime and Eureka lemon, and quick decline in sweet on sour orange (Table 1). NM83 is biologically mild, with no evidence of stem pitting, stunting, seedling yellows, corking, vein clearing or quick decline in all indicator plants used in this study (Table 1).

Clones N1, N5 and N9 were identical, and they clustered with N3, N4, N6, N7, N8 and N10; all nine clones caused moderate stunting

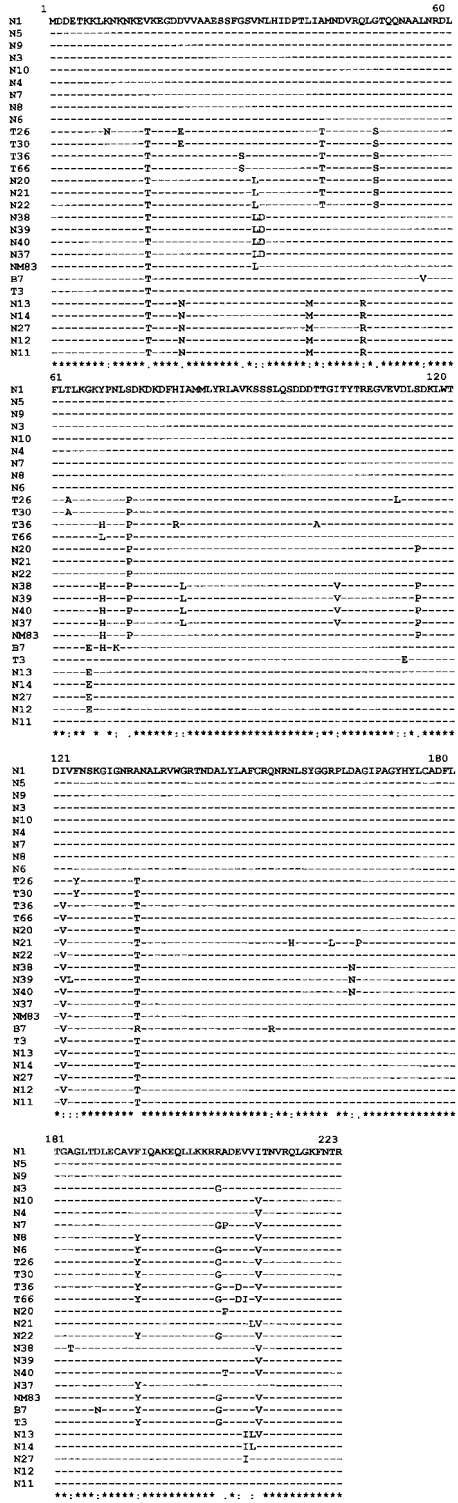

Fig. 1. Multiple alignment of deduced amino acid sequences of the coat protein genes of New Zealand isolates of citrus tristeza virus and isolates T3, T26, T30, T36 and T66 from Florida and the South African Nartia isolate B7. The asterisks and dashes indicate identical amino acids. Dots indicate substitution with different but similar amino acids.

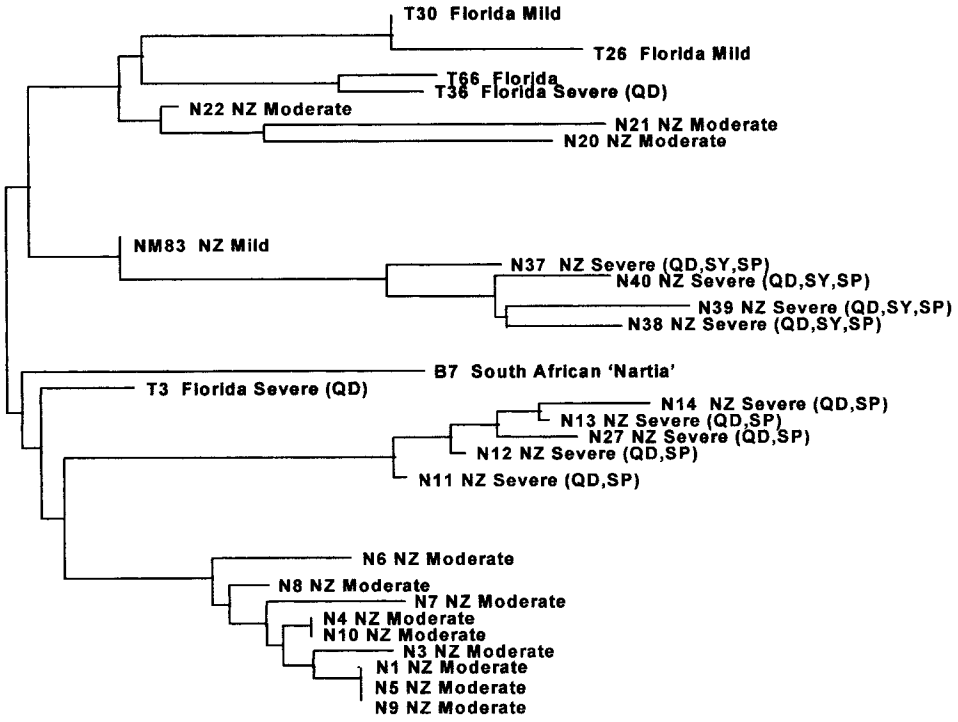

Fig. 2. Dendrogram showing the relationships of the deduced amino acid sequences of the coat protein genes of New Zealand isolates of citrus tristeza virus and isolates T3, T26, T30, T36 and T66 from Florida and the South African Nartia isolate B7. Tree constructed using Tajima and Nei (7) correction, neighbour joining, mid-point rooting.

and moderately severe stem pitting in Mexican lime. N1, N5 and N9 differed from N3, N4, N6, N7, N8, N9 and N10 by one to three amino acids. These isolates were all collected from three Tahiti lime trees in a germplasm collection at the Kerikeri Research Centre.

The third distinct domain consisted of four isolates from Calamondin and one isolate from Parent Navel from the Bay of Plenty region. These five isolates caused severe stem pitting on Mexican lime, severe quick decline, stunting and moderate seedling yellows (Table 1). N11, N12, N13, N14 and N27 differ from each other by one to two amino acids (Fig. 1). When compared with the Florida and South African isolates, N1 and N3 through to N14 and N27, were phylogenetically distinct from T26, T30, T36 and T66, but were more closely linked to B7 and T3.

Clones N20, N21 and N22 clustered together, these isolates caused moderately severe stem pitting in Mexican lime and moderate quick decline in sweet on sour orange. These three isolates were more closely related to the Florida isolates T26, T30, T36 and T66 than they were to the other New Zealand isolates (Fig. 2). However, given that T26 and T30 are distinctly different from the other two Florida isolates T36 and T66 in their symptomatology, the phylogenetic relationship between the New Zealand isolates N20 to N22 and these four Florida isolates is tenuous at best.

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