UC Riverside

International Organization of Citrus Virologists Conference Proceedings (1957-2010)

Title
Evidence for Trifoliate Resistance Breaking Isolates of Citrus Tristeza Virusin New Zealand

Permalink
https://escholarship.org/uc/item/9x93b3dr

Journal
International Organization of Citrus Virologists Conference Proceedings (1957-2010), 14(14)

ISSN
2313-5123

Authors
Dawson, T. E.
Mooney, P. A.

Publication Date
2000

DOI
10.5070/C59x93b3dr

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Evidence for Trifoliate Resistance Breaking Isolates of Citrus Tristeza Virus in New Zealand

T. E. Dawson and P. A. Mooney

ABSTRACT. Fifteen isolates of citrus tristeza virus (CTV) have been identified in New Zealand citrus which are infectious after transmission from trifoliate orange. These isolates were identified during routine virus screening for other citrus viruses following filtering through trifoliate orange. Bark patches from *Citrus excelsa* infected with transmissible CTV isolates were budded onto visually and isozyme tested, *Ctv*-R, true-to-type trifoliate orange seedlings. Clean buds of Mexican lime and Madam Vinous were then budded both above and below the inoculation site. Symptoms of vein clearing and stem pitting were produced on the Mexican lime and Madam Vinous indicators. Stem pitting and leaf curling symptoms were produced on some trifoliate orange rootstock shoots. Double stranded (ds) RNA extractions were then obtained from bark material of the components of the composite plant. Reverse transcription polymerase chain reaction (RT-PCR) was performed on the extracted dsRNA, using primers specific for the coat protein sequence. Ten PCR products were obtained then sequenced and compared with each other and with known coat protein sequences of the Florida T36 and T3 and the South African B7 strains.

In studies for citrus viruses other than tristeza, latent citrus tristeza virus (CTV) can cause severe symptoms in the indicator plants, which can mask the symptoms of the virus of interest. It was, therefore, necessary to eliminate CTV from the field source material prior to screening for other viruses. CTV elimination was achieved using a filtering step through trifoliate orange (12). The success of this technique is based upon resistance or immunity that true-to-type trifoliate orange has to CTV (3, 12, 13).

CTV is widespread in New Zealand (NZ) citrus orchards but the status of other viruses is unknown. When screening for the presence of viruses other than CTV, test buds from commercial orchard plants grown in the Kerikeri district and known to be viroid free, were budded on to either trifoliate orange Rubidoux or Flying Dragon seedlings in order to filter out CTV. Buds from basal shoots on these trifoliate orange plants were then used to inoculate the selected indicator plants. After checking the cause of unexpected symptom expression on these indicator plants, the presence of CTV was confirmed by double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) and double-stranded (ds) RNA analysis. This obviously raised the possibility that there are isolates of CTV in New Zealand that were able to replicate and be transmissible through trifoliate orange.

This paper reports on the studies to investigate the movement of CTV through trifoliate orange and the partial characterization of these trifoliate orange transmissible isolates.

MATERIALS AND METHODS

Biological Characterization. Initial indexing was carried out using the following indicators: Madam Vinous sweet orange, Mexican lime, Duncan grapefruit, Eureka lemon. Three plants of each indicator were graft-inoculated with three bark patches. Madam Vinous sweet orange buds from plants that had previously been inoculated with CTV isolates, were budded onto Brazilian sour orange indicator seedlings to test for quick decline. Symptom expression on the indicator plants were assessed 4 to 5 mo and 9 mo after plant inoculation.

Transmission through trifoliate orange. Seed of trifoliate orange Rubidoux was obtained from commercial sources. The trifoliate orange seedlings were initially culled on visual symptoms, then isozyme tested (8), and samples of trifoliate orange sent to Citrus Research and Education Centre, Florida, for checking for presence of the CTV resistance gene using SCAR markers (1).

Buds obtained from basal rootstock shoots of trifoliate orange plants that had been previously budded with commercial citrus cultivars were budded onto either *Citrus excelsa* or sour orange seedlings. Inoculum buds from the *C. excelsa* seedlings, which had tested CTV positive by DAS-ELISA (Sanofi, CTV polyclonal antibodies), were T-budded onto tested true-to-type trifoliate orange Rubidoux seedlings 12 cm above the base of the stem. Each inoculum source was replicated on three plants. After 3 wk, wrapping tapes were removed and inoculations checked for survival; 3 wk later virus-free indicator buds of Mexican lime and Madam Vinous were budded 5 cm above and 5 cm below the inoculation sites, respectively. The indicator bud tapes were removed 3 wk later, and buds checked for survival; at this time the trifoliate orange stem was cut back above the Mexican lime bud. The buds of the two indicators and a bud of the trifoliate orange rootstock were allowed to sprout and grow (Fig. 1). After 5 mo growth, the shoots were examined for symptoms. Bark was collected separately from Madam Vinous and Mexican lime indicator shoots and from the trifoliate orange shoot for dsRNA analysis. No ELISA were carried out as dsRNA analysis was performed on the component parts of the composite plant. The peeled shoots were examined for signs of stem pitting.

The trial was carried out in an insect-proof glasshouse with mean maximum temperature of 28°C and mean minimum temperature of 18°C.

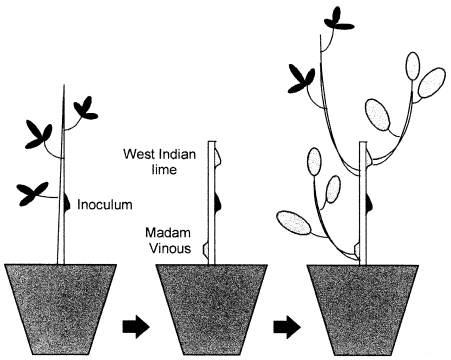

Fig. 1. Indexing methodology for testing CTV replication and movement in trifoliate orange Rubidoux seedlings. Inoculum source was from *Citrus excelsa* plants previously inoculated by trifoliate orange buds.

Extraction of CTV specific dsRNA. DsRNA analysis was carried out by phenol/chloroform/isoamyl alcohol extraction of nucleic acids from 3 to 5 g of fresh bark tissue, followed by purification by CF11 cellulose chromatography, concentrated by ethanol precipitation, and analyzed by polyacrylamide gel 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. 4.5 µl of sample dsRNA was heat-treated under three drops of paraffin oil for 5 min at 94°C then chilled on ice. The cDNA synthesis and amplification of the CTV coat protein gene followed 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 precipitated by the addition of $^{1}/_{10}$ vol. of 3M sodium acetate and 2.5 vol. of absolute ethanol, and overnight incubation at -20°C. The PCR product was collected by

centrifugation and the pellet washed with 70% ethanol, dried under vacuum and resuspended in 20 µl sterile water. An aliquot (10%) of the PCR product was checked on 1.2% agarose/TBE gels and visualized by ethidium bromide staining. Amplified cDNA 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).

Sequence analysis. The purified coat protein gene (CPG) PCR products were sequenced directly in a commercial laboratory using the forward CTV CPG primer and the Applied Biosystems International protocol. Sequence analysis was performed using GCG PileUp and Clustal V sequence analysis programs (2, 6) on these sequences and compared to those of T3, T36 and B7. A dendrogram showing the relationship of the deduced amino acids was constructed using Tajima and Nei correction, neighbour joining and mid-point rooting (11).

RESULTS

The biological properties of the CTV isolates used in this study are given in Table 1, all isolates had tested positive for CTV by DAS-ELISA. The symptoms produced by these isolates, including those that appear transmissible through trifoliate orange, ranged from mild to severe quick decline, seedling yellows and stem pitting.

The biological symptoms expressed in the composite plant (Fig. 1) are summarized in Table 2. The biological properties of isolate NS43 were not determined, but the inoculation material was from a pummelo tree showing severe stem pitting symptoms. The biological symptoms produced by the isolates on the test

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

	Biological characteristics					
		Seedling yellows	Stunting	Stem pitting		
Isolate	Quick decline	EL[z]	DG[y]	MV[x]	DG	ML[w]
NS2	—[v]	—	—	—	—	—
NS9	—	—	—	Mild	Moderate	Severe
NS11	+	Severe	Moderate	Moderate	Mild	Severe
NS13	+	Mild	Severe	Mild	—	Severe
NS16	—	—	—	Mild	Mild	Moderate
NS19	+	Severe	Severe	Severe	—	Moderate
NS21	+	Severe	Severe	Moderate	—	Moderate
NS23	+	Severe	Mild	Severe	Mild	Severe
NS24	+	Mild	Moderate	—	—	Moderate
NS25	+	Severe	Severe	Mild	—	Severe
NS27	—	—	—	—	Mild	Moderate
NS29	+	Severe	Severe	Severe	Mild	Severe
NS34	—	—	—	—	—	Severe
NS36	+	Moderate	Severe	—	—	Severe
NS43	nd	nd	nd	nd	nd	nd

[z]EL = Eureka lemon
[y]DG = Duncan grapefruit
[x]MV = Madam Vinous
[w]ML = Mexican lime
[v]— = no symptoms

TABLE 2
BIOLOGICAL SYMPTOMS OF COMPOSITE PLANTS INOCULATED WITH 15 NEW ZEALAND ISOLATES OF CITRUS TRISTEZA VIRUS[z].

	Biological symptoms of trifoliate orange transmissible CTV isolates					
	Madam Vinous		Mexican Lime		Trifoliate Orange	
Isolate	SP[y]	VC[x]	SP	VC	SP	LC[w]
NS2	Mild	+	Mild	+	Mild	+
NS9	Mild	+	Mild	+	—[v]	—
NS11	Mild	+	Mild	+	—	—
NS13	Mild	+	Mild	+	—	—
NS16	Mild	+	Mild	+	—	—
NS19	Mild	+	Mild	+	—	—
NS21	—	—	—	—	—	—
NS23	Mild	+	Mild	+	Moderate	+
NS24	—	—	—	—	—	—
NS25	—	+	Mild	+	—	—
NS27	nd[u]	nd	Mild	+	—	—
NS29	Mild	+	Mild	+	Mild	+
NS34	—	+	Moderate	+	—	—
NS36	nd	nd	Mild	+	—	—
NS43	Mild	+	Mild	+	—	—

[z]Composite plant as in Fig. 1.
[y]SP = Stem pitting
[x]VC = Vein clearing
[w]LC = Leaf curling
[v]— = no symptoms
[u]nd = Not determined

Mexican lime and Madam Vinous shoots on the composite plant, were similar but milder than those recorded in the full biological characterization screening. However, stem pitting on Madam Vinous sweet orange and Mexican lime shoots on the composite plants inoculated with isolate NS2 also occurred. Several Madam Vinous buds on the composite plant, being sub-dominant, did not grow. Three of the isolates, NS2, NS23, NS29, caused stem pitting on the trifoliate orange shoots.

DsRNA profiles of isolates recovered from Mexican lime and trifoliate orange (Fig. 1) are CTV as PCR products using CTV CPG specific primers were subsequently obtained. The presence of dsRNA in the trifoliate orange shoots suggests that the virus is replicating in this tissue, or long distance movement of the replicative form has occurred. The presence of CTV in the indicator shoots indicates that the virus particles have moved both acropetally and basipetally.

CTV-specific dsRNA extracted from the Mexican lime indicator and trifoliate orange shoots was used to clone the CTV CPG from several isolates following transmission through trifoliate orange. The nucleotide sequences have been successfully determined for isolates NS2x, NS16x, NS25x, NS34x and NS36x recovered from trifoliate orange shoots and NS19, NS25, NS27, NS29 and NS43 recovered from the Mexican lime shoots on the test plants (Fig. 1). The presence of more than one CPG sequences obtained indicates that more than one strain was present in the isolates.

The only matched pair of sequences was NS25 and NS25x, where NS25 was recovered from Mexican lime shoots, and NS25x

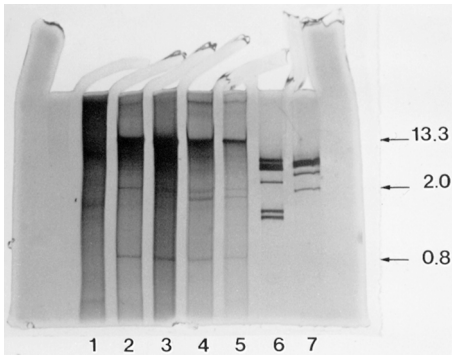

Fig. 2. DsRNA profiles of isolates recovered from Madam Vinous, Mexican lime and Trifoliate orange. 1 = isolate NS29 Mexican lime and Madam Vinous; 2 = isolate NS2 Mexican lime and Madam Vinous; 3 = isolate NS2 Trifoliate orange; 4 = isolate NS25 Mexican lime and Madam Vinous; 5 = isolate NS25 Trifoliate orange; 6 and 7 = molecular weight markers.

from the trifoliate orange shoots on the same composite plant. These two sequences were identical, suggesting that the CTV isolate inoculated onto the trifoliate seedling moved into the indicator shoots and the new growth of the trifoliate orange. We failed to obtain readable sequences from the other paired isolates, resulting from mixed stains in the PCR product.

There was 91% direct sequence similarity between the New Zealand isolates at the nucleotide level (data not shown) and 94% similarity at the amino acid level (Fig. 3). The majority of divergent amino acid residues were found to be functionally conserved substitutions (Fig. 3).

Using GCG PileUp, the phylogenetic relationship was determined between the amino acid sequences of the CPG of the New Zealand CTV clones NS16x, NS2x, NS25, NS25x, NS29, NS19, NS34x, NS27, NS43 and NS36x, the Florida T36 and T3 isolates and the South African B7 isolate (Fig. 4). Two phylogenetic groupings were observed. The New Zealand clones NS16x, NS2x, NS25, NS25x, NS29, NS36x clustered in one group with the Florida quick decline isolate T36 coming off separately. The second domain consisted of the New Zealand isolates NS19, NS34x, NS27 and NS43 clustering together, with the Florida T3 and the South African B7 isolates clustering together on a branch off this grouping.

All the CPGs of the CTV isolates which replicate and move through trifoliate orange had a thymine (T) at position 371 in the nucleotide sequence and phenylalanine (F) at amino acid position 124 (Fig. 3). Consequently, the MCA13 epitope is present in all of the New Zealand CTV isolates. This included NS2x, a mild New Zealand CTV isolate, which did not produce any symptoms on indicator plants and only mild stem pitting in the composite plants.

Samples of trifoliate orange tissue from the composite plants showing symptoms were analyzed using SCAR markers linked to the resistance gene (1) at CREC, Lake Alfred, Florida, and were all found to possess the resistance allele.

DISCUSSION

This is the first report of CTV replicating and translocating in Rubidoux seedlings. The assumptions to date have been that: (a) CTV is not able to replicate in true-to-type trifoliate orange, (b) short distance accumulation may occur around the site of inoculation, and (c) movement may occur acropetally by passive transport through the phloem to a susceptible genotype where it unloads, replicates and spreads uniformly (9). The results of this study do not support these premises, in that we frequently extracted dsRNA of a range of CTV isolates separately from the composite plant, indicating that CTV movement was acropetally and basipetally into both the susceptible indicator shoots and the new shoots of trifoliate orange.

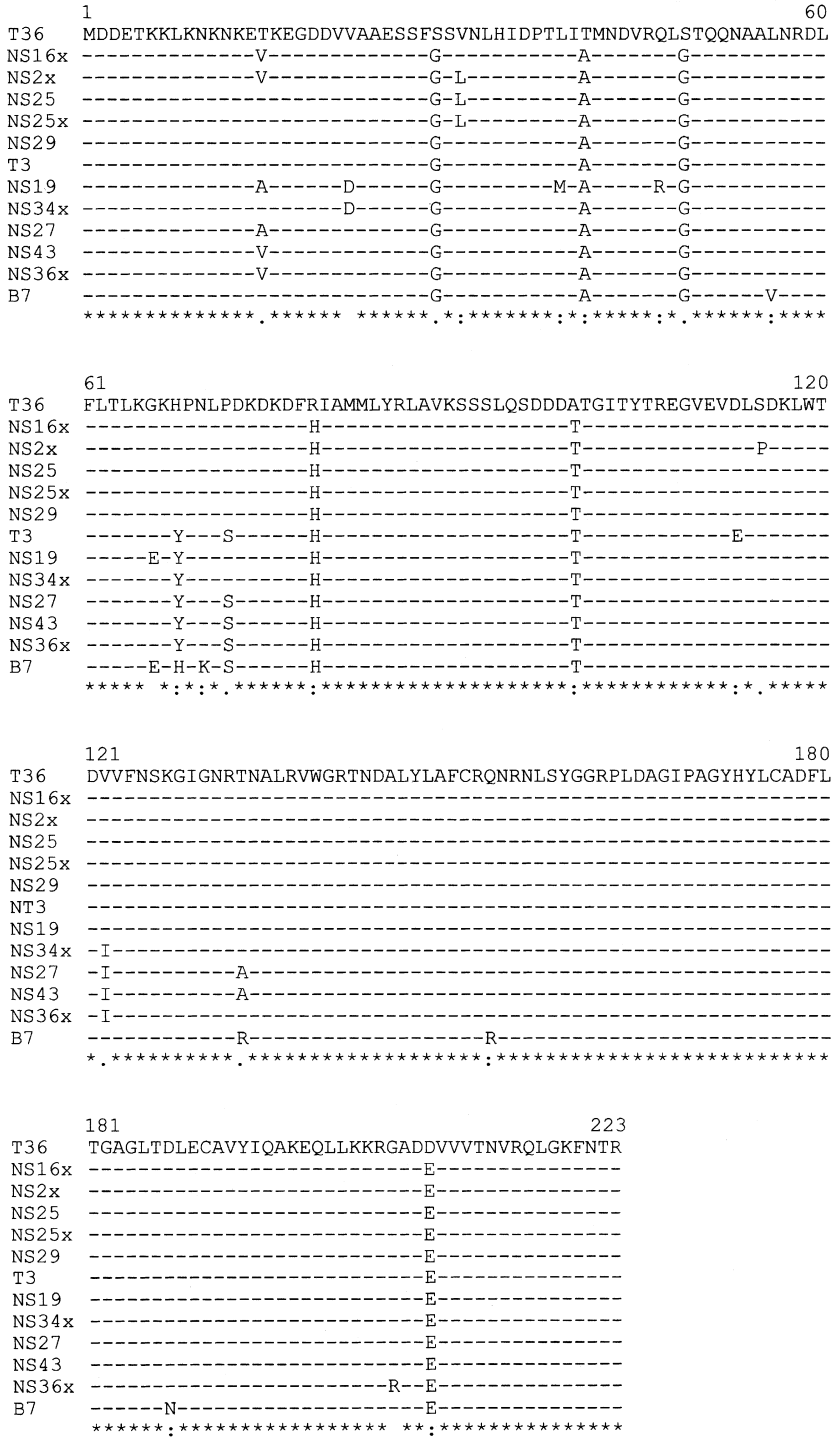

Fig. 3. Multiple alignment of deduced amino acid sequences of the coat protein gene of New Zealand CTV isolates with those of T3, T36 and B7. The asterisks and dashes indicate identical amino acids. Dots indicate substitution with different but similar amino acids.

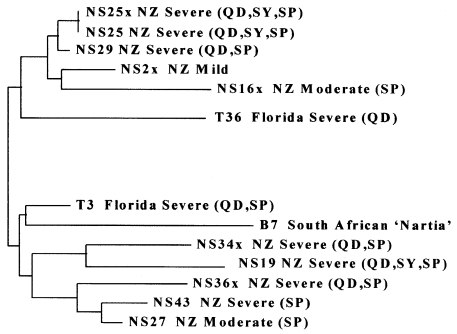

Fig. 4. Dendrogram showing the relationships of the deduced amino acid sequences of CPGs of New Zealand isolates and isolates T3 and T36 from Florida and the South African 'Nartia' strain B7.

In the present study, CTV replication and movement occurred from inoculated trifoliate orange plants, to susceptible host, then to clean trifoliate orange plants where replication and movement still occurred. This, in our opinion, indicates that movement of the CTV in the trifoliate was active rather than that of passive movement.

In the recent report by Mestre and his co-workers (9) of passive movement of CTV through the phloem of Flying Dragon on Rough lemon seedlings, virus detection was based upon the presence of virus particles using DTBIA and DAS-ELISA. These researchers also stated that CTV isolates able to replicate in trifoliate orange had not been found. However, the isolate NS25x in this study not only moved into the new trifoliate shoots, but CTV specific dsRNA was also isolated from the trifoliate orange shoots.

Sequence analysis of the transmissible isolates show that phenylalanine (F) is present in all isolates at amino acid position 124, this includes NS2x, a mild New Zealand CTV isolate. Kano et al. (7) also observed phenylalanine at this position in two of their Japanese mild isolates, previously this region has been used to distinguish between mild and severe strains. The results of both Kano and his colleagues and this report suggest that this is no longer the case.

The resistance in trifoliate orange has been linked to one or two dominant genes at the Ctr and/or Ctm loci, respectively (5, 9, 10). CTV replication close to the site of inoculation and short distance movement within trifoliate orange has been reported in the literature (9, 10, 13). This movement has been linked to the presence and combination of the Ctr and Ctm genes as either homozygous or heterozygous genotypes. Individuals homozygous Ctrrr, in combination with all genotypes of the Ctm gene, supported virus replication and movement, while individuals heterozygous Ctr-Rr and homozygous Ctm-mm, suppress virus replication but allow short distance movement of the virus particles. All other combinations inhibited both replication and movement (9).

In these trials the culling of zygotic seedlings was in agreement with Khan and Roose (8) where visual symptoms were insufficient diagnosis for complete identification. A further 13% were culled using isozyme analysis, and with subsequent SCAR marker testing, the presence of the resistance gene in the remaining plants was confirmed. Consequently, the replication and movement of a range of CTV isolates in our test plants could not be attributable to these plants being homozygous Ctr-rr, as the test plants were isozyme tested as nucellar and SCAR tested for the presence of *Ctv*-R resistance gene.

The presence of stem pitting on the trifoliate orange shoots appear to indicate that CTV is replicating within and infecting these plants, even with the confirmed Ctr-R status, thus indicating that some breakdown in resistance has occurred.

ACKNOWLEDGMENTS

This research was supported in full by funding from the New Zealand Foundation for Research Science and Technology. Expert technical advice and encouragement was gratefully received from Dr. Patricia Barkley (Department of Agriculture, NSW, Australia), Dr. C. L. Niblett (University of Florida, Gainesville, USA) and Dr. Richard Forster (HortResearch, Mt. Albert, New Zealand). Sequences of the Florida isolates T3 and T36 and the South African B7 were provided by Dr. C. L. Niblett. We thank Dr. Fred Gmitter, Jr. (Citrus Research and Education Center, University of Florida, Lake Alfred, Florida, USA.) for the testing for the resistance allele, and also thank Dr. Ross Crowhurst (HortResearch, Mt Albert, New Zealand) for his assistance with the deduced amino acid pileup and dendrogram construction.

LITERATURE CITED

1. Deng, Z., S. Haung, S. Xiao, and F. G. Gmitter, Jr.
   1997. Development and characterization of SCAR markers linked to the citrus tristeza virus resistance gene from *Poncirus trifoliata.* Genome 40: 697-704.
2. Devereux, J. P. Haerbeli, and O. Smithies
   1984. A comprehensive set of sequence analysis programmes for the VAX. Nucleic Acid Res. 12: 387-395.
3. Garnsey, S. M., H. C. Barrett, and D. J. Hutchison
   1987. Identification of citrus tristeza virus resistance in citrus relatives and it potential applications. Phytophylatica 19: 187-191.
4. Gillings, M., P. Broadbent, J. Indsto, and R. F. Lee
   1993. Characterisation of isolates and strains of citrus tristeza closterovirus using restriction analysis of the coat protein gene amplified by the polymerase chain reaction. J. Virol. Methods 44: 305-307.
5. Gmitter Jr., F. G., S.Y. Huang, S. Xiao, X. L. Hu, S. M. Garnsey, and Z. Deng
   1996. A localized linkage map of the citrus tristeza virus resistance gene region. Theor. Appl. Genet. 92: 688-695.
6. Higgins, D. G., A. Gleasby, and A. R. Fuchs
   1992. Clustal V: Improved software for multiple sequence alignment. Comp. Appl. Biosci. 8: 189-191.
7. Kano, T., T. Hiyama, T. Natsuaki, N. Imanishi, S. Okuda, and H. Ieki
   1998. Comparative sequence analysis of biologically distinct isolates of citrus tristeza virus in Japan. Ann. Phytopathol. Soc. Jpn. 64: 270-275.
8. Khan, I. A. and M. L. Roose
   1988. Frequency and characteristics of nucellar and zygotic seedlings in three cultivars of trifoliate orange. J. Amer. Soc. Hort. Sci. 113: 105-110.
9. Mestre, P. F., M. J. Asins, E. A. Carbonell, and L. Navarro
   1997. New Gene(s) involved in the resistance of *Poncirus trifoliata* (L) Raf. to citrus tristeza virus. Theor. Appl. Genet. 95: 691-695.
10. Mestre, P. F., M. J. Asins, J. A. Pina, E. A. Carbonell, and L. Navarro
    1997. Molecular markers flanking citrus tristeza resistance gene from *Poncirus trifoliata* (L.) Raf. Theor. Appl. Genet. 94: 458-464.
11. Tajima, F. and M. Nei
    1984. Estimation of evolutionary distance between nucleotide sequences. Mol. Biol. Evol. 1: 269-285.
12. Tanaka, H., S. Yamada, and J. Nakanishi
    1971. Approach to eliminating tristeza virus from citrus trees using trifoliate orange seedlings. Bull. Hort. Res. Sta. Japan, Section B No 11: 157-165.
13. Yoshida, T.
    1993. Inheritance of immunity to citrus tristeza virus of trifoliate orange in some citrus intergeneric hybrids. Bull. Fruit Tree Res. Sta. No 25: 33-43.