J Gen Plant Pathol (2006) 72:46–51
DOI 10.1007/s10327-005-0249-5

VIRAL AND VIROID DISEASES

Ali Barzegar · Haleh Hashemi Sohi
Heshmatollah Rahimian

Characterization of *Citrus tristeza virus* isolates in northern Iran

Received: February 23, 2005 / Accepted: July 11, 2005

Abstract The biological and molecular properties of four *Citrus tristeza virus* (CTV) isolates isolated from infected Satsuma trees imported from Japan, and growing in citrus groves in northern Iran (Mahdasht orchards, Mazandaran Province), were investigated. CTV-infected samples were collected from sweet orange trees and grafted onto Alemow (*Citrus macrophylla* Wester) seedlings. On indicator plants, these isolates produced various symptoms including vein clearing and stem pitting on Mexican lime, Alemow, and *Citrus hystrix*, and yellowing and stunting on sour orange and grapefruit seedlings. Citrus samples were also surveyed for CTV using serological tests. The coat protein (CP) gene of these isolates was amplified using specific primers, yielding an amplicon of 672 bp for all isolates. Sequence analysis showed 98%–99% sequence homology of Iranian isolates with the Californian CTV severe stem-pitting isolate SY568 and 97%–98% homology with the Japanese seedling yellows isolate NUagA. The Iranian isolates were compared by restriction fragment length polymorphism (RFLP) analysis of the CP amplicon for further classification.

Key words *Citrus tristeza virus* · Coat protein gene · Iranian isolates · Nucleotide sequencing · RFLP · Biological properties

A. Barzegar
Department of Biology, Faculty of Science, Razi University, Kermanshah, Iran

A. Barzegar · H.H. Sohi
Plant Biotechnology Department, National Institute of Genetic Engineering and Biotechnology, 15 Shahid Shafiee Alley, Ghods Street, Enghelab Avenue, Tehran, Iran

H. Rahimian (✉)
College of Agriculture, Mazandaran University, Khazarabad Street, Sari, Iran
Tel. +98-15-1382-2574; Fax +98-15-1382-2577
e-mail: rahimian.h@gmail.com

Introduction

Citrus tristeza virus (CTV), a member of the genus *Closterovirus* of the family *Closteroviridae*, has a single-stranded, positive sense RNA genome of 19.3 kb, which is organized into 12 open reading frames (ORFs) and encodes up to 17 proteins (Karazev et al. 1995). The virus particles are flexuous, about 2000 × 11 nm in size (Lee and Bar-Joseph 2000). The RNA is enclosed by two coat proteins; one covers 95% of the virion, and the other encapsidates the terminus of the virion (Febres et al. 1996).

Different methods have been used to differentiate and characterize the CTV isolates (Niblett et al. 2000). The nucleotide sequence of the coat protein (CP) gene of diverse strains of CTV has been determined, and sequence variability has been found to be related to the types of symptoms caused by CTV strains (Cevik et al. 1996; Penaranda et al. 1996). Restriction fragment length polymorphism (RFLP) patterns for the CP gene are used to distinguish CTV strains with different biological properties (Gillings et al. 1993, 1996; Roy et al. 2003). Recent molecular studies, including the complete sequencing of the genome of several isolates, revealed extensive differences among CTV isolates (Karasev et al. 1995; Suastika et al. 2001).

CTV causes one of the most economically important diseases of citrus trees. The spread of CTV occurs through the infected budwood and by aphids (Lee and Bar-Joseph 2000). The severity of symptoms and the extent of damage depend on the virus strain, the scion/rootstock combination, and the presence of vectors in the affected areas (Bar-Joseph and Lee 1989).

With over 200 105 ha of primarily sweet orange and mandarins (*Citrus reticulata* Blanco) under cultivation, Iran is among the major citrus-producing countries in the world. CTV was introduced into Iran via the import of 55 000 infected Satsuma mandarins (*Citrus unshiu* Marc.) on trifoliate orange (*Poncirus trifoliata* (L.) Raf.) rootstock from Japan to the Mahdasht orchards (Sari, Mazandaran Province, northern Iran) in the late 1960s (Bove 1995;

Rahimian 1994). The presence of CTV was initially recorded in 1977 in northern Iran (Ebrahim-Nesbeat and Nienhaus 1978), but the virus remained nontransmissible by the existing aphid species for about three decades (Ebrahimi et al. 1988). In 1997, the first presumptive evidence for the onset of natural spread of the virus was obtained when several declining sweet orange [*Citrus sinensis* (L.) Osb.] trees on sour orange (*Citrus aurantium* L.) rootstock were observed in the vicinity of the originally infected Satsuma trees (Rahimian et al. 2000). Transmissibility of a CTV isolate by the melon aphid (*Aphis gossypii* Glov.) in the Mazandaran region was subsequently demonstrated (Rahimian and Zarei 2002). The present study was undertaken to biologically and molecularly characterize four naturally transmitted CTV isolates from northern Iran. The RFLP patterns of these isolates were also analyzed and compared with other known CTV isolates in the world.

Materials and methods

Virus isolates

CTV isolates (M1, M2, and MP2) in this study were all recovered from mature, naturally infected sweet orange trees at various stages of decline near Mahdasht orchard (Sari, Mazandaran Province). The infected trees were several hundred meters away from the originally infected Satsuma mandarin trees on trifoliate orange rootstocks imported from Japan. An isolate (M1S) transmitted by *Aphis gossypii* from an infected sweet orange tree to a Mexican lime [*Citrus aurantifolia* (Christm.) Swing.] seedling under screenhouse conditions was also included in this study (Rahimian and Zarei 2002). All isolates were graft-inoculated onto Alemow (*Citrus macrophylla* Wester) seedlings and maintained in the greenhouse. Biological features of CTV isolates were determined by grafting budwood onto indicator plants. The citrus species and cultivars used as indicator plants were grown from seeds, except for sweet oranges (cv. Thomson navel), which were 6- to 12-month-old buddings of nucellar origin. Plants were grown in a heat-sterilized composite mix of bark shavings and sandy loam soil (1:1 v/v) in 4-l pots. Plants were kept in a greenhouse at 20°–30°C and fertilized regularly. Three or four plants were graft-inoculated with each inoculum using three pieces of bark strip from each donor tree. One seedling of each test plant was left uninoculated as control. Two to 3 weeks after graft-inoculation, the plants were cut back to stimulate a flush of new growth and examined weekly for typical symptoms of CTV for about 24 months. The plants were periodically sprayed with aphidicides and acaricides.

Serological assays

Collected samples were checked for CTV infection by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using polyclonal antibody (Garnsey and Cambra 1991). Graft-inoculated indicator plants were tested for infection by direct immunoprinting-ELISA using monoclonal antibodies 3DF1+3CA5 approximately 2 months after graft-inoculation (Cambra et al. 2000).

RNA extraction, cDNA synthesis, and polymerase chain reaction (PCR) amplification

Nucleic acids from about 0.25 mg of young bark and leaf midrib tissues were extracted by the method described of Hung et al. (2000). The nucleic acids were then resuspended in 30 µl TE buffer. Reverse transcription for synthesis of the first cDNA strand was carried out using the RevertAid first strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. To 5–8 µl of isolated RNA template, 20 pmol of the antisense primer (L28: 5′-CGCGGATCCTCAACGTGTGTTGAATTTC-3′) was added, the volume adjusted to 12 µl with double-distilled H_2O, and the mixture was incubated at 70°C for 5 min and then chilled on ice. Then, 4 µl of 5X reaction buffer (250 mM Tris-HCl pH 8.3, 250 mM KCl, 20 mM $MgCl_2$, and 50 mM dithiothreitol), 1 µl of 20 u/µl ribonuclease inhibitor (Fermentas) and 10 nM dNTP mix were added, and the reaction was incubated for 5 min at 37°C. After the addition of 1 µl of reverse transcriptase (RevertAid 200 u/µl, Fermentas), incubation was continued at 42°C for 60 min. PCR amplification was performed following cDNA synthesis in a 50-µl reaction containing 5 µl 10X PCR buffer (500 mM KCl, 200 mM Tris-HCl pH 8.4, 50 mM $MgCl_2$), 1.5 mM $MgCl_2$, 200 µM dNTP, 0.3 µM of each forward (U28: 5′-GGTTGGATCCATGGACGACGAAACAAAG-3′) and reverse primers, two units of *Taq* DNA polymerase, and 1 µl cDNA. Amplification included an initial denaturation at 94°C for 4 min, followed by 30 cycles of 1-min denaturation at 94°C, 1-min annealing at 59°C, and 1-min elongation at 72°C. A final elongation step of 20 min at 72°C was added.

Cloning, sequencing, and sequence analysis

The primer pairs U28 and L28 were designed for amplification of the 672-bp CTV coat protein gene. Amplified PCR products were separated by electrophoresis on a 1% agarose gel and directly ligated to pTZ57R/T vector (Fermentas) following purification using a High Pure PCR Product Purification kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. At least three clones of each PCR fragment were sequenced in both directions at MWG (Ebersberg, Germany) using automated ABI systems (Applied Biosystems) and standard M13 primer pairs. The acquired sequences were analyzed and compared with those of the CP gene sequence of other CTV isolates available from databases (Table 1) using the BLAST (NCBI) program (Altschul et al. 1990). A multiple sequence alignment was generated by the Clustal W program (Thompson et al. 1994). Phylogenetic trees were constructed using MegAlign program ver. 5.00 from the DNASTAR package (DNASTAR, Madison, WI, USA) (Burland 2000).

Restriction digestion and RFLP profile

The RFLP profile of the RT-PCR amplified CP gene of four isolates from Iran was determined with *Hin*fI and *Rsa*I restriction enzymes to partially discriminate and assess their variation (Gillings et al. 1993). The digested products were separated in a 12% tris-borate-EDTA polyacryamide gel (Sambrook et al. 1989) and visualized by silver staining (Brandt et al. 1996). To analyze the results of restriction analysis further, the four Iranian and other exotic isolates (Table 1) were mapped and compared using Mapdraw program ver. 5.00 (DNASTAR; Burland 2000).

Results

Reactions of host plants

CTV infection was detected in both original and graft-inoculated samples by either polyclonal antiserum in DAS-ELISA or monoclonal antibodies in direct immunoprinting-ELISA. All Mexican lime, Alemow, and *Citrus hysterix* seedlings inoculated directly with buds from declining field trees (M1, M2, and MP2) had new leaves with vein clearing and moderate to severe stem pitting and stunting of new growth. Some Mexican lime seedlings developed vein corking and dieback as well. Alemow seedlings also had occasional vein corking. The aphid-transmitted isolate (M1S) also induced vein corking and dieback in Mexican lime. Sour orange and grapefruit seedlings inoculated with the field isolates (M1, M2, MP2) as well as with the aphid-transmitted isolate (M1S) had severe yellowing and stunting of new growth. In some sour orange seedlings, the yellows reaction was followed by vein clearing and severe leaf stunting and cupping, and the growth of the subsequent flushes was severely arrested within 12 months of inoculation. Sweet orange budding on sour orange rootstocks grew poorly and was chlorotic, and developed decline symptoms by 9–12 months after inoculation.

Table 1. *Citrus tristeza virus* coat protein gene sequences used in restriction fragment length polymorphism, sequence analysis, and phylogenetic analysis

Isolates	Country	Accession number
Y568	CA, USA	AF001623
T36	FL, USA	U16304
T385	Spain	Y18420
VT	Israel	U56902
NUagA	Japan	AB046398
CTV-B	India	AF501867
13C	Portugal	AF184113
Cheju Island	South Korea	AF249279
PB61	Australia	AJ297702
Qaha	Egypt	AY340974
Jordan	Jordan	AY550252

PCR amplification, cloning, and sequence analysis

PCR amplification of the CP gene produced a fragment of the same size for all isolates in 1% agarose gel. The sequence database search matched these products to the 672-bp CP gene with ATG and TGA as the start and stop codons, respectively. Multiple sequence alignment and resulting sequence identity showed 98%–99.9% homology among the CP gene sequences of the four Iranian isolates tested (Fig. 1). The highest level of homology was found among Iranian isolates and SY568 (98%–99%), Cheju Island, and NUagA (97%–98%). A phylogenetic tree constructed from the nucleotide sequences of Iranian and other isolates showed a close relationship between Iranian isolates and the California SY568 isolate (Fig. 2). There was

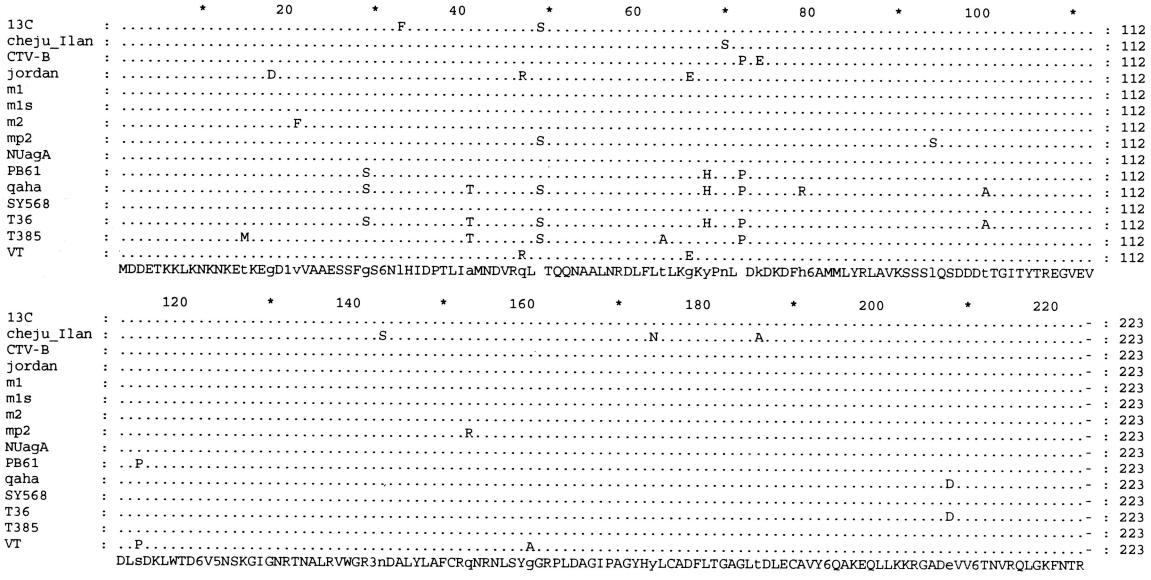

Fig. 1. Multiple alignment of deduced amino acid sequences of the coat protein (CP) genes of Iranian *Citrus tristeza virus* (CTV) isolates and other isolates from various geographical origins

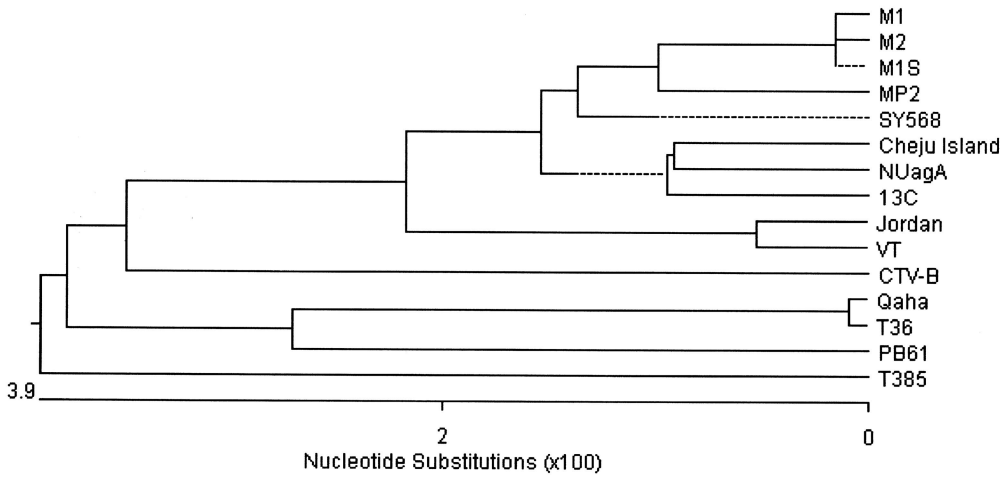

Fig. 2. Phylogenetic tree constructed by neighbor-joining method and Clustal W program showing the genetic relationship among CP gene sequences of Iranian and other exotic CTV isolates

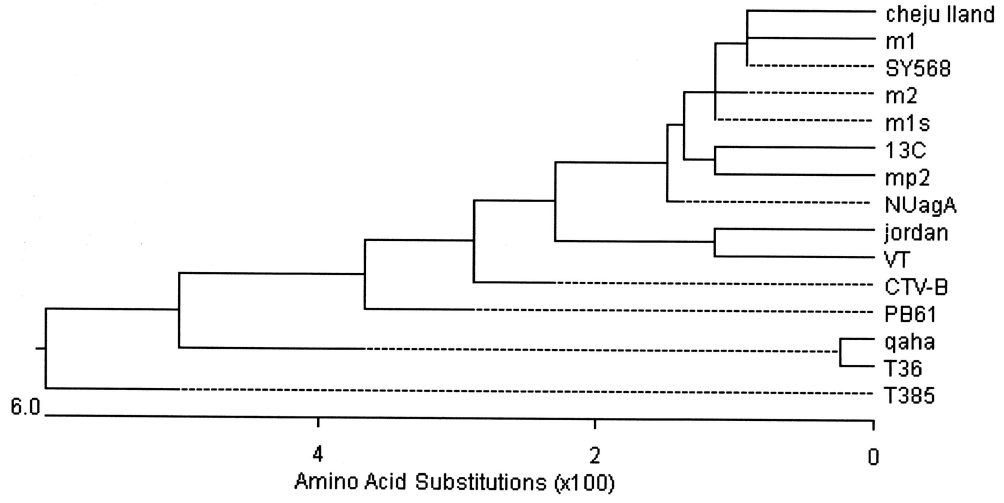

Fig. 3. Phylogenetic tree constructed by neighbor-joining method and Clustal W program showing the genetic relationship among deduced CP amino acid sequences of Iranian and other exotic CTV isolates

98%–99.7% homology among Iranian isolates at the CP gene amino acid sequence level. The deduced CP gene amino acid sequence of the Iranian CTV isolates had high similarity (97%–99%) to SY568, Cheju Island, 13C, and NUagA isolates (Fig. 3). Interestingly, the mild T385 isolate was separately clustered in a main branch with the least similarity to Iranian isolates. CP genes of all Iranian isolates had a thymidine base (T) at the 371 position of the nucleotide sequence corresponding to phenylalanine (F) at position 124 in the amino acid sequence. In both phylogenetic trees, the Iranian MP2 isolate, recovered from a region far from the other isolates, had more differing sequences with three amino acid substitutions, including $S_{49,94}$ and R_{153}, relative to the other Iranian isolates (Fig. 1).

Restriction digestion and RFLP profile

Each of the *Hin*fI and *Rsa*I restriction enzymes created four RFLP groups (Figs. 4, 5). Complementary analysis based on the acquired sequences showed that with *Hin*fI, all four Iranian isolates produced a common restriction pattern that included six fragments of 299, 171, 73, 49, 42, and 38 bp; these isolates comprised the RFLP group I along with SY568, VT, 13C, Jordan, CTV-B, and Cheju Island isolates (Figs. 4, 5). RFLP group II with four restriction sites contained the NUagA, and the mild T385 isolates. The T36 and Qaha strains were placed in RFLP group III, and PB61 comprised the fourth RFLP group with two restriction sites. Similarly, four RFLP groups with two to five restriction sites at positions 147, 313, 396, 577, and 581 were also identified using the *Rsa*I restriction enzyme (Figs. 4, 5). The Iranian MP2 isolate with five restriction sites produced RFLP group I. The 13C, Cheju Island, NUagA, SY568, T36, Qaha, Jordan, PB61, and VT isolates with four restriction sites comprised the RFLP group II. Group III, with three restriction sites, contained M1, M1S, M2, and T385 strains. RFLP group IV with two restriction sites included the CTV-B isolate.

Discussion

In this study of biological and molecular properties of four Iranian CTV isolates from northern Iran, all the isolates

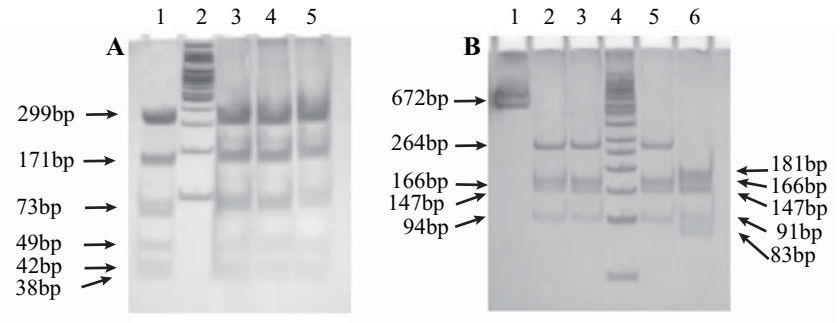

Fig. 4. **A** Restriction digestion of CP gene of four Iranian CTV isolates by *Hin*fI. Lanes *1*, *3*, *4*, *5*, MP2, M1S, M1, and M2, respectively. Lane *2*, 100-bp ladder (Fermentas). **B** Restriction digestion of CP gene by *Rsa*I. Lane *1*, amplified uncut CP gene. Lane *4*, 50-bp DNA ladder. Lanes *2*, *3*, *5*, and *6*, digested products of M2, M1, M1S, and MP2, respectively

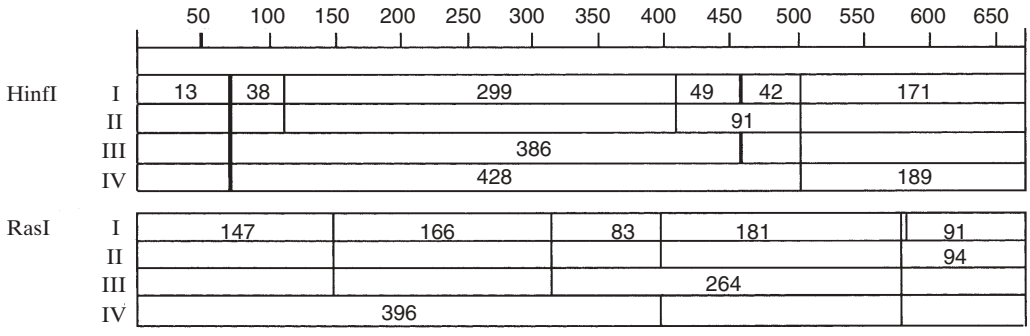

Fig. 5. Comparison of restriction fragment length polymorphism (RFLP) profiles of the CP gene sequences of Iranian and other exotic CTV isolates created using *Hin*fI and *Rsa*I endonuclease. Four RFLP groups (I to IV) were identified using each restriction enzyme. *Vertical lines* in *upper* and *lower* boxes represent the *Hin*fI and *Rsa*I restriction sites, respectively. The resulting digested products were indicated between restriction sites in base pairs

produced symptoms such as stem pitting on Mexican lime, Alemow, and *Citrus hystrix*. Severe yellowing and stunting of new growth occurred on sour orange and grapefruit seedlings and the strains were considered to be severe.

The phylogenetic tree based on both the CP gene nucleotide sequence and its deduced amino acid of Iranian isolates and other exotic isolates revealed that all the Iranian isolates were closely related to the SY568, Cheju Island, and NUagA isolates (>97% identity), and were least similar to T385 mild strain. The close relationships of the Iranian CTV isolates to the Californian SY568 severe strain confirmed a previously described association between symptom severity and CP gene sequence variability (Cevik et al. 1996; Penaranda et al. 1996; Yang et al. 1999). The deduced amino acid sequence of the MP2 isolate had four amino acid substitutions relative to the other three isolates (Fig. 1).

Pappu et al. (1993a,b) found that phenylalanine (F) at CP position 124, which is responsible for MCA-13 monoclonal antibody reactivity, is conserved among severe CTV isolates that cause either decline, stem-pitting, or seedling yellows in citrus. On the other hand, some Japanese mild isolates containing phenylalanine (F) at 124 react with MCA-13 (Kano et al. 1998). Our results showed that the CP gene of Iranian severe isolates contained thymidine (T) at position 371 corresponding to phenylalanine (F) at position 124.

The RFLP grouping of these isolates and other exotic isolates showed the occurrence of four RFLP groups for each of the *Hin*f1 and *Rsa*I enzymes. Iranian isolates had a common RFLP pattern that included five restriction sites with *Hin*fI that were similar to California SY568 isolate, Israel VT isolate, Portugal 13C isolate, Jordan, and South Korea Cheju Island isolate. When *Rsa*I was used to group these isolates, all the Iranian isolates except MP2 had three restriction sites and were placed in a group with the T385 mild strain (Fig 5). The presence of Iranian severe isolates and T385 in a group shows that unlike *Hin*f1, *Rsa*I is not a good enzyme for assessing the correlation between RFLP grouping and symptom severity of CTV isolates (Gillings et al. 1993). Nevertheless, the resulting RFLP grouping shows that, for whatever reason, *Rsa*I may be a suitable complementary marker for discriminating these isolates because it differentiates the MP2 isolate from other Iranian isolates and from exotic isolates. Moreover, the MP2 isolate has a unique *Rsa*I restriction site at position 581 of the nucleotide sequence. The MP2 isolate recovered from a geographically different region had more differences in its nucleotide sequence and a distinct RFLP profile (using *Rsa*I) relative to the other Iranian isolates. These data show that the geographical distribution of CTV in relation to aphid transmission might result in increased genetic divergence (Albiach-Marti et al. 2000; Ayllon et al. 1999; d'Urso et al. 2000).

This study reveals the presence of severe strains of CTV in northern Iran with some biological and molecular properties similar to those of California SY568 and Japanese

NUagA. The presence of severe strains of CTV in northern Iran where sour orange still remain the dominant rootstock, places the citrus industry of this entire region at high risk for an imminent, destructive outbreak of tristeza, especially with the presence of the vector *Aphis gossypii* in this area.

Acknowledgments The authors thank Esmat Jourabchi for her skillful technical assistance. We also thank Dr. Sadeghi for his assistance in providing the software. Monoclonal antibodies (3DF1 and 3CA5) were kindly provided by Mariano Cambra, Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain. GenBank accession numbers: AY190048, AY490208, AY490207, and AY490206.

References

Albiach-Marti MR, Guerri J, Hermoso De Mendoza A, Laigret F, Ballester-Olmos JF, Moreno P (2000) Aphid transmission alters the genomic and defective RNA populations of *Citrus tristeza virus* isolates. Phytopathology 90:134–138

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Ayllon MA, Rubio L, Moya A, Guerri J, Moreno P (1999) The haplotype distribution of two genes of citrus tristeza virus is altered after host change or aphid transmission. Virology 255:32–39

Bar-Joseph M, Lee RF (1989) Citrus tristeza virus. AAB description of plant viruses No. 353 Commonw. Mycol Inst/Assoc Appl Biol Kew, Surrey, England

Bove JM (1995) Virus and virus-like disease of citrus in the near-east region. Food and Agriculture Organization, Rome, pp 149–173

Brandt J, Bylsma FW, Gross R, Stine OC, Ranen N, Ross CA (1996) Trinucleotide repeat length and clinical progression in Huntington's disease. Neurology 46:527–531

Burland TG (2000) DNASTAR's lasergene sequence analysis software. Methods Mol Biol 132:71–91

Cambra M, Gorris MT, Roman MP, Terrada E, Garnsey SM, Camarasa E, Olmos A, Colomer M (2000) Routine detection of citrus tristeza virus by direct immunoprinting-ELISA method using specific monoclonal and recombinant antibodies. In: Proc 14th Conf Inter Organ Citrus Virol, Riverside, CA, pp 34–41

Cevik B, Pappu SS, Pappu HR, Tight D, Benscher D, Futch SH, Rucks P, Lee RF, Niblett CL (1996) Molecular cloning and sequencing of coat protein genes of citrus tristeza virus isolated from Meyer lemon and Homely Tangor trees in Florida. In: Proc 13th Conf Intern Organ Citrus Virol, Riverside, CA, pp 47–53

d'Urso F, Ayllon MA, Rubio L, Sambade A, Hermoso de Mendoza A, Guerri J, Moreno P (2000) Contribution of uneven distribution of genomic RNA variants of citrus tristeza virus (CTV) within the plant to changes in the viral population following aphid transmission. Plant Pathol 49:288–294

Ebrahimi Y, Anvari F, Darjani R, Shakholleslami K (1988) Non transmission of tristeza virus by aphids in northern Iran during last eighteen years. In: Proc 10th Conf Intern Organ Citrus Virol, Riverside, CA, pp 65–67

Ebrahim-Nesbeat F, Nienhaus F (1978) Occurrence of citrus tristeza virus in Iran. Z Pflanzenk Pflanzen 85:308–312

Febres VJ, Ashoulin L, Mawassi M, Frank A, Bar-Joseph M, Manjunath KL, Lee RF, Niblett CL (1996) The p27 protein is present at the end of citrus tristeza virus particles. Phytopathology 86:1331–1335

Garnsey SM, Cambra M (1991) Enzyme-linked immunosorbent assay (ELISA) for citrus pathogens. In: Roistacher CN (ed) Graft-transmissible diseases of citrus. Handbook for detection and diagnosis. Food and Agriculture Organization, Rome, pp 193–216

Gillings M, Broadbent P, Indsto J, Lee RF (1993) Characterization 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 44:305–317

Gillings M, Broadbent P, Indsto J (1996) Restriction analysis of amplified CTV coat protein cDNA is a sensitive and rapid method for monitoring and controlling CTV infections. In: Proc 13th Conf Intern Organ Citrus Virol, Riverside, CA, pp 25–37

Hung TH, Wu ML, Su HJ (2000) A rapid method based on the one-step reverse transcriptase-polymerase chain reaction (RT-PCR) technique for detection of different strains of citrus tristeza virus. J Phytopathol 148:469–475

Kano T, Hiyama T, Natsuaki T, Imanishi N, Okuda S, Ieki H (1998) Comparative sequence analysis of biologically distinct isolates of citrus tristeza virus in Japan. Ann Phytopathol Soc Jpn 64:270–275

Karasev AV, Boyko VP, Gowda S, Nikolaeva OV, Hilf ME, Koonin EV, Niblett CL, Cline K, Gumpf DJ, Lee RF, Garnsey SM, Lewandowski DJ, Dawson WO (1995) Complete sequence of citrus tristeza virus RNA genome. Virology 208:511–520

Lee RF, Bar-Joseph M (2000) Tristeza. In: Timmer LW, Garnsey SM, Graham JH (eds) Compendium of citrus diseases, 2nd edn. APS, St Paul, MN, pp 61–63

Niblett CL, Genc H, Cevik B, Halbert S, Brown L, Nolasco G, Bonacalza B, Manjunath KL, Febres VJ, Pappu HR, Lee RF (2000) Progress on strain differentiation of citrus tristeza virus and its application to the epidemiology of citrus tristeza disease. Virus Res 71:97–106

Pappu HR, Pappu SS, Manjunath KL, Lee RF, Niblett CL (1993a) Molecular characterization of a structural epitope that is largely conserved among severe isolates of a plant virus. Proc Natl Acad Sci USA 90:3641–3644

Pappu HR, Pappu SS, Niblett CL, Lee RF, Civerolo E (1993b) Comparative sequence analysis of the coat protein of biologically distinct citrus tristeza closterovirus isolates. Virus Genes 7:255–264

Penaranda J, Acosta O, Guzman-Barney M, Alegria A, Pappu HR, Pappu SS, Manjunath KL, Febres VJ, Lee RF, Niblett CL (1996) Incidence and characterization of mild and severe isolates of citrus tristeza virus from Colombia. In: Proc 13th Conf Inter Org Citrus Virol, Riverside, CA, pp 71–77

Rahimian H (1994) Strains of citrus tristeza virus in Mazandaran. Iran J Plant Pathol 30:35–36

Rahimian H, Zarei A (2002) Characteristics of some aphid-transmitted isolates of *Citrus tristeza virus* in the north of Iran. Proceedings of the 15th Iran Plant Protection Congress, pp 257–258

Rahimian H, Alavi V, Shaygan J, Hadizadeh A (2000) Spread of citrus tristeza virus by *Aphis gossypii* in the north of Iran. Iran J Plant Pathol 36:103

Roy A, Ramachandran P, Brlanskey RH (2003) Grouping and comparison of Indian citrus tristeza virus isolates based on coat protein gene sequences and restriction analysis patterns. Arch Virol 148:707–722

Sambrook J, Fritsh E, Maniatis T (1989) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Suastika G, Natsuaki T, Terui H, Kano T, Ieki H, Okuda S (2001) Nucleotide sequence of Citrus tristeza virus seedling yellows isolate. J Gen Plant Pathol 67:73–77

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties, and weight matrix choice. Nucleic Acids Res 22:4073–4080

Yang ZN, Mathews DM, Dodds JA, Mirkov TE (1999) Molecular characterization of an isolate of Citrus tristeza virus that causes severe symptoms in sweet orange. Virus Genes 19:131–142