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Grouping and comparison of Indian citrus tristeza virus isolates based on coat protein gene sequences and restriction analysis patterns

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Summary. *Citrus tristeza virus* (CTV) is an aphid-transmitted closterovirus, which causes one of the most important citrus diseases worldwide. Isolates of CTV differ widely in their biological properties. CTV-infected samples were collected from four locations in India: Bangalore (CTV-B), Delhi (CTV-D), Nagpur (CTV-N), and Pune (CTV-P), and were maintained by grafting into Kagzi lime (*Citrus aurantifolia* (Christm. Swing.). All isolates produced typical vein clearing and flecking symptoms 6–8 weeks after grafting. In addition, CTV-B and CTV-P isolates produced stem-pitting symptoms after 8–10 months. The CTV coat protein gene (CPG) was amplified by RT-PCR using CPG specific primers, yielding an amplicon of 672 bp for all the isolates. Sequence analysis of the CPG amplicon of all the four Indian isolates showed 93–94% nucleotide sequence homology to the Californian CTV severe stem pitting isolate SY568 and 92–93% homology to the Japanese seedling yellows isolate NUagA and Israeli VT p346 isolates. In phylogenetic tree analysis, Indian CTV isolates appeared far different from other isolates as they formed a separate branch. Comparison among the Indian isolates was carried out by restriction analysis and restriction fragment length polymorphism (RFLP). Specific primers to various genome segments of well-characterized CTV isolates were used to further classify the Indian CTV isolates.

Introduction

Citrus tristeza virus (CTV), an aphid-transmitted closterovirus, has been the most important viral pathogen of citrus for the last ninety years [2]. Diseases caused by CTV have been reported from various parts of India, but are not well characterized.

In India, the citrus growing areas are distributed into four geographic zones: Northwest, Northeast, Central and South. CTV, though present in the Northwest zone, shows negligible spread due to very low populations of the most efficient aphid vector, *Toxoptera citricida* (Kirkaldy) [1]. In the Central zone states of Maharashtra and Madhya Pradesh, CTV is present either singly or in mixed infections with the huanglongbing bacterium (HLB). In the Northeast where *T. citricida* is prevalent, CTV is a major problem. In the South zone, tristeza is also one of the major diseases and occurs as a mixed infection with citrus yellow mosaic badnavirus (CYMBV), citrus ringspot virus (CRSV), and/or with the HLB [1].

In this study, we compared the four Indian CTV isolates from the South, Central and Northwest geographical zones based on the homology of their coat protein gene (CPG) nucleotide and amino acid sequences. We also analyzed the RFLP patterns and genotypes of these four isolates and compared these results with other known CTV isolates of the world.

Materials and methods

Virus isolates

Four isolates of CTV representing three geographical zones in the citrus growing belt of India were used in this study: CTV-B, the Bangalore isolate from the South zone, CTV-N, a Nagpur isolate, and CTV-P, a Pune isolate from the Central zone, and CTV-D, a Delhi isolate from Northwest zone. All of the isolates originated from Kagzi lime (*Citrus aurantifolia* (Christm. Swing.) except for isolate CTV-N, which was from a Mosambi sweet orange (*C. sinensis* (L.) Osb.) tree. The biological activity of the virus isolates was determined by grafting budwood on 5-month-old healthy Kagzi lime, Mosambi sweet orange, sour orange (*C. aurantium* L.), and grapefruit (*C. paradisi* Macf.), Etrog citron (*C. medica* L.), Nagpur mandarin (*C. reticulata* Blanco), rough lemon (*C. jambhiri* Lush), Rangpur lime (*C. limonia* Osb.), trifoliate orange (*Poncirus trifoliata* (L.) Raf.), and *Severinia buxifolia* plants. Seedlings of Kagzi lime were used for maintenance and multiplication of the virus isolates.

Serological assays

CTV inoculated plants were checked for infection using double antibody sandwich (DAS)-ELISA [4] using polyclonal antibody 1052 approximately 2 months after graft inoculation. All ELISA positive plants were further tested using monoclonal antibody MCA-13 in DAS-ELISA tests [14].

Nucleic acid extractions, reverse transcription and PCR amplification

Total RNA from CTV infected and healthy leaves and bark of Kagzi lime plants was isolated using the RNeasy Plant Mini Kit (QIAGEN, Valencia, CA) following manufacturer's protocol. All isolates were evaluated with the multiple molecular marker method of Hilf and Garnsey [7] for determination of genotype relationship to the Florida isolates, T30, T36, T3, and VT Israel isolate. The isolated RNA was used to synthesize the first strand cDNA. Ten to 15 μl of RNA were added to 2 μl of 10 μM of the antisense primer, microfuged for 10 sec, incubated for 10 min at 70 °C, and instantly chilled on ice for 5 min. A mixture of 10X 1st strand buffer, 0.1 M DTT, 10 mM dNTP (Promega, Madison, WI) was prepared and incubated for 2 to 3 min at 42 °C and removed to room temperature (24 °C). Twenty units of Superscript^{TM} II RNase H-Reverse transcriptase (Invitrogen, Carlsbad, CA) and 40 units of r-RNasin (Promega) were

added, and microfuged for 10 sec. The mixture was then equally distributed to each tube containing the template RNA, and the total contents of 50 μl was gently mixed. The tube was incubated at 50 °C for 1 h followed by 72 °C for 15 min and then held at 4 °C for 10 min. The prepared cDNA was purified using a QIAquick PCR purification kit (QIAGEN). Three to five μl of purified cDNA was amplified in a 50 μl reaction volume containing 5 units of Taq DNA polymerase (Promega), 2.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Promega) and 10 mM each of sense and antisense primers. PCR was performed in a thermal cycler (Model HBPX 110, PCR Express, Hybaid Limited, Middlesex, UK) using the following parameters; one cycle at 94 °C for 2 min, 30 cycles at 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 45 sec, followed by one cycle at 72 °C for 5 min. PCR products were analyzed by electrophoresis on 0.8% agarose gel containing 200 ng of ethidium bromide per ml.

Cloning, sequencing and sequence analysis

The primer T36CP, which amplifies the CPG of all the CTV isolates [7], was used to amplify the CPG of the four Indian isolates. Amplified products were purified after electrophoresis using a gel purification kit (QIAGEN). Purified DNA fragments were ligated into a pGEM-T Easy Vector System II using the Original TA Cloning Kit (Promega). The clones were sequenced in both directions by the dideoxynucleotide chain termination method using the T7 and SP6 primers following standard protocol at the DNA Sequencing Core Lab, Gainesville, FL [15]. For sequence determination, at least three clones of each isolate were sequenced and analyzed using the computer program CLUSTAL X [17] and GeneDoc version 2.6.002. The phylogenetic relationships of the CPG sequences with nine exotic CTV isolates (Table 1) and other Indian isolates [10] were generated using the program TreeView (Win32) version 1.6.6.

Nucleotide sequence accession numbers

The complete nucleotide CPG sequence of Indian isolates CTV-B, CTV-D, CTV-N, and CTV-P were deposited in the GenBank database under accession numbers AF501867 to AF501870, respectively.

Restriction analysis

Selected recombinant plasmid DNA's were subjected to digestion using *Eco*RI restriction enzyme at 37 °C for 2 h and then electrophoresed in 0.8% agarose gel to determine the insert

Table 1. CTV coat protein gene sequences from gene bank used in RFLP, sequence analysis, and phylogenetic tree relationships

CTV isolates	Country	Accession number
T36	FL, U.S.A.	U16304
T30	FL, U.S.A.	AF260651
T385	Spain	Y18420
SY568	CA, U.S.A.	AF001623
p346	Israel	U56902
NUagA	Japan	AB046398
PB61	Australia	AJ297702
Cheju	South Korea	AF249279
28C	Portugal	AF184118

size. The insert of 672 bp was cut and purified using a gel purification kit (QIAGEN). In order to determine the restriction sites and generate a restriction map, the gel purified insert was digested with *Bst*EII, *Eco*RI, *Eco*RV, *Hin*dIII, and *Pvu*II restriction enzymes. The products were resolved using polyacrylamide gel electrophoresis and fragment sizes were assessed in comparison with 100 bp MW markers (Promega). The amplified CPG also was digested with *Hin*fI and *Rsa*I restriction enzymes, the product separated in a 10% TBE polyacrylamide gel [6], and visualized by staining with silver nitrate [9]. To further confirm the restriction analysis results, the four Indian isolates and the known nine exotic and four Indian CTV isolates CPG sequences were mapped and compared using the computer program SDSC Biology Workbench 3.2 (http://workbench.sdsc.edu/).

Results

Biological activity of the CTV isolates

The four CTV isolates produced variable host reactions on different citrus species ranging from vein clearing, flecking, bushy, chlorosis, yellowing, stem pitting, and stunting (Table 2). All the isolates produced vein flecking in Kagzi lime,

Table 2. Biological activity of CTV isolates by graft inoculation on different host species

Host species	Symptoms*			
	CTV-B	CTV-D	CTV-N	CTV-P
Etrog citron (*Citrus medica* L.)	St, SP	St	St, B	St
Grapefruit (*C. paradisi* Macf.)	St, SP	St, B	St, Yl	St, B
Kagzi lime (*C. aurantifolia* (Christm. Swing.)	Fl, SP	VC, Fl	VC, Fl	VC, Fl, SP
Nagpur mandarin (*C. reticulata* Blanco)	Sl	Sl	Sl	Sl
Rangpur lime (*C. limonia* Osb.)	Sl	Sl	Sl	Sl
Rough lemon (*C. jambhiri* Lush)	Sl	Sl	Sl	Sl
Sour orange (*C. aurantium* L.)	Ch, SP	Ch, St	St, Yl	St, SP
Sweet orange (*C. sinensis* (L.) Osb.)	SP	Sl	St	St, SP
Trifoliate orange (*Poncirus trifoliata* (L.) Raf.)	Sl	Sl	Sl	Sl
Severinia buxifolia	Sl	Sl	Sl	Sl

**B* Bushy; *Ch* Chlorosis; *Fl* Flecking; *Sl* Symptomless; *SP* Stem Pitting; *St* Stunting; *VC* Vein Clearing; *Yl* Yellowing

but the isolates CTV-D, CTV-N, and CTV-P also caused vein clearing. Isolates CTV-B and CTV-P produced stem pitting in Kagzi lime, sour orange, and sweet orange seedlings. Isolate CTV-B also produced stem pitting on grapefruit and Etrog citron. A seedling yellows reaction was produced by CTV-N in grapefruit and sour orange. Five hosts *viz*., Rangpur lime, trifoliate orange, and *S. buxifolia*, Nagpur mandarin, and rough lemon were symptomless. Nagpur mandarin, *S. buxifolia*, and rough lemon were negative in ELISA.

Serological assays

All four Indian isolates were detected using CTV polyclonal antibody 1052 and CTV monoclonal antibody MCA-13.

Molecular profile of Indian CTV isolates

Molecular profiles of the four Indian isolates were obtained using the 11 sets of primers described by Hilf and Garnsey [7] and were compared to the standard genotype isolates (Florida decline isolate T36, Florida mild isolate T30, Florida severe isolate T3, and the Israeli severe VT isolate B199). In addition, Florida CTV isolate, FS627 containing a mixture of three genotypes T36, T30, and VT, also was used for comparative studies (Table 3). The molecular profile of CTV-P and CTV-N were identical and were a mixture of T30 and VT genotypes. CTV-D contained only the VT genotype and the CTV-B produced a non-standard profile of T36k17, T30k17, and VTk17 molecular markers (Table 3).

Cloning, sequencing and sequence analysis

Comparison of CPGs of Indian isolates with the CPGs of other isolates from different geographic areas was determined using sequence analysis. The 672 bp

Table 3. Molecular marker profiles of Indian CTV isolates, according to Hilf et al. [8]

	RT-PCR Markers										
Isolates	T36CP	T36POL	T36-5'	T36k17	T30POL	T30-5'	T30k17	VTPOL	VT-5'	VTk17	T3k17
T36	(+)	(+)	(+)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
T30	(+)	(-)	(-)	(-)	(+)	(+)	(+)	(-)	(+)	(+)	(-)
T3	(+)	(-)	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(+)
B199(VT)	(+)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)	(-)
CTV-B	(+)	(-)	(-)	(+)	(-)	(-)	(+)	(-)	(-)	(+)	(-)
CTV-D	(+)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)	(-)
CTV-N	(+)	(-)	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)
CTV-P	(+)	(-)	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)
FS627	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)

RT-PCR amplified cloned product was sequenced for each of the four Indian isolates. The analysis revealed the product contained the complete CPG (223 amino acids) (Fig. 1). The predicted amino acid sequence based on the nucleotide sequences of all the Indian isolates is shown in Fig. 2. The comparative analysis showed a high degree of homology in nucleotide and amino acid sequence among the different isolates. All the Indian isolates showed 93–94% nucleotide sequence homology to the California severe stem pitting isolate SY568 and 92–93% homology to the Japan seedling yellows isolate NUagA and Israeli VT isolate p346. There was 97–98% similarity at nucleotide level among the four Indian isolates in this study. Homology of these isolates also were compared with sequence available information on the other Indian isolates [10] and showed a 97–98% homology with Indian isolates B194, B220, and B227, and 93% with isolate B165.

A phylogenetic tree, generated using nucleotide sequences from the Indian and other CTV isolates, produced four main clusters (Fig. 3). The four studied Indian isolates and Indian isolates B194, B220, and B227 occurred in the same cluster and are well separated from all the other characterized CTV isolates. One cluster included the mild isolates T30 and T385; another cluster was occupied by isolates T36 and PB61, and the fourth cluster included B165, p346, SY568, NUagA, 28C, and Cheju Island isolates. The other characterized Indian CTV isolates B194, B220, and B227 most closely resembled the four Indian isolates in the study; however, Indian isolate B165 was in a different cluster (Fig. 3).

The deduced amino acid sequence comparison of the Indian isolates showed 96–98% homology to California severe stem pitting isolate SY568, 95–97% homology with Japan seedling yellows NUagA isolate, and 95–96% with Israeli VT isolate p346. There was 95–99% similarity at amino acid level among all the four Indian isolates studied. CPGs of the Indian isolates CTV-B, CTV-N, and CTV-P had a thymidine (T) at position 371 of the nucleotide sequence, corresponding to phenylalanine (F) at amino acid position 124 (Figs. 1, 2). The CTV-D isolate had cytosine (C) at 371st nucleotide position, which represents serine (S) as the 124th amino acid (Figs. 1, 2).

A dendrogram tree was constructed for showing the clustering relationship among the deduced amino acid sequences of the CPG of the four Indian CTV isolates studied. CTV-D, CTV-N, and CTV-P showed 95–97% similarity at the amino acid level, but CTV-B was more closely related to the Californian isolate SY568 and the Japanese isolate NUagA, and occupied a different branch (Fig. 4).

Restriction analysis

Restriction analysis of the PCR amplified CTV CPGs was performed using specific enzymes. The resultant RFLPs were used to determine variation among isolates and their relatedness to other CTV strains. The restriction enzymes *Bst*EII, *Eco*RI, *Eco*RV, *Hin*dIII, and *Pvu*II were used. The *Bst*EII site was common for all the isolates and produced 283 bp and 389 bp fragments. All the four Indian isolates have a single restriction site for *Eco*RV and *Hin*dIII and exhibited 157, 515 bp and

Fig. 1 (*continued*)

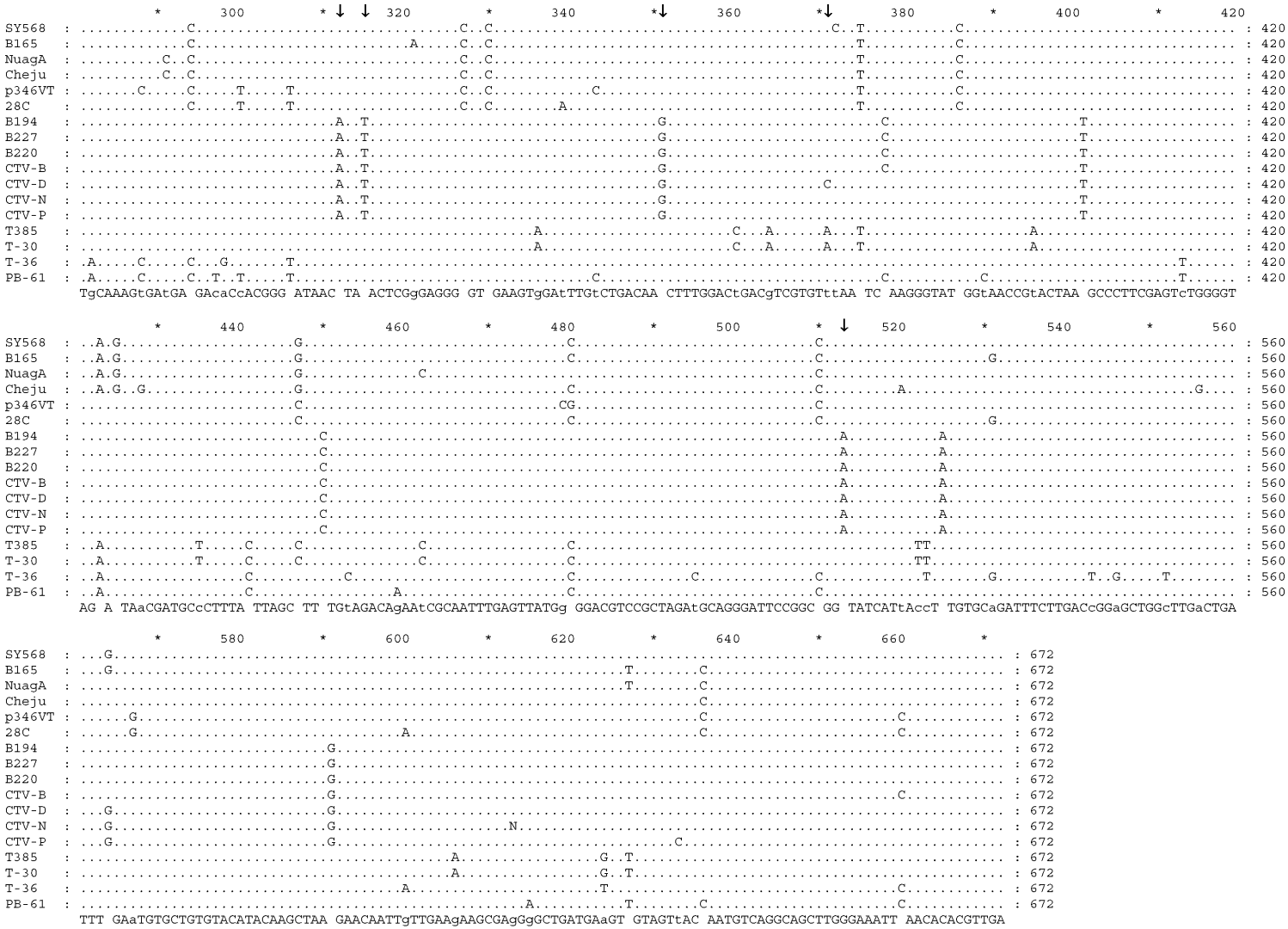

Fig. 1. Nucleotide sequence alignment of the coat protein gene regions of the genomic RNAs of citrus tristeza virus isolates from India and other geographic areas. Nucleotides that differ from the consensus sequence are shown; dots indicate where sequence identity occurs. Arrows denote positions where the presence of a particular nucleotide either created or deleted some restriction sites which were conserved for specific genotypes

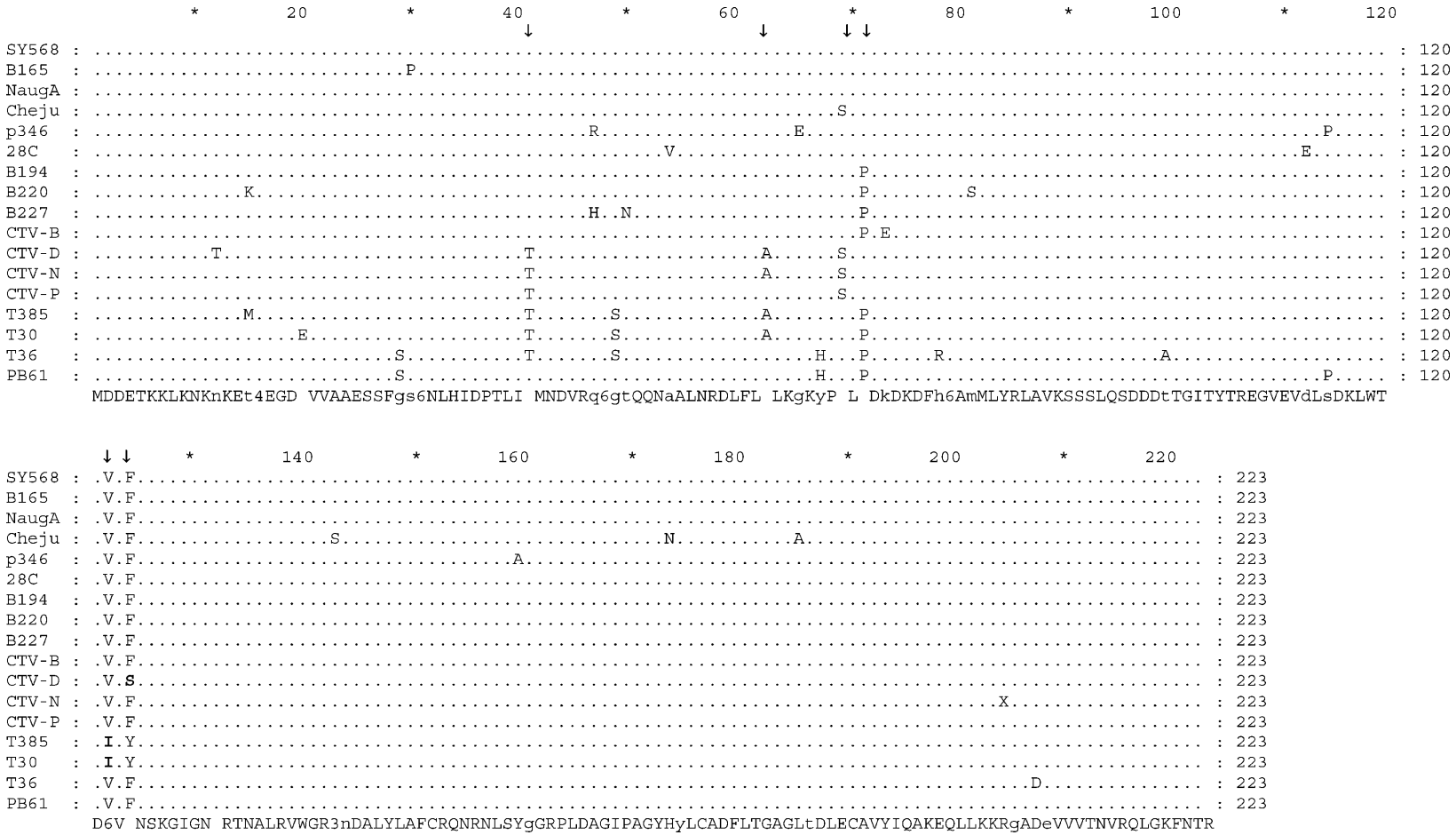

Fig. 2. Alignment of amino acid sequences of the CPGs of citrus tristeza virus isolates from India and other geographic areas, as predicted based on the nucleotide sequences shown in first 375 nt in Fig. 1. Dots indicate where sequence identity occurs. Arrows denote the positions where specific amino acids were conserved for specific genotypes

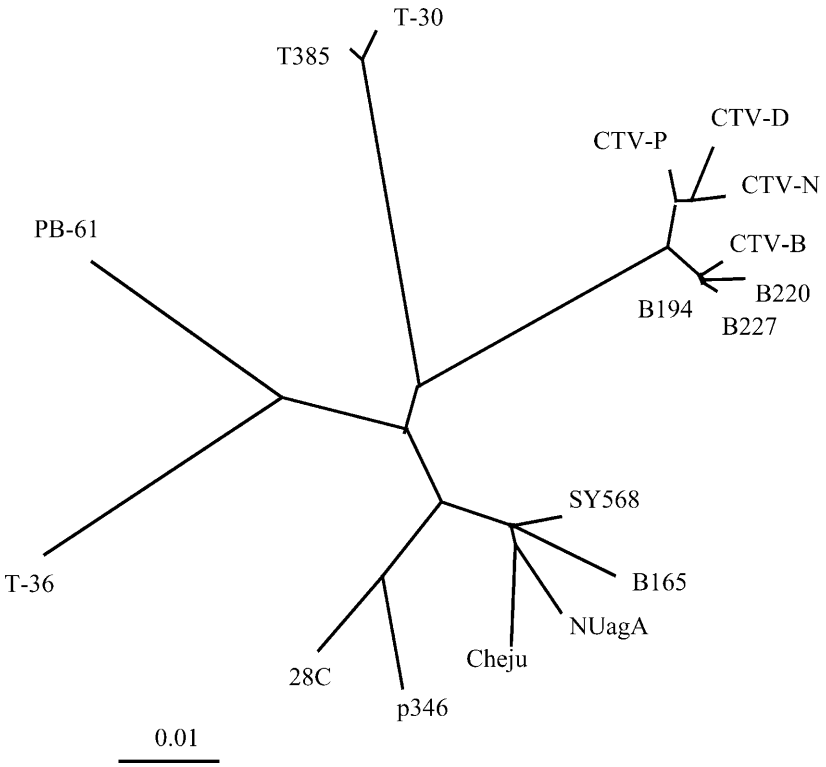

Fig. 3. Dendrogram showing the genetic relationships among coat protein gene of Indian and other exotic CTV isolates based on the nucleotide sequences

322, 350 bp products, respectively; whereas the other isolates did not have these two restriction sites (Fig. 5). In restriction analysis using the enzyme *Pvu*II, CTV-N was different from the three Indian and other isolates, producing two bands of 530 bp and 142 bp (Fig. 5). Restriction with *Eco*RI enzyme failed to digest the amplified fragment from any of the Indian isolates.

Restriction products of the insert of the four Indian test isolates were resolved using *Hin*fI and *Rsa*I in polyacrylamide gel electrophoresis, and individual bands smaller than 150 bp were observed. To confirm the RFLP patterns, the Indian isolates CPG sequences were compared with the CPG sequences of different geographical CTV isolates available in gene bank (http://www.ncbi.nlm.nih.gov) and listed in Table 1. Two to five restriction sites were found with *Hin*fI digestion and the digested products ranged in size from 38 to 299 bp (Fig. 6). Four RFLP groups identified by *Hin*f1 were designated when the test isolates were compared with other isolates listed in Table 1 (Fig. 6). *Hin*fI group 1 isolates have five *Hin*fI restriction sites at the 74, 112, 411, 460, 502 nucleotide positions and consisted of all the Indian isolates and California isolate SY568, Israel VT isolate p346, Portugal isolate 28C, and the South Korean isolate from Cheju island. Group 2 isolates with four restriction sites at 74, 112, 411, 502 nucleotide positions contained the mild isolates T30 and T385 and the Japan

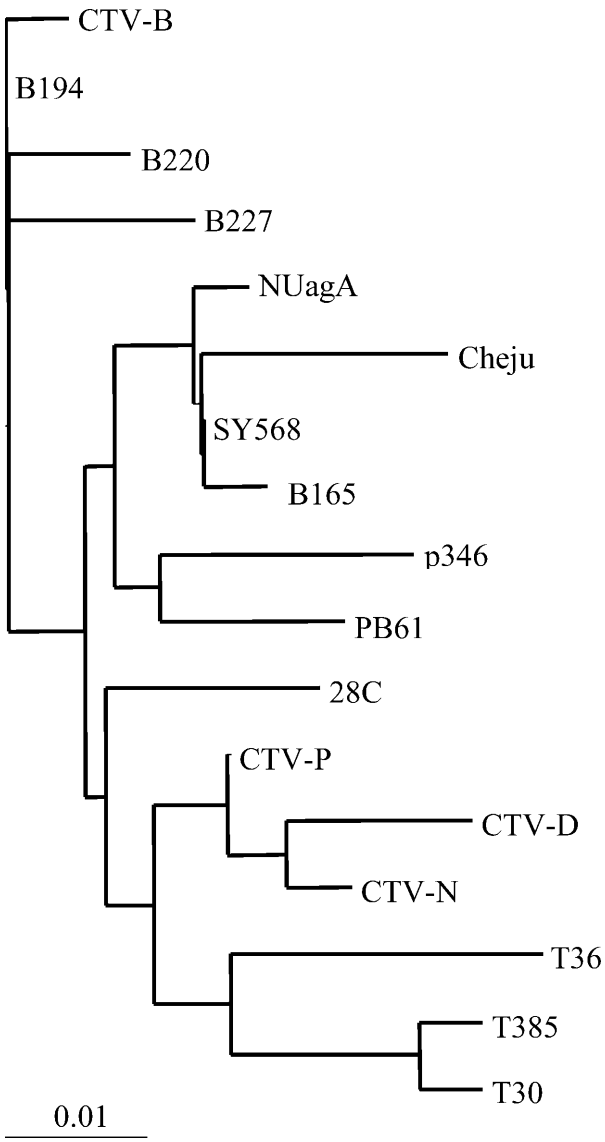

Fig. 4. Dendrogram showing the relationships of the deduced amino acid sequences of the coat protein genes of Indian and other CTV exotic isolates

isolate NUagA. Florida decline isolate T36 with three restriction sites was in group 3, and the Australian stem pitting isolate PB61 with two restriction sites was in group 4.

Two to four restriction sites were found with *Rsa*I digestion and the digested products ranged in size from 83 to 397 bp (Fig. 6). In the classification of the *Rsa*I products, three RFLP groups were identified. *Rsa*I group 1 had two restriction sites and contained all the Indian isolates used in the present study except previously studied isolate B165. *Rsa*I group 2 with three restriction sites contained mild isolates T30 and T385, and *Rsa*I group 3 with four restriction sites included isolate B165 and the remaining isolates (Table 1).

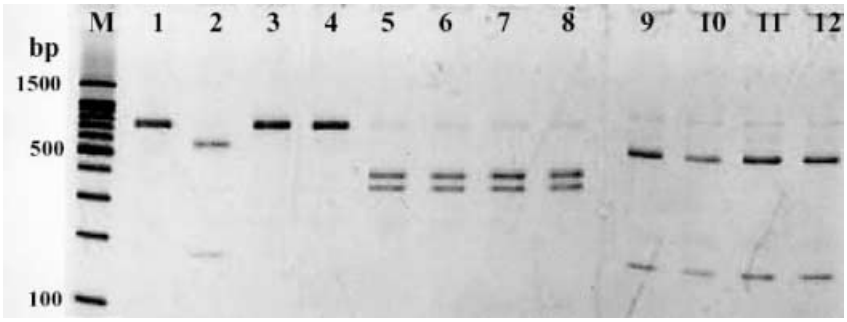

Fig. 5. Restriction digest of the PCR amplified product of CPG of the citrus tristeza virus isolates; CTV-P, CTV-N, CTV-D, and CTV-B from India using *Pvu*II, *Hin*dIII, and *Eco*RV endonucleases. Lanes *1–4* were digested with *Pvu*II, lanes *5–8* were digested with *Hin*dIII, and lanes *9–12* were digested with *Eco*RV. Isolate CTV-P digestion products are shown in lanes *1*, *5*, and *9*, isolate CTV-N digests are in lanes *2*, *6*, and *10*, isolate CTV-D digests are in lanes *3*, *7*, and *11*, and isolate CTV-B digests are in lanes *4*, *8*, and *12*. *M* is a 100 bp ladder, (Promega, Madison, WI)

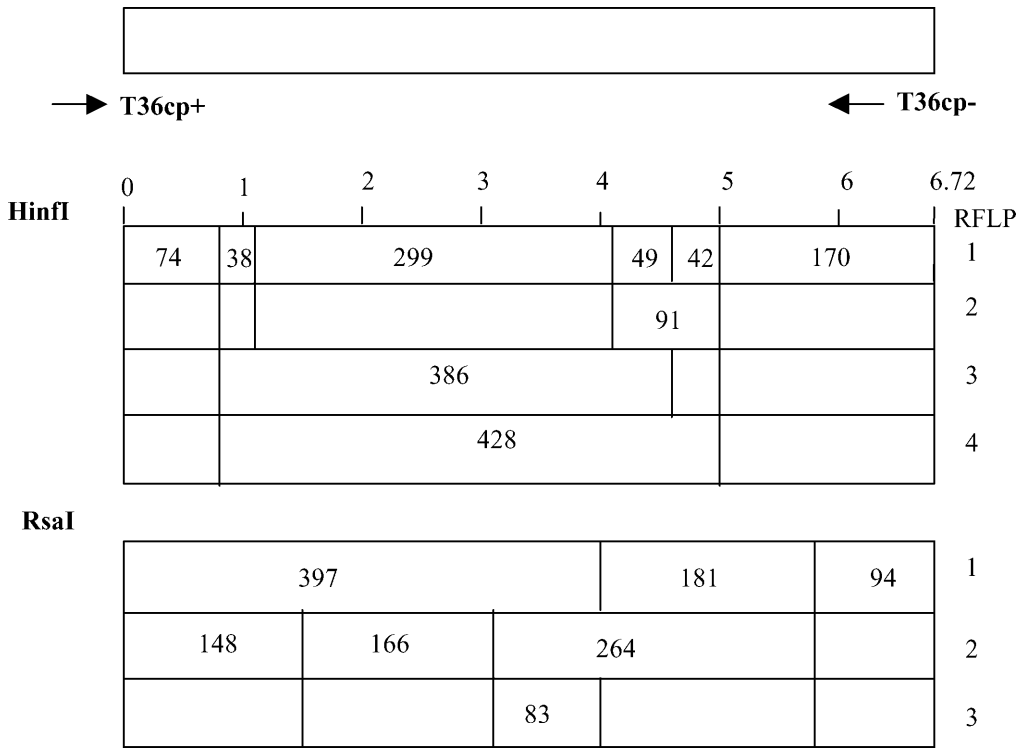

Fig. 6. Comparison of the Indian CTV isolates with other exotic CTV isolates on the basis of RFLP restriction maps. The four RFLP groups of the CTV coat protein gene defined by *Hin*fI and three RFLP groups defined using *Rsa*I digestion. Vertical lines in upper and lower box represent *Hin*fI and *Rsa*I restriction sites, respectively. The size in base pairs, of the selected restriction fragments is given between the restriction sites generating those fragments. The RFLP groups defined by *Hin*fI and *Rsa*I digestion are given on the right of the figure (Scale: 1 = 100 bp)

Discussion

Differences were found in host range tests with the four Indian isolates. All isolates produced vein flecking symptoms in Kagzi lime, indicative of CTV presence. In addition, isolates CTV-B and CTV-P produced stem pitting in Kagzi lime, sour orange and sweet orange. Isolate CTV-B also produced stem pitting symptoms in grapefruit and Etrog citron. Isolate CTV-N produced a seedling yellows reaction in sour orange and grapefruit. Isolate CTV-D produced mild to no symptoms in most of the hosts. According to the reaction on these various host plants, CTV-B and CTV-P are considered a severe and mild stem pitting isolates, respectively. CTV-N is considered as seedling yellows isolate and CTV-D as mild isolate.

The primers developed by Hilf et al. [7, 8] to determine genotypes based on the genomes of four CTV isolates *viz*., Florida decline isolate T36, Florida mild isolate T30, Florida severe isolate T3, and the Israeli severe VT isolate were used to classify the Indian CTV isolate genotypes. VT and T30 genotype mixtures were found in CTV-N and CTV-P isolates, but in the CTV-D isolate only the VT genotype was observed. CTV-B isolate was the only type of isolate that did not fit into any of the genotypes as defined by Hilf et al. [7], and thus it appears to be a group of unexpected genotype mixtures.

The T36cp, the common coat protein gene primers, amplified all the Indian CTV isolates and these products were cloned and sequenced. The CPG sequence of all the four isolates was most closely related to the California stem pitting isolate SY568 at the nucleotide and amino acid level. The nucleotide clustal dendrogram showed that all the Indian isolates created a new branch in the phylogenetic tree (Fig. 3). Amino acid sequence(s) dendrogram in tree view showed that CTV-B occupied a different branch with Japanese NUagA and Californian SY568 isolates, whereas the other three Indian isolates occupied another branch in the phylogenetic tree (Fig. 4).

However, unlike the results of studies on CTV isolates from Florida and Spain [3, 13], the association between biological characteristics and the CPG sequences of the Indian isolates was not as clear. All the Indian isolates except CTV-D contained a thymidine (T) at position 371 of the nucleotide sequence corresponding to phenylalanine (F) at amino acid position 124, and therefore, reacted with the MCA-13 antibody in ELISA. The presence of amino acid tyrosine (Y) at the same position gave negative results for mild isolates in MCA-13 ELISA [12]. Indian isolate CTV-D had cytosine (C) at nucleotide position 371, which represents serine (S) at amino acid 124. Nikolaeva et al. [11] mapped the MCA-13 epitope to the region between amino acid 118 to 128. Previously, the linear epitope for MCA-13 was suggested to be located close to and probably include amino acid 124 of the CP [12]. Indian isolate CTV-D reacted with the MCA-13 antibody even though it doesn't have tyrosine (Y) or phenylalanine (F) at amino acid position 124 that is present in most severe and mild CTV isolates. Valine (V) is the common amino acid for CTV-D and other severe CTV isolates at amino acid position 122. The amino acid isoleucine (I) present in amino acid position 122 of the mild CTV isolates, T30 and T385, was not present either in

CTV-D or other severe CTV isolates. So, not only amino acid 124, but also amino acid 122 is responsible for a positive MCA-13 reaction. Even though CTV-D reacts positive with MCA-13 in ELISA, it is different from the other Indian isolates as well as the exotic isolates described in the Table 1 (Fig. 2).

The restriction analysis of CTV-CPG cloned DNA helped to group the CTV isolates. The restriction sites of *Bst*EII were common for all the CTV isolates, but restriction sites of *Eco*RV and *Hin*dIII were present in the Indian isolates only. Nucleotide sequence at the 513th position showed the presence of adenine (A) instead of guanidine (G) and created the *Eco*RV restriction site. The presence of guanidine (G) instead of adenine (A) at the 351st position of the nucleotide sequence created the *Hin*dIII restriction site in all the Indian isolates (Fig. 5). CTV-N is the only isolate having the *Pvu*II endonuclease restriction site at 142nd nucleotide position. The Indian isolates did not have the *Eco*RI restriction site, which has been reported in the Brazilian CB3-104 isolate [16].

In RFLP studies, digestion of the amplified cDNA with *Hin*fI and *Rsa*I revealed considerable polymorphisms between the Indian and other exotic CTV isolates. Comparison between the Indian isolates and the nine exotic CTV CPG sequences revealed four RFLP groups for *Hin*fI and three for *Rsa*I. In the classification of the *Hin*fI RFLP pattern, the Indian isolates were placed in group 1 which also contained Californian isolate SY568, Israeli p346, Portugal 28C, and South Korean Cheju island isolates. The Indian isolates were found to belong to *Rsa*I RFLP pattern; group 1 consisted of all the Indian isolates under study and have two restriction sites at 397th and 578th position.

Gillings et al. [5, 6] observed seven RFLP groups for the CTV strains with *Hin*fI sites and four RFLP groups with the *Rsa*I sites. In the current study, all the test isolates along with nine exotic isolates from different geographical regions of the world were compared, and only four groups were found for *Hin*fI and three for *Rsa*I. Previous RFLP experimental results [5, 6] were based on measurement of restricted band sizes from 4% NuSieve 3:1 agarose gel (FMC). Bands above 150 bp were easy to resolve and simple to analyze; however, the presence of numerous smaller fragments and interfering, overlapping bands hindered accurate results. In the present study, data on actual number of RFLP patterns was based on sequences that assisted in analysis of the correct band size. All of the Indian isolates are closely related to some of the exotic isolates like, Californian SY568, Israeli p346, and Japanese NUagA, with some similar biological or molecular data but are not specifically related to any one of them. It is believed that such ambiguous relationship of the Indian isolates to other exotic isolates may contribute significantly to the variability in CTV populations.

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