Analyses of 3' half genome of citrus tristeza virus reveal existence of distinct virus genotypes in citrus growing regions of India

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Abstract Citrus tristeza virus (CTV, genus *Closterovirus*) is one of the most serious pathogens responsible for huge loss of citrus trees worldwide. Four Indian CTV isolates, Kat1 (*C. reticulata*/Central India), D1 (*C. sinensis*/North India), B5 (*Citrus limettoides*/South India) and G28 (*C. lemon*/Northeast India) collected from different regions of India were characterized based on sequencing of 3' half genome (~ 8.4 kb) comprising 10 open reading frames (ORFs2–11) and 3' UTR and the sequences were submitted to NCBI database as Acc. No KJ914662, HQ912022, HQ912023 and KJ914661, respectively. The present and previously reported Indian isolates Kpg3 and B165 were analyzed and compared with other Asian and international CTV isolates. The Indian CTV isolates had 92–99% nt identities among them. The phylogenetic analysis generated overall ten genogroups/lineages. Of them, all the Asian isolates fell into seven genogroups, whereas the Indian isolates into four. Indian isolates Kat1, D1 and Kpg3 grouped together, termed “Kpg3Gr”, along with Florida

severe isolate T3. The Indian isolates B5, and G28 were found to be two distinct and separate lineages, indicating that these isolates are two new CTV entities. Based on phylogenetic analysis, Kpg3Gr was identified as “Indian VT” subtype which is distinct from the Asian and the Western VT subtype within diversified VT genotype. The recombination detecting-program, RDP4 detected Indian isolates Kat1, B5, B165 and G28 as recombinants, where G28 as strong recombinant. The present study determined the occurrence of at least four CTV genotypes, B5 (distinct), B165 (T68 type) G28 (distinct) and Kpg3Gr in citrus growing regions of India.

Keywords Citrus tristeza virus · 3' Half CTV genome · Sequence analysis · Genetic diversity · Recombination · Phylogenetic relationship

Introduction

Citrus tristeza virus (CTV, genus *Closterovirus*, family *Closteroviridae*) is one of the most serious pathogens responsible for the loss of over 100 million citrus trees globally either killing or rendering unproductive over the past century [21]. CTV, a phloem inhabiting, long flexuous virus measuring 2000 × 11 nm in size, is transmitted by several aphid species, predominantly by brown citrus aphid (*Toxoptera citricida*) in a semi-persistent manner. The virus infects nearly all the citrus species, cultivars and citrus relatives [15]. Three major host reactions caused by the virus include seedling yellows, stem pitting and quick decline; of which the last two are significant problems for citrus cultivation. Disease severity and symptom expression depend on various factors that include citrus species/cultivar infected the rootstock on which the scion is

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grafted, the virus strain, mixture of strains and climatic conditions [4, 21].

CTV contains positive sense ssRNA genome of ~ 19.3 kb which is organized into 12 ORFs encoding at least 19 putative proteins along with two UTRs at 5' and 3' end termini [14, 26]. The ORF 1 expresses one large polyprotein (ORF1a) containing helicase, methyltransferase, and duplicated protease domains, as well as the RdRP (ORF1b) via a C1 frameshift [27]. The ORFs 2–11 are expressed through co-terminal sub-genomic RNAs and encode the major coat protein p25 (ORF 7) and minor coat protein p27 (ORF 6); suppressors of RNA silencing p20 (ORF 10), and p23 (ORF 11); a heat shock protein (HSP 70) homolog p65 (ORF4) and p61 (ORF 5) both required for virion assembly; and three proposed host range determinant proteins p33 (ORF 2), p13 (ORF 9) and p18 (ORF 8); and a membrane anchored protein p6 (ORF 3) required for systemic invasion in host [16, 26, 27, 28, 33]. Complete genome analysis shows that the 3' half CTV genome comprising ORFs 2–11 are conserved showing 88–92% nt identity and the 5' half comprising ORF1a and 1b are divergent with 72–97% nt identity [6]. Sequence analysis using complete genomes of several CTV isolates worldwide recognized six CTV genotypes VT, T36, T30, T3, RB, T68 and one recombinant entity HA16-5 [12, 20]. Of them VT genotype has been described as biologically severe, T30 as mild and T36 as intermediate CTV strain [3]. It has also been suggested that homologous and non-homologous recombination are major factors in the evolution CTV variants in India and other countries [5, 18, 20, 23, 30].

Citrus is cultivated almost in all the geographical regions of India and CTV occurs in all the economically important citrus cultivars grown in India. CTV incidence varies from 26.3% in Vidarbha (Central India), 47.1–56.0% in Northeast (Assam, Meghalaya, Sikkim and the Darjeeling hills), 36–50% in South (Andhra Pradesh and Karnataka) and 16–60% in North and Northwest India (Uttarakhand, Delhi and Punjab) [7]. It has destroyed more than one million citrus trees in recent past in India [1, 9]. Extensive genetic diversities among Indian CTV isolates has been reported earlier analysing nucleotide sequences of 5'ORF1a fragment and CP gene of more than 104 isolates [8, 11, 24, 29, 32]. Complete genome of two Indian CTV isolates, Kpg3 of Northeast Himalayan hill region of India [6] and B165 from South India [23] have been sequenced, and both the isolates are reported to be different from each other. The isolate Kpg3 is described as a decline inducing [6], and isolate B165 as severe and stem pitting CTV isolate [23].

In the present study, four Indian CTV isolates collected from different geographical regions of India, viz. Kat 1 (Central), D1 (North), B5 (South) and G28 (Northeast)

were biologically characterized and about 8.4 kb of the 3' half genome comprising 10 ORFs (ORFs 2–11) and 3' terminal UTR were sequenced, analyzed and compared with previously reported Indian CTV isolates Kpg3 of Northeast and B165 of South India, other Asian and internationally recognised CTV isolates to assess genetic variability and phylogenetic relationships.

Materials and methods

CTV isolates

Four CTV samples/isolates, viz. Kat1 causing poor growth of Nagpur mandarin (*C. reticulata*) of Katol of Vidarbha (Central India), D1 causing chlorosis and stunting of sweet orange (*C. sinensis*) of Delhi (North India), B5 causing vein clearing and chlorosis of sweet lime (*Citrus limetoides*) of Bangalore (South India) and G28 causing vein clearing, stunting and chlorosis of Assam lemon (*C. lemon*) of Assam (Northeast India) were studied. All the isolates were maintained in Kagzilime/Mexican lime (*C. aurantifolia*) through bud wood grafting in insect-proof greenhouse. CTV infection in the samples was confirmed by Direct antigen coated- enzyme linked immuno sorbent assay (DAC- ELISA) using specific antisera [9]. For biological indexing the present CTV isolates were inoculated on Kagzilime, sweet orange, Darjeeling mandarin (*C. reticulata*) and rough lemon (*C. jambhiri*) seedling through bud wood grafting and maintained in insect-proof greenhouse. Three to four successful graft unions were maintained and biological reactions (symptomatology) were recorded after six months of inoculation.

cDNA synthesis, cloning and sequencing of CTV genome

Total plant RNA was extracted from 50 mg tender bark tissues of the CTV infected Kagzilime plants using SV Total RNA isolation system (Promega, Madison) according to manufacturer's protocol. The first strand cDNA was synthesized using reverse primers according to the methods mentioned earlier [8]. A set of thirteen pairs of specific and degenerate primers designed earlier by Biswas [6] were used for amplification of the 3' half of CTV genome (~ 8.4 kb) comprising ten ORFs (ORF 2–11) and 3' UTR. The PCR was performed in a final volume of 50 µl reaction mixture using the method described previously [8]. The amplicons were purified using QIAquick PCR Purification Kit (Qiagen, Maryland), cloned into the T&A cloning vector system (RBC, UK) and grown in *E. coli* strain DH5α using standard method [25]. Two clones of each isolate were sequenced in both the directions using the vector

derived primers M13F and M13R in AB 13130 Genetic analyzer (Chromous Biotech, Bangalore). The nucleotide sequences were assembled by multiple sequence alignment using the overlap sequence, 80–100 nt, of adjacent sequence. The consensus sequences obtained by DNA STAR SeqMan software were used for further analysis.

Nucleotide sequence identity matrix and phylogenetic analysis

Multiple sequence alignment was carried out with CLUSTAL X version 1.81 [34]. The sequence annotations were done using the reference CTV isolates, Kpg3 of India [6] and T30 of Florida [2]. The sequence identity matrix was generated using Sequence Demarcation Tool (SDT) version 1.2 [22]. The neighbourjoining phylogenetic tree was constructed using maximum likelihood parameter of MEGA 6.0 [31]. The ORFs were identified using NCBI-ORF finder and protein domains using BLASTp search in the NCBI Conserved Domain Database. Recombination analysis was carried out using the recombination detection program version 4.55 (RDP4), implementing seven algorithms viz. RDP, Geneconv, BootScan, Maxchi, Chimera, SisScan and 3Seq [17]. Default settings were used with a standard Bonferroni-corrected highest acceptable *P* value cut off of 0.05. The recombination events involving same major parent, detected by two or more algorithms were considered to be putatively positive evidence of recombination.

The sequences of the present CTV isolates Kat1, D1, B5 and G28, two previously reported Indian CTV isolate Kpg3 (VT type) and B165 (T68 type), seven other Asian isolates T1 (RB type), T5, A18, CT11A (VT type), CT14A (T68 type), AT1 (VT type) and NuagA (VT type) and six internationally recognized CTV isolates (genotypes), VT, T36, T30, T3, NZRB-TH30 and T68-1 and one recombinant entity, HA16-5 (as genotype, not determined) were taken for sequence analysis, (Table 1) [12, 35].

Results

Biological reaction of CTV isolates

The biological reactions of the present CTV isolates, Kat1, D1, B5 and G28 were studied using a set of different citrus species through graft inoculation (Table 2). All the isolates induced vein clearing in Kagzilime which is a recognized indicator host for CTV; stunting with or without chlorosis in sweet orange; poor growth without distinct leaf or foliage symptoms in both the Rough lemon and Darjeeling mandarin seedlings. However, biological property of the present CTV isolates will be clear when biological

indexing will be carried out using more number of citrus species. Indian isolate Kpg3 originated from the Darjeeling hills has been described as a decline inducing CTV isolate based on biological indexing by Biswas et al. [6]. The CTV isolate, B165 originated from South India has been described to be a more severe and also stem pitting isolate by Roy and Brlansky [23]. However, in the present study, stem pitting symptom was not observed either in the field from where the isolates were collected or in the greenhouse inoculated plant. Therefore, it is concluded that the present isolates could be decline inducing CTV isolates of India.

Cloning and sequencing of 3' half of CTV genome

Two clones of each PCR products obtained from CTV genome using specific primer pairs were sequenced and analysed. The sequence of overlapping regions of two clones containing adjacent sequence fragments was identical (99–100%) that confirmed that the CTV isolates, Kat1, D1, B5 and G28 studied contain sequences of same isolates, respectively. The assembled consensus sequences of 13 clones for each of the present CTV isolates showed that all the isolates contained total of ~ 8.4 kb. The assembled sequences were analysed and submitted in NCBI GenBank database [Acc. Nos KJ914662 (Kat1), HQ912022 (D1), HQ912023 (B5) and KJ914661 (G28)] (Table 1). The sequence analysis showed that genome organization of the present CTV isolates are typical with the other CTV isolates reported earlier [6, 14, 23].

Sequence analysis and phylogenetic relationship

The sequence analysis of the 3' half 8.4 kb genome showed that the present CTV isolates had 92–99% nt identities among themselves (Fig. 1). A range of 89–99% nt identities were observed among all the Asian, and 91–99% among the Indian CTV isolates (Table 3). The isolates, D1, Kat1 and Kpg3 were closely related with each other showing 98–99% nt identities and they were considered as members of Kpg3 Gr. The isolate B5 was different showing 93–95% nt identity with Indian and 89–96% with other CTV isolates. The isolates G28 and B165 had 91–93% nt identity with other Indian isolates. However, the isolate G28 differed from isolate B165 by 91% nt identity. The isolate B165 was related with VT type isolates AT1, CT11A, NuagA and VT by 96–97% nt identity; and T68 type isolates T68-1 and CT14A by 97–99% nt identity. The Indian isolate B5 and Kpg3Gr were closely related (96% nt identity) with T3 isolate. The isolate G28 was found to be distinct showing 91–93% nt identity with the Indian and 90–94% nt identity with other CTV isolates. The sequence variation in individual ORFs of the CTV genome was determined (Table 3). There were 83–100% nt identity per

Table 1 The sequences of *Citrus tristeza virus* used for comparative analysis in the present study

CTV isolate	Source host and country of origin	3' half CTV genome size (nt)	Accession No.
Indian			
Kat 1	<i>C. reticulata</i> (Nagpur mandarin), India	8387	KJ914662
D1	<i>C. sinensis</i> (Sweet orange), India	8392	HQ912022
B5	<i>Citrus limmetoides</i> (Sweet lime), India	8392	HQ912023
G28	<i>C. lemon</i> (Assam lemon), India	8390	KJ914661
Kpg3	<i>C. reticulata</i> (Darjeeling mandarin), India	8390	HM573451
B165	<i>C. reticulata</i> (Ellendale Mandarin), India	8393	EU076703
Other Asian			
T1	<i>C. grandis</i> (Pummelo), Taiwan,	8388	JX 266712
T5	<i>C. grandis</i> (Pummelo), Taiwan	8389	JX266713
A18	<i>C. reticulata</i> (Mandarin),Thailand	8392	JQ798289
CT11A	<i>C. Sinensis</i> (Sweet orange), China	8392	JQ061137
CT14A	<i>C. sinensis</i> (Sweet orange), China	8392	JQ911663
AT-1	<i>C. Sinensis</i> (Sweet orange), China	8392	JQ911664
NuagA	Unknown host, Japan,	8392	AB046398
International			
VT (VT type)	Unknown host, Israel	8391	U56902
T36 (T36 type)	Unknown host, FL (USA)	8391	UL6304
T30 (T30 type)	Unknown host, FL (USA),	8398	AF260651
T3 (T3 type)	<i>C. sinensis</i> (Sweet orange), FL (USA)	8393	KC525952
NZRB-T30 (RB Type)	<i>Poncirus trifoliata</i> (Trifoliate orange), New Zealand	8392	FJ525434
T68-1(T68 Type)	<i>C. macrophylla</i> , USA	8392	JQ965169
HA16-5 (recombinant entity)	Unknown host, Hawaii (USA)	8382	GQ454870

Table 2 Biological reactions of different *Citrus tristeza virus* isolates collected in the present study

Citrus host	Symptoms caused by CTV isolates				
	Kat1	D1	B5	G28	Kpg3*
Kagzi lime (<i>C. aurantifolia</i>)	Vcl	Vcl	Vcl	Vcl, Vcr, Dec	Vcl, Vfl, Vcr
Darjeeling mandarin (<i>C. reticulata</i>)	Asy, Pg	Asy, Pg	Asy, Pg	Asy, Pg	Asy, Pg
Rough lemon (<i>C. Jambhiri</i>)	–	Asy	–	Asy	Asy, Pg
Sweet orange (<i>C. sisensis</i>)	St, Cl	St	St	St, Chl	St, Chl

Vcl: vein clearing, Vcr: vein corking, Vfl: vein flecking, Dec: decline type, Asy: asymptomatic, Pg: poor growth, St: stunting, Chl: chlorosis; –: not tested

*Biswas et al. [6]

ORF (ORF 2-11) among all the CTV isolates, 84–100% among the Asian, and 88–100% for Indian CTV isolates.

Based on ORF wise sequence analysis, the Kpg3Gr and B5 were related with CTV isolates A18 and HA16-5 for ORF 2 (95–96% nt identity), and isolate T3 for ORFs 2-6 (95–99%). The Kpg3Gr was related with HA16-5 for ORFs7-9 (97–99%) and T3 for ORFs 10-11 (96–99%). The isolate G28 is related with NZRB-TH30 for ORF 2 (94%) and T3 for ORF 3 (98%). For ORFs 4-6, isolate G28 was related with HA16-5 by 94–98% nt identity. The isolates B5 and G28 were related with isolate T3 for ORF 7 and 9

(98–99%) and with A18 for ORF7 and 8 (97–98%). The isolate B5 was related with VT and T68-1 by 97% for ORF10-11. The isolate G28 was related with HA16-5 and A18 showing 98% nt identity for ORF 10 and 11.

The phylogenetic analysis using 3' half genome generated overall ten CTV genogroups (Gr-I to Gr-X) (Fig. 1). All the Asian CTV isolates fell into seven genogroups, whereas the Indian isolates into four genogroups. Two Indian CTV isolate Kat1 and D1 grouped with Indian decline inducing CTV isolate Kpg3 and Florida severe isolate T3. Another two Indian isolates B5, and G28 were

Fig. 1 Nucleotide sequence analysis for 3' half genome (~ 8.4 kb sequence) of *Citrus tristeza virus*; **a** pairwise nt identity generated by SDT, **b** phylogenetic tree generated by MEGA6 using maximum

likelihood method. The strains/genotypes are highlighted with bold letter, *grouping based on complete genome (Harper, 2013) and **grouping based on 3' half genome (in the present study)

Table 3 Nucleotide sequence variation and phylogenetic relationship among *Citrus tristeza virus* isolates based on individual ORF

ORF of CTV	Among all the CTV isolates analysed		Among the all the Asian CTV isolates analysed		Among all the Indian CTV isolates analysed	
	No. of phylogenetic group generated	Range of nucleotide sequence variation (%)	No. of phylogenetic group generated	Range of nucleotide sequence variation (%)	No. of phylogenetic group generated	Range of nucleotide sequence variation (%)
ORFs 2-11 (assembled)	10	89–99	7	89–99	4	91–99
ORF 2	9	84–99	7	84–99	3	88–99
ORF 3	6	83–100	5	90–100	2	92–100
ORF 4	6	88–99	4	89–99	3	92–99
ORF 5	9	87–99	7	88–99	3	88–98
ORF 6	8	87–100	6	88–100	3	88–100
ORF 7	8	89–99	6	89–99	3	91–99
ORF 8	6	89–100	4	90–100	3	90–100
ORF 9	11	88–99	7	88–99	3	88–99
ORF 10	6	87–100	3	88–100	3	91–100
ORF 11	10	87–99	7	90–99	4	90–98

distinct as two new entities. The other Asian isolates fell into four genogroups, where isolate A18 and T5 were two new entities. Citrus was believed to be originated in

Southeast Asia [10] and CTV is also believed to be co-evolved with its citrus host [10]. As the present study determined that the Asian CTV isolates are extensively

diverse, it is concluded that Southeast Asia is the centre of origin of CTV. Similar opinion of the origin of CTV has also been reported earlier [2].

Recombination analysis

Recombination detecting-program, RDP4 detected recombination events in 18 isolates out of 20 isolates tested. The recombination events showed sequences of same major parent detected by two or more algorithms in RDP4 were considered as true recombination events. Overall 29 true recombination events were detected, where 18 events were detected among the Asian CTV isolates including Indian isolates. Of six Indian isolates, four isolates Kat1, B5, G28 and B165 were recombinants and two isolates Kpg3 and D1 were non-recombinants (Fig. 2). Interestingly, all the other Asian isolates tested were recombinants. Among Indian isolates, G28 was strong recombinant with three recombination events followed by Kat1 and B5 with two events. Equal number of two recombination events was detected in three other Asian isolates T1, T5 and CT14A.

Discussion

The present study reports at least four CTV genotypes, B5 (distinct), B165 (T68 type), G28 (distinct) and Kpg3Gr (Indian VT subtype) occurring in India, and extensive genetic diversity among the Indian and the Asian CTV isolates. Indian isolate Kpg3 is reported as VT type on complete genome analysis [6]. The VT genotypes shows two subtypes, the Asian (AT1, CT11A and NUagA) and the Western subtype (Israel VT and other US isolates), where Kpg3 placed separately as bridge between the Asian and Western subtypes [12]. For complete genome analysis, CTV isolates Kpg3 and T3 are different [12], but our study shows Kpg3Gr (Kpg3, Kat1 and D1) and T3 are similar (96% nt identity); it is due to T3 like sequence insertion in the 3' half genome of Kpg3 as shown by Harper [12]. For this fact, Kpg3 isolate has been a separate VT subtype in the phylogenetic tree generated by Harper [12]; thus Kpg3Gr is termed as "Indian VT" subtype revealing existence of Asian, Indian and Western CTV subtypes within the VT genotype. As T3 is a severe isolate of Florida [13], and Kpg3 is a decline inducing isolate [6], the Kpg3Gr may be biologically similar.

Complete genome analysis shows the India isolate B165 is a T68 type [12] but our 3' half genome analysis shows B165 and T68-1 are closely related with VT type. The VT-like sequence insertion in the 3' half genome of T68 genotype (B165 and T68-1) shown by earlier Harper [12] suggested that the CTV isolates VT, B165 and T68-1 are diversified from a unique lineage of ancestral proto CTV

sequences. The p23 (ORF11) is reported to be pathogenicity determinant of CTV that induces seedling yellowing in infected citrus plant [2]. The CTV isolates VT and B165, both, cause seedling yellowing in the infected citrus [19, 23]. The present study shows that p23 gene of VT, B165 and T68-1 had 96–99% nt identity among them. Therefore, it was suggested that pathogenicity/disease severity of B165 and VT may be attributed to p23 gene of CTV.

CTV is known to undergo frequent recombination resulting in evolution of new genotypes [5, 6, 12, 18, 20, 23]. The present study also indicated that recombination is an important factor for origin of CTV variants in India. Frequent recombination with multiple events is also common for evolution of the Asian CTV variants. Previously, isolate Kpg3 is reported to be a recombinant [6]. Numbers of recombinant Indian CTV isolates also have been reported analysing CP gene and ORF1a fragments [5, 32]. Most of the CTV isolates did not show recombination events in CP, p20 and p23 genes and majority of the CTV isolates showed recombination events in HSP70, p33 and p6 genes indicating that CP, p20 and p23 genes are unique, and HSP70, p33 and p6 genes are selectively favored sites for recombination. The recombination analysis shows that B5 and G28 both contain sequence mixture of the Asian and international CTV isolates; AT-1/VT for B5 and B5/HA16-5 for G28. Thus, the isolates B5 and G28 have been placed into two separate branches in the phylogenetic tree as two distinct entities. Most of the isolates under VT, T68 and RB genotypes are common donors for recombination of the Asian CTV isolates. Indian CTV isolate G28 is strong recombinant because it has originated from multiple parents with three recombination events involving HSP70, p61, CP, CPm and p18 genes with indication of G28 as distinct Indian CTV isolate. The recombinant CP gene of G28 is originated from the Asian CTV isolates and interestingly, recombinant CP gene of T36 is also originated from Asian CTV isolates. This finding indicates that episodic diversification events might separated the G28 and T36 genotypes from an ancient proto CTV sequence.

In conclusion, the present study determined occurrence of at least four CTV genotypes, B5 (distinct), B165 (T68 type), G28 (distinct) and Kpg3Gr (Indian VT subtype) indicating genome plasticity and strain profiling of CTV in India. However, the existence of more number of genotypes is likely to be expected with the availability of large number of CTV sequences from citrus growing regions of India. The present study shows the Asian CTV is more diverse and clear sequence difference between the Indian and other Asian CTV groups. It is also revealed that frequent recombination with multiple events is the major factor for evolution of new CTV genotypes in the citrus

Fig. 2 Graphical representation of recombination events with donor sequences associated with recombination of *Citrus tristeza virus*; the 3' half genome (~ 8.4 kb) of CTV with 10 ORFs outlined at the top

with sequence scale (Kb). Parenthesis denotes major donor(s). Recombinant regions are represented by shaded box with the minor donor(s)

growing regions of Asia. Indian isolates have genetic relationships with many CTV genotypes known worldwide; supporting the hypothesis that CTV has coevolved with the citrus crops in Southeast Asia.

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