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Molecular characterization of tobacco curly shoot virus infecting tomato (*Solanum lycopersicum L.*) in India

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ABSTRACT: Tomato is an important vegetable crop cultivated through out India and leaf curl disease is the major constraint for production. The disease has previously been shown to be associated with begomoviruses and betasatellites. A distinct monopartite begomovirus was found associated with tomato plants showing sever leaf curl, interveinal chlorosis and stunting symptoms in polyhouse grown tomatoes in Pantnagar, Uttarakhand, India. The complete DNA-A component was amplified through rolling circle amplification (RCA) using Phi29 DNA polymerase and characterized. The DNA-A of the four isolates as comprised of 2,758 nucleotides, encoding six open reading frames (ORFs) with genome organization typical of an old world monopartite begomovirus. The begomovirus showed highest sequence identity of (89.3 to 95.8%) to tobacco curly shoot virus (TbCSV) and some other begomoviruses (59.6 to 88.1%) reported from India in tomato. Based on sequence comparison and phylogenetic analysis these isolates are strains of tobacco curly shoot virus. Recombinant analysis of the four isolates revealed that some of the isolates are recombinants of Ageratum enation virus (AEV) and tobacco curly shoot virus isolates. This is new begomovirus infecting tomato in addition to the known 16 begomoviruses occurring in India. Information on the distribution and prevalence of the different begomovirus species and recombinant forms comprising the disease complex is crucial in guiding tomato breeding programs in the search for stable and durable sources of resistance.

Keywords: Rolling circle amplification, RCA, RDP, *Solanum lycopersicum,* Tobacco curly shoot virus

INTRODUCTION

Tomato (*Solanum lycopersicum* L) is one of the most economically important vegetable crops in India. Its production is constrained by tomato leaf curl disease (ToLCD). Although incidence of tomato leaf curl disease in India was first reported from northern region (Vasudeva and Sam Raj, 1948), this disease is now widely spread in almost all tomato growing regions of the country. Tomato leaf curl disease epidemics are increasing every year in intensive tomato growing areas causing (27-100%) yield loss, depending on the stage of infection (Muniappa *et al*., 2000). The disease is transmitted by the whitefly *Bemisia tabaci* (Homoptera:Aleyrodidae), with symptoms consisting of leaf curling, vein clearing, reduction in leaf lamina, vein enation and, overall stunting of plants (Chowda Reddy *et al*., 2012). The incidence of diseases caused by begomoviruses in tomato has increased dramatically in recent years in India. The first report of begomovirus infection on tomatoes in the country date back to the 1950's (Vasudeva and Sam Raj, 1948). However, in the late 1990's and early 2000's an extremely diverse begomovirus complex has emerged in India, coinciding with the introduction and spread of a new biotype of the whitefly vector *Bemisia tabaci* biotype B (Banks *et al*., 2001; Chowda Reddy *et al*., 2012; Rekha *et al*., 2005; Shankarappa *et al*., 2007). At least fifteen different begomovirus (family *Geminiviridae*, genus *Begomovirus*) species associated with ToLCD have been reported in India (Brown *et al*., 2012; Fauquet *et al*., 2008), and mixed infections in natural epidemics can be frequent (Delatte *et al*., 2005; García-Andrés *et al*., 2007). The existence of mixed infections is a cause of concern because it is a prerequisite for recombination to occur, a frequent phenomenon among geminiviruses (Padidam *et al*., 1999, Kirthi *et al*., 2002), with unpredictable pathological consequences.

The members of the genus Begomovirus have typical geminate particles encapsidating a single stranded circular DNA genome, infect dicotyledonous plants and are transmitted by the whitefly *B. tabaci*. They have either a bipartite genome with two single-stranded (ss) DNA components designated as DNA A and DNA B, each one about 2.7 kb in size, or a monopartite genome with one ssDNA component analogous to DNA A (Briddon *et al*., 2012). The genomes of monopartite (DNA A components of bipartite) begomoviruses are typically <2800 nucleotides in length and encode genes bidirectionally

from a non-coding intergenic region which contains promoter elements and the origin (ori) of virion-strand DNA replication. The virion-strand ori consists of a predicted hairpin structure containing the absolutely conserved (for geminiviruses) nonanucleotide (TAATATTA↓C) loop sequence and repeated motifs upstream known as iterons. The virion-sense strand encodes the coat protein (CP) that is required for insect transmission and movement in plants) and V2 protein that is to be involved in virus movement in plants. (Rojas *et al*., 2001). The complementary-sense strand encodes the replication-associated protein (Rep) the only virus-encoded gene product required for viral DNA replication, which is a rolling circle replication initiator protein that recognizes the iterons and nicks within the nonanucleotide sequence to initiate replication (Hanley-Bowdoin *et al*., 2004), the transcriptional activator protein (TrAP) which up-regulates the late [virion-sense] genes bipartite begomoviruses, is a suppressor of post-transcriptional gene silencing (PTGS; Yang *et al*., 2007) and also overcomes virus induced hypersensitive cell death (Hussain *et al*., 2007; Mubin *et al*., 2010), the replication enhancer protein (REn) that is involved in establishing an environment conducive for virus replication (Pedersen and Hanley-Bowdoin, 1994) and the C4 protein, the function of which remains unclear but for some viruses is a pathogenicity determinant and a suppressor of PTGS; Gopal *et al*., 2007; Saeed *et al*., 2008; Vanitharani *et al*., 2004). DNA B encodes two ORFs, nuclear shuttle protein (NSP, BV1) on the viral-sense strand and movement protein (MP, BC1) on the complementary-sense strand (Hanley-Bowdoion *et al*., 1999).

A small, circular, single-stranded DNA (1.3kb) referred to as DNA β is found to be associated with DNA A of old world monopartite begomoviruses. Betasatellites induce severe symptoms in plants and are dependent on DNA A for replication, encapsidation and transmission (Briddon *et al*., 2008; Briddon and Stanley, 2006; Sivalingam and Varma, 2012). They have a highly conserved structure, consisting of a single gene encoded in the complementary-sense (known as β C1), a region of sequence rich in adenine (A-rich) and an approximately 100–150 nucleotide sequence (known as the satellite conserved region) conserved between all betasatellites that contains a predicted hairpin structure containing a loop with the nonanucleotide sequence with similarity to the origin of virion-strand DNA replication of the geminiviruses (Briddon *et al*., 2003). The âC1 protein has DNA binding properties and is involved in overcoming host defenses (by suppression of PTGS), eliciting symptoms and possibly mediates virus movement in plants (Cui *et al*., 2005; Kon *et al*., 2007; Saeed *et al*., 2005, 2007). In addition to betasatellites, monopartite viruses are found to be associated with another type of satellite known as alphasatellite, which encodes one replication initiation protein similar to those of nanoviruses in the viral strand. How these alphasatellites are involved in pathogenesis has not been deciphered (Brown *et al*., 2012).

Despite the efforts to manage ToLCD, viral disease problems continue to emerge in tomato, invading new areas every year. During January 2013, a severe ToLCD was observed in a tomato grown in polyhouses near Pantnagar. Form this severely infected tomato plants, we cloned and characterized DNA-A and we report a new monopartite begomovirus in tomato prevalent in northern India based on comprehensive biological and molecular analyses and following International Committee on Taxonomy of Viruses (ICTV) norms.

MATERIALS AND METHODS

Sample collection and DNA isolation: Symptomatic young leaves were collected from infected tomato plant and total DNA was isolated by a modified CTAB method as follows. One gram of fresh leaf tissue was ground into a fine powder in liquid nitrogen using a mortar and pestle, 100mg of powder was added to a 2.0-ml screw cap microcentrifuge tube (Fisher Scientific, USA) and 1ml of CTAB buffer (2% CTAB, 2% PVP-40, 100mMTris-HCl, pH 8.0, 1.4MNaCl, 20mMEDTA, and 1.0% 2-mercaptoethanol) and vortexed thoroughly for few minutes. The homogenate was incubated at 65°C for 10 min, followed by addition of equal volume of chloroform-isoamyl alcohol (IAA) (24:1 v/v) mixed and centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant (500µl) was transferred to a 1.5 ml microcentrifuge tube containing 3/4th volume of isopropanol, and the mixture was incubated at "20°C for 2 to 3 hours or overnight at 4 °C. After the incubation sample was centrifuged at 10,000*g* for 30min. The supernatant was discarded and pellet was air-dried and dissolved in 100µl of 20mMTris-HCl, pH 8.0. The pooled DNA samples were treated with RNase and incubate for 1 hr at 37°C. To the sample mixture, equal volume of chloroform-isoamyl alcohol (IAA) (24:1 v/v) was added, mixed and centrifuged at 10,000*g* for 10 min at 4°C. The supernatant was discarded and pellet was washed with 70% ethanol by centrifugation at 15,000*g* for 5 min, air-dried and dissolved in 100µl of 20mM Tris-HCl, pH 8.0.

The final elute was adjusted to 80-100µl with sterile water and stored at "20 °C for short term (d"2 months) or "80°C for long term(e"3months) for further use. The quality of the extracts was measured using a NanoDrop® ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and visualized by electrophoresis through 1.5% agarose gels.

Rolling circle amplification and cloning of viral genome: The viral genomic DNA was amplified through rolling-circle amplification (RCA) (Haible *et al*., 2006). Amplification of circular DNA was performed using 10-20ng of total nucleic acid as template. The reaction mixture contained 2µl of reaction buffer (10X), 2µl of random hexamer primers (500 mM Fermentas catalog no. SO142) and 2µl of dNTPs (10 mM). The mixture was denatured for 3 min at 94°C and was then cooled down to room temperature for 5 min, after which 4 µl of pyrophosphatase (0.1 U/ µl, Fermentas catalog no. EF 0221) and 0.7µl of ϕ29 DNA polymerase 10 U/µl (Fermentas catalog no. EP0092) was added. The reaction mixture was incubated for 18-20 h at 30°C and was inactivated at 65°C for 10 min. Concatamers obtained through RCA were digested with restriction endonucleases *BamHI, EcoRI, HindIII* and *KpnI* to release 2.7kb fragments of full-length DNA A and which were cloned in the vector pUC18 following standard protocol (Sambrook *et al*., 1989). The clones were confirmed by restriction digestion with *BamHI, EcoRI, HindIII*, and *KpnI* and positive clones were confirmed by sequencing. The universal degenerate primers for DNA-B (Rojas *et al*., 1993), and specific primers based on DNA-A intergenic region were used to detect presence of DNA-B in infected plant sample.

Sequence analysis of viral genome DNA-A

Three clones in each case were sequenced by automated DNA sequencer ABI PRISM3730 (Applied Biosystems) at Eurofins India Ltd, Bangalore, India. Full-length sequences of DNA A were analyzed using BioEdit version 7.0.9. Open reading frames (ORF) were predicted using GENERUNNER (Hartings Software Inc., Hastings, NY, USA; http://www.generunner.net). The sequence results were analyzed using NCBI (www.ncbi.nlm.nih.gov) blast search, followed by multiple sequence alignments using ClustalX (Thompson *et al*. 1997) and Bioedit Sequence Alignment Editor (version 5.0.9) (Hall, 1999), to determine percentage sequence similarities with other species, which showed maximum similarities in the blast search. Full-length genome of selected Begomovirus species, phylogenetic trees were generated by MEGA 5.0 software (Tamura *et al*. 2011), using the neighbor joining method with 1000 bootstrapped replications, to estimate evolutionary distances between all pairs of sequences simultaneously. Phylogenetic trees were constructed using the neighbour joining algorithm of Clustal X and displayed, manipulated and printed using Tree view (Page, 1996).

Detection of recombination events

To unmask probability of natural recombination as cause of origin of begomovirus associated with tomato leaf curl disease, the viral sequence was analyzed using the recombination detection program, RDP version 3 (Martin *et al*., 2010). Defaults RDP settings were used with a P-value cut off of 0.05 and the standard Bonferroni correction. Only internal references were allowed with a parental cutoff 70–100%. For general recombination detection, sequences were considered circular, consensus daughters were found and sequences were polished. For bootscan analysis, 200 boot replicates with 95% cutoff percentage was taken and for GENECONV analysis g-scale parameter was set to 1 (Martin *et al*., 2010).

RESULTS

Sample collection, detection and cloning of viral genome

Fifty one leaf samples collected from polyhouse grown tomatoes at Pantnagar, Uttarakand, India, showing severe leaf curling, vein clearing, yellowing, reduced leaf size and stunting were analyzed, Of the 51 samples tested, 48 samples gave 1.2-bp amplicons with PAR1v722 and PAL1c1960 primers (Deng *et al*., 1994, Rojas et al., 1993). From partial sequencing data, PCR amplicons were identified as belonging to AEV, TbCSV. No amplification was seen with specific primers for ToLCNDV, ToLCGuV, ToLCBaV or ToLCKaV. The begomovirus species detected in tomato grown in polyhouse indicated 31 samples of TbCSV (60.78%), 18 samples of AEV (35.3%) with the incidence of 27.6 to 68.5% tomato leaf curl disease in five different polyhouses. Viral genomic DNA of infected plants was enriched by rolling-circle amplification (RCA), and the RCA product was digested individually with *BamHI, EcoRI, HindIII* and *KpnI*. Clear bands of 2.7 kb, the expected unit genome length of begomoviruses were detected in BamHI and HindIII treated samples (Fig. 1). Of the twelve samples analyzed, only eight showed clear RCA products. These 2.7- kb fragments were purified and cloned in the pUC18 vector and linearized with the corresponding enzymes. The cloned components were

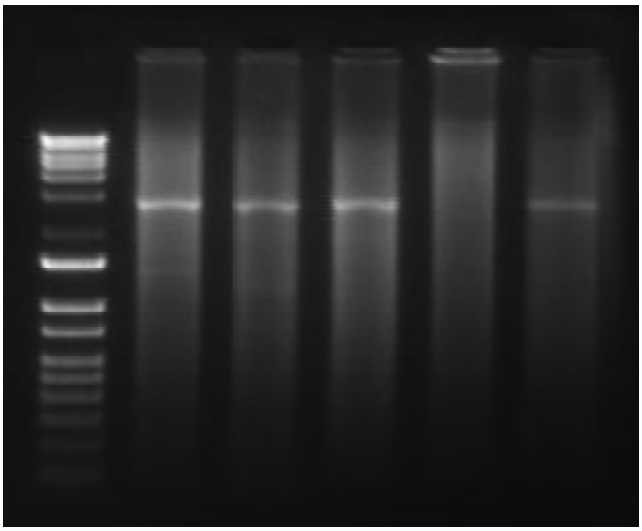

Fig. 1. Gel photograph showing restriction digests of the RCA products by different enzymes. Lane 1: BamHI, 2: HindIII, 3: EcoRI, 4: XbaI, 5:Kpn1, M:10 kb DNA Ladder (Fermentas).

confirmed by restriction digestion and further confirmed by partial sequencing.

Genome organization of TbCSV

The full-length nucleotide sequences of begomovirus genome (DNA A component) obtained from isolates TCb1, TCb8, TCh8 and TCh82 were determined to be 2758 bp in length (NCBI accessions JX457341, JX457342, JX467692 and JX467693). Attempts to amplify a begomovirus genome DNA B component from these isolates using the two primer pair's specific to DNA-B were uniformly negative. This may indicate that the virus associated with these isolates is distinct monopartite virus. Analysis of the two begomovirus components showed the presence of a predicted hairpin structure with the sequence TAATATTA↓C forming part of the loop. This structure is typically part of the origin of virion-strand replication of geminiviruses. The final adenine nucleotide of the nonanucleotide sequence was by convention, used to start nucleotide numbering. Further examination of the sequences using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) showed the presence of six predicted genes with a coding capacity greater than 12kDa, two in the virion-sense (encoding the coat protein (CP) and precoat protein AV2) and four in the complementary sense (encoding the replication associated protein (Rep), the transcriptional activator protein (TrAP), the replication enhancer protein (REn) and AC4 (PTGS Suppressor), diverging from a non-coding sequence (the intergenic region) that contains the predicted hairpin structure. This arrangement of genes is typical of the genomes (or DNA A components) of begomoviruses originating from the Old World.

Sequence comparisons and analysis

The complete nucleotide sequences of the begomoviruses cloned from isolates TCb1, TCb8, TCh8 and TCh82, have 99.4 to 99.8% sequence identity among these isolates. An initial comparison of the four sequences obtained with sequences in the databases using BLAST indicated them to be more similar to viruses previously identified in tomato originating from china. Multiple alignment and pair-wise sequence comparison of complete nucleotide sequences of the 27 begomoviruses infecting occurring in India has shown highest nucleotide similarities of 89.3 to 95.8% with tobacco curly shoot virus isolates. Based on the presently applicable species demarcation threshold 89% for begomoviruses (Fauquet *et al*., 2008), It is concluded that that the four begomoviruses are isolates of the same species. Among the tomato infecting begomoviruses in India, isolates TCb1, TCb8, TCh8 and TCh82 have 74.6 to 88.1% homology, where as with other begomoviruses they have nucleotide sequence homology of 59.7 to 75.5% (Table 1). The highest level of identity of 89.3 to 95.8% with tobacco curly shoot virus indicates that present isolates are a strains of TbCSV infecting tomato in North India. Multiple alignment and Comparison of individual proteins with begomoviruses has indicated that TCb1, TCb8, TCh8 and TCh82 have highest nucleotide similarities of 97.2 to 100% with TbCSV coat protein (CP), 93.5 to 100% with replication associated protein (Rep), 89.7 to 100% with transcriptional activator protein (TrAP), and 87.3 to 100% with replication enhancer protein (REn). Where as with the non coding IR sequence has 97.2 to 100% (Table 1).

Phylogenetic relationships of TbCSV

A phylogenetic analysis of the begomovirus sequences obtained in the study known sequences of begomoviruses associated with ToLCD and selected other sequences are shown in Fig. 2. A phylogenetic tree, based upon an alignment of the full-length sequences of DNA A component of selected begomoviruses present in India, shows that sequences of TCb1, TCb8, TCh8 and TCh82 to be most closely related to isolates of TbCSV, but the tomato isolates from India formed a separate sub group along with TbCSV(Y1)(AF240675) from china infecting tobacco. The relative positions of these isolates are well supported by boot -strapping.

Detection of recombination

Table 1 Pairwise comparisons of percent nucleotide identities between the genomic components and amino acid sequence identities of encoded genes four isolates of TBCSV infecting tomato with selected begomoviruses from NCBI the database

Begomovirus species	Full genome	IR	AV1	AV2	AC1	AC2	AC3	AC4
1. HG00365	93.1-93.4	78.5-78.9	99.2-99.6	96.8	96.8	91.9-91.6	93.2-97	89.8-90.1
2. JX457342	99.4-100	76.7-77.0	99.6-100	100	100	100	100	42.0-100
3. JX457341	99.4-99.8	99.6-100	99.6-100	100	100	100-99.7	94.0-100	94.2-100
4. JX467693	99.4-99.8	99.6-100	99.6-100	100	100	100-99.7	92.5-100	95.0-100
5. JX467692	99.4-99.6	99.6-100	99.6-100	100	100	99.7-100	92.5-100	94.2-100
6. HQ407395	89.3- 92.2	75.1-75.5	98.8-99.2	96.8	96.8	96.7	87.3-89.5	90.1-92.4
7. GU001879	95.6-95-8	85.7-86.0	98.8-99.2	97.4	97.4	95.0-94.7	93.2-95.5	95.0-98.9
8. GU199584	92.4-92.6	78.2-92.6	98.0-98.4	93.5	93.5	91.4-91.1	93.2-95.5	89.6-94.2
9. GU199583	93.0-93.1	78.5-78.9	97.6-98.0	95.4	95.4	92.5-92.2	90.2-91.0	84.2-88.6
10. AF240675	93.5-93.7	85.3-85.7	98.8-99.2	94.1	94.1	90.0-89.7	87.3-91.7	95.0-98.9
11. AJ420318	95.5-95.7	85.7-86.0	99.2-99.6	97.1	97.1	95.2-95.0	93.2-95.5	95.0-98.9
12. AJ971266	95.6-95.7	85.7-86.0	99.2-99.6	96.8	96.8	94.7-94.4	93.2-95.5	95.7-97.9
13. AJ457986	93.5-93.7	80.2-80.6	98.4-98.8	97.4	97.4	92.2-91.9	92.5-94.7	90.7-94.2
14. JN387045	94.4-94.7	86.3-86.7	97.2-97.6	92.7	92.7	96.1-95.8	90.2-95.5	95.0-97.9
15. JN176565	84.2-84.4	72.0-72.3	97.6-98.0	28.9	28.9	83.6-83.3	73.8-76.8	75.0-75.7
16. AM884015	71.2-71.3	50.4-50.8	89.8-100	70.4	70.4	63.8-64.1	74.4-75.8	74.2-78.1
17. GU112084	72.6-72.7	52.1-52.4	91.7-92.1	75.5	75.5	75.0 -74.7	75.8-76.6	75.0-76.4
18. EU862323	74.6-74.8	61.8-62.2	61.7	70.9	70.9	77.5-77.2	65.6-68.6	65.7-67.0
19. U38239	84.7-84.8	71.7-72.0	96.0-96.4	90.7	90.7	85.5-85.3	82.8-85.8	85.0-87.4
20. GU732204	83.7-83.9	62.7-63.1	97.2-97.6	88.7	88.7	86.0-87.8	83.5-85	85.7-88.4
21. AY190290	81.4-81.5	71-71.4	80.0-80.4	88.2	88.2	85.8-85.5	76.8-79.1	75.0-83.2
22. EF068246	72.7-72.8	50.4-50.8	91.7-92.1	74.7	74.7	75-74.7	74.4-75.8	74.2-75.1
23. GQ994095	89.9-90.0	70.5-70.9	98.0-98.4	94.5	94.5	88.3-88	92.5-94.7	82.4-83.5
24. DQ339117	77.1-77.2	68.4-68.7	96.8-97.2	92.4	92.4	76.7-76.4	75.1-76.6	74.9-75.2
25. AM992618	63.3-63.4	49.1-51.4	50.1	52.5	52.5	71.8-71.5	41.7-41	53.5-54.6
26. HM461863	78.6-78.8	48.5-48.9	97.6-98.0	88.5	88.5	79-78.7	78.3-77.6	76.477.5
27. DQ629103	82.4-82.6	70.4-70.8	93.3-93.7	87.1	87.1	84.4-84.2	70.1-73.1	75.7-77.3
28. JN135234	76.2-76.3	71.1-71.5	95.3-95.7	89.3	89.3	72.5-72.2	67.1-70.8	76.4-78.9
29. JX436472	87.5-87.8	76.7-77.0	99.2-99.6	94.1	94.1	81.4-81.1	88.8-92.5	86.4-87.2
30. AY754814	78.9-79.1	56.0-56.3	90.6-91.0	74.2	74.2	83.9-83.6	76.1-79.8	75.0-76.0
31. EU910141	80.7-80.9	61.8-62.1	96.0-96.4	90.4	90.4	86.6-86.7	82.8-84.3	83.5-84.4
32. AF188481	88.0-88.1	68.1-68.5	98.8-99.2	91.8	91.8	87.5-87.2	83.5-85.8	85.0-87.2
33. AJ575819	73.2-73.4	48.1-48.3	91.4-91.7	77.8	77.8	77.8-77.5	65.6-67.9	75.7-76.0
34. JN663865	81.0-81.1	60.1-60.5	96.4-96.8	86.6	86.6	83.3-83.1	74.6-76.8	84.2-85.2
35. HQ588901	59.6-59.7	34.0-34.3	36.6	37.5	36.5	41.2-41.2	35.8-36.5	36.0-43.2
36. FN645902	78.2-78.4	70.9-71.2	82.4-82.8	83.3	90.4	74.5-74.2	82.8-83.5	85.0-86.8
37. HQ698591	85.1-85.4	64.9-65.2	98.4-98.8	72.1	89	86.2	76.1-79.8	86.0-87.4
38. GQ220850	75.4-75.5	40.7-40.9	95.7-96.0	74.0	73.3	76.6	70.8-73.1	75.0-75.6
39. GU111999	71.1-71.2	45.9-46.0	41.4-41.7	87.1	72.1	86.3	67.9-70.1	83.5-84.1
40. AF241479	72.4-71.5	8.5-48.7	93.3-93.7	48.5	74.0	86.6	70.1-71.6	73.1-74.0
41. HM007104	83.0-83.1	72.2-72.6	95.7-96.0	96.8	87.1	85.8-85.5	70.8-73.1	85.0-86.7

Table 2. Break point analysis of TbCSV and their putative parental sequences

DNA-A	Break point			P-Values					
	begin-end	Major Parent	Minor parent	RDP	GENECOV	Max Chi	Chimera	Si Scan	3Seq
TbCSV.TCb1	93-932	ToLCRnV [GQ994095]	TbCSV.WSF1[HQ407395]	$1.241X10^{-6}$	$2.935X10^{-5}$	$5.91X10^{-7}$	$1.436X10^{-8}$	$2.573X10^{-7}$	$1.769X10^{-8}$
	2358-2707	TbCSV.Y-T8 [HG00365]	TbCSV[IN:var:FB:12].JQ733557	$8.14X10^{-15}$	$1.22X10^{-4}$	$1.523X10^{-9}$	$3.401X10^{-10}$	$1.607X10^{-11}$	$6.198X10^{-16}$
TbCSV.TCh82	2358-2707	ToLCRnV [GQ994095]	TbCSV.WSF1[HQ407395]	$1.241X10^{-6}$	$2.935X10^{-5}$	$5.91X10^{-7}$	$1.436X10^{-8}$	$2.573X10^{-7}$	$1.769X10^{-8}$
	93-932	TbCSV.Y-T8 [HG00365]	TbCSV[IN:var:FB:12].JQ733557	$8.14X10^{-15}$	$1.22X10^{-4}$	$1.523X10^{-9}$	$3.401X10^{-10}$	$1.607X10^{-11}$	$6.198X10^{-16}$
TbCSV.TCb8	93-932	ToLCRnV [GQ994095]	TbCSV.WSF1[HQ407395]	$1.241X10^{-6}$	$2.935X10^{-5}$	$5.91X10^{-7}$	$1.436X10^{-8}$	$2.573X10^{-7}$	$1.769X10^{-8}$
	2358-2707	TbCSV.Y-T8 [HG00365]	TbCSV[IN:var:FB:12].JQ733557	$8.14X10^{-15}$	$1.22X10^{-4}$	$1.523X10^{-9}$	$3.401X10^{-10}$	$1.607X10^{-11}$	$6.198X10^{-16}$
TbCSV.TCh8	93-932	TbCSV.WSF1[HQ407395]	TbCSV.WSF1[HQ407395]	$1.241X10^{-6}$	$2.935X10^{-5}$	$5.91X10^{-7}$	$1.436X10^{-8}$	$2.573X10^{-7}$	$1.769X10^{-8}$
2358-2707		TbCSV.Y-T8[HG00365]	TbCSV[IN:var:FB:12].JQ733557	$8.14X10^{-15}$	$1.22X10^{-4}$	$1.523X10^{-9}$	$3.401X10^{-10}$	$1.607X10^{-11}$	$6.198X10^{-16}$

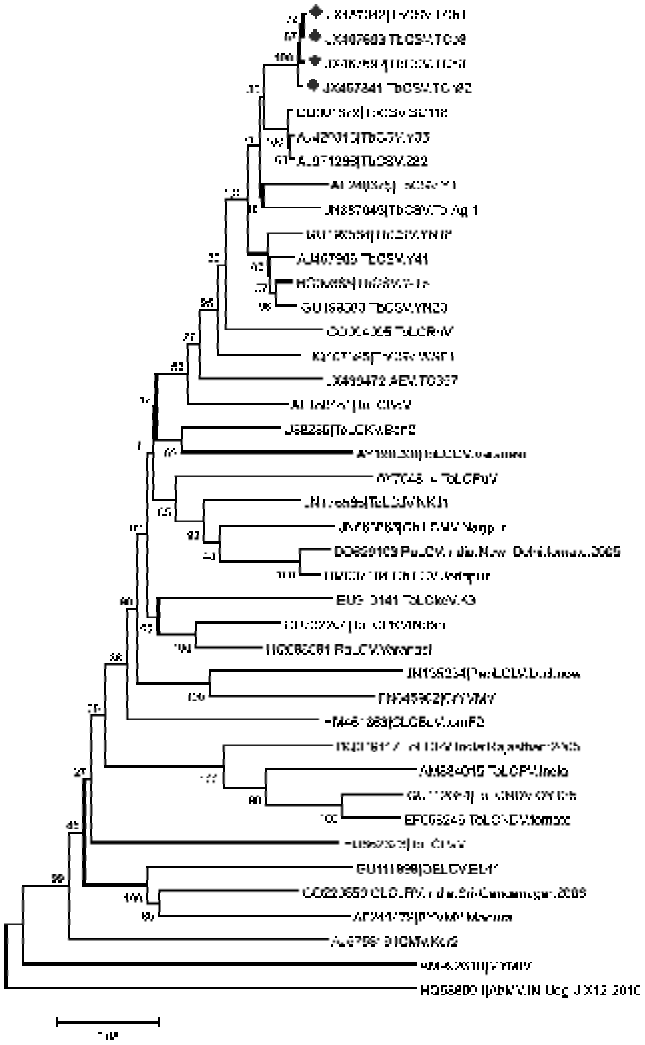

Fig. 2. Phylogenetic tree of complete nucleotide sequences of the DNA-A of the Tobacco curly shoot virus (TBCSV) isolates originating from Pantnagar with selected other begomoviruses available in the databases. The TBCSV isolates originating from pantnagar is highlighted. The number at the nodes indicates the bootstrap confidence values (1000 replicates). For each isolate the database accession numbers are given.

Recombination is a common feature in the evolutionary history of many begomoviruses. To determine whether the begomovirus identified here shows an evidence of recombination, RDP3 analysis was conducted based on alignments with full-length sequences of selected begomoviruses and the closest identity with two begomoviruses such as Ageratum enation virus and tomato leaf curl Ranchi virus along with other available in the NCBI database. The results of this are shown in (Fig. 3) with the details, including p-values, given in Table

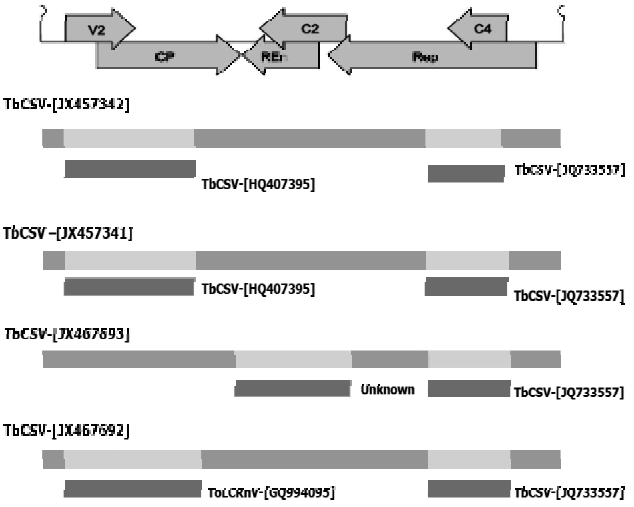

Fig. 3. Analysis for recombination among selected begomoviruses associated with ToLCD. Recombinant fragments in the sequences of TbCSV isolates were identified using RDP3 analysis of a Clustal W alignment. The default X-Over settings was used for recombination analysis. Recombinant sequences are identified as dark lines below the sequence (identified above each full-length sequence line).

3. The analysis showed TbCSV isolates from tomato to consist of the virion-sense sequences of ToLCRnV and the complementary-sense sequences of AEV and other isolates of TbCSV covering in the C1 region and C4 region. The isolate TbCSV-(JX467692) was found to be recombinant with ToLCRnV-(GQ994095) as major and TbCSV-(JQ733557) as minor parents. The recombination breakpoints were determined are shown in the (Table 2).

DISCUSSION

The tomato leaf curl disease (ToLCD) is one of the most devastating diseases of tomato (*Solanum lycopersicum* L.) and mixed infections in natural epidemics can be frequent (Delatte *et al*., 2005; García-Andrés *et al*., 2007). The existence of mixed infections is a cause of concern because it is a prerequisite for recombination to occur, a frequent phenomenon among geminiviruses (Padidam *et al*., 1999), with unpredictable pathological consequences. In India, diseases caused by whitefly-transmitted begomoviruses are on increase, affecting the cultivation and economic yield of vegetables, including, tomato, pepper and chilli. Increased overlapping cultivation of closely related solanaceous and cucurbitaceous crops has resulted in an expanded host range of existing begomoviruses and has led to the emergence of new strains and recombinants. This is especially true for tomato, which is grown in diverse

agroclimatic zones throughout the year, thus perpetuating the virus inoculum and vector population (Varma *et al*., 2011, Ramappa *et al*., 1998). Considerable progress has been made in characterizing tomato begomoviruses in southern, western and northern India. At present, a total of 16 tomato infecting begomovirus species are known to be associated with ToLCD in India (Brown *et al*. 2012; Fauquet *et al*. 2008). Tomato leaf curl Bangalore virus (ToLcBav) (Muniyappa *et al*., 2000), Tomato leaf curl Karnataka virus (ToLCKaV) (Chatchawankanphanich and Maxwell 2002), and Tomato leaf curl Kerala virus (ToLCKeV) (Pasumarthy *et al*., 2010) from southern India (Hong and Harrison, 1995; Kirthi *et al.* 2002). Whereas Ageratum enation virus (AEV) (Swarnalatha *et al*., 2013), Croton yellow vein mosaic virus (Chowda Reddy *et al*., 2005; Pramesh *et al*., 2013) Papaya leaf curl virus (PaLCV), Tomato leaf curl New Delhi virus (ToLCNDV) (Srivastava *et al*. 1993), Tomato leaf curl Gujarat virus (ToLcGuV) (Chakraborty *et al*. 2003; Jyothsna *et al*., 2013), Tomato leaf curl Palampur virus (ToLCPaV) (Kumar *et al*., 2008), Tomato leaf curl Pune virus (ToLCPuV), Tomato leaf curl and Rajasthan virus (ToLCRaV) from northern India (Padidam *et al*, 1995, Chakraborty, 2008), Cotton leaf curl Burewala virus (CLCuBV) (Kumar *et al*., 2013), Tomato leaf curl Ranchi virus (ToLCRnV) (Kumari *et al*., 2011b), Tomato leaf curl Patna virus (ToLCPV) (Kumari *et al*., 2011a) and tomato leaf curl Joydebpur virus (ToLCJV) (Tiwari *et al*., 2012) have been reported from eastern India (Kumari *et al*., 2009, 2010, 2011b).

With an increasing number of samples analyzed from different locations, it is now evident that both monopartite and bipartite begomoviruses are distributed throughout India. Information regarding tomato begomoviruses from the eastern region of India – eastern U.P., Bihar, Jharkhand, West Bengal and the far north-eastern region comprising Assam, Manipur, Meghalaya and Mizoram is very limited. We surveyed the Pantnagar district and samples were collected from five poly houses and analyzed, results showed the presence of Tobacco curl shoot virus (TbCSV) causing tomato leaf curl disease in tharai region of Uttarakhand. All of the samples from Pantnagar that were tested showed the presence of AEV and TbCSV based on sequencing of genomic components of amplicons obtained with PAR1v722 and PAL1c1960 primers (Deng et al. 1994) and RCA. The Samples were also subjected to PCR amplifications with primers specific for ToLCNDV, ToLCBaV, ToLCGuV, ToLCKaV and betasatellites and were negative. Preliminary data generated in the present study indicate the predominant occurrence of TbCSV. Two tomato begomoviruses, ToLCPaV and ToLCRnV, have been reported from the neighboring state of Bihar. Interestingly sequence analysis of ToLCRnV has shown that this virus is a strain or isolate more closely related to TbLCSV (Kumari *et al*., 2011b).

Among begomoviruses, mixed infection and recombination serves as a key factor for evolution of a novel species. Indian tomato-infecting begomoviruses are also well documented for having one or more recombination events for their origin (Padidam *et al*., 1999; Kirthi *et al*., 2002; Chatchawankanphanich and Maxwell, 2002; Chakraborty *et al*., 2003). High probability of recombination, resulting into emergence of a new pathogenic population, has been reported in geminiviruses (Hou and Gilbertson, 1996; Padidam *et al*., 1999). In addition, recombination has also been reported to affect the host specificity leading to mobility to different unusual hosts (Varsani *et al*., 2008). Moreover, the recombination predictions, in which AEV (JX436472), ToLRnV (GQ994095) and TbCSV isolates (HG00365, HQ407395) were detected as probable parents for present TbCSV isolates, indicating that recombination can occur between virus species and isolates during mixed infection.

TbCSV reported first from china infecting oriental tobacco and tomato (Xie *et al*., 2002; Zhou *et al*., 2003; Li *et al*., 2004), occurs in different hosts throughout china such as ageratum (AJ971266, HG003650), *Mirabilis jalapa* (GU199584) and *Alternanthera philoxeroides* (GU199583), pepper (Qing *et al*., 2010), Its occurrence in Northern India was first observed on beans with high incidence at Varanasi, Uttar Pradesh (Venkataravanappa *et al*., 2012), subsequently it was also noticed on wild sunflower (Gen Bank acc.no. HQ407395). It is expected to spread easily throughout the north India and Indian subcontinent, as its land mass is continuous without any obstacles such as mountains or oceans. The peculiar feature of TbCSV is that it is a typical monopartite begomovirus and does not require a betasatellite to infect and produces disease symptoms in indicator hosts or the primary host, but intensifies symptoms in some hosts (Cui *et al*., 2004; Li *et al*., 2005).

Annual crops such as tomato are re-infected with geminiviruses every growing season from sources that must include other crops and weeds, as well as related hosts such as chilli, potato, and brinjal. These plants act as reservoirs of both the viruses and insect vectors in the off season. This annual cycle between crop and reservoir hosts is a stringent bottleneck that potentially

reduces the genetic diversity of the viruses in the crop. Each year only the best adapted viruses survive the bottleneck and spread within the crop. It is only recently that researchers have come to realize that weeds may harbor a far greater diversity of viruses and their satellites, that actually appears in the crop, and that it is possible that genetic changes (including for example component exchange and recombination) within weeds plays a major role in virus diversification. Information on the distribution and prevalence of the different begomovirus species and recombinant forms comprising the disease complex is crucial in guiding tomato breeding programs in the search for stable and durable sources of resistance. This type of study is also an important piece of information to understand natural genetic and population dynamic processes driving the emergence and continuing evolution of this unique virus complex. In addition, Brazil is considered to be one of the major diversity centers of the neotropical bipartite begomovirus cluster. However, few studies are available describing the evolutionary mechanisms associated with the upsurge of so many species and quasi-species in this group of viruses on Solanum hosts in India.

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