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A recombinant *Tobacco curly shoot virus* causes leaf curl disease in tomato in a north-eastern state of India and has potentiality to *trans*-replicate a non-cognate betasatellite

S. Shilpi · Alok Kumar · S. Biswas · Anirban Roy · Bikash Mandal

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Abstract Leaf curl disease is a serious constraint in tomato production throughout India. Several begomoviruses were reported from different parts of the country; however, identity of begomovirus associated with leaf curl disease in tomato in north-eastern states of India was obscured. In the present study, the complete genome of an isolate (To-Ag-1) of begomovirus was generated from a leaf curl sample collected from Tripura state. However, no DNA-B and betasatellite were detected in the field samples. The genome of To-Ag-1 isolate contained 2,755 nucleotides that shared 94.7 % sequence identity with *Tobacco curly shoot virus* (TbCSV) and 71.3–90.1 % sequence identity with the other tomato-infecting begomoviruses occurring in the Indian subcontinent. Several inter-specific recombination events among different tomato-infecting begomoviruses from India and intra-specific recombination among different isolates of TbCSV reported from China were observed in the genome of To-Ag-1 isolate. Agroinoculation of the virus alone produced leaf curl symptoms in tomato and *Nicotiana benthamiana.* However, co-inoculation with a non-cognate betasatellite, Croton yellow vein mosaic betasatellite (CroYVMB) with the TbCSV resulted in increased severity of the symptoms both in tomato and *N. benthamiana*. Systemic distribution of the TbCSV and CroYVMB was detected in the newly developed leaves of tobacco and tomato, which showed ability of TbCSV to trans-replicate CroYVMB. The present study for the first time provides evidence of occurrence of TbCSV in tomato in north-eastern region of India and showed increased virulence of TbCSV with a non-cognate betasatellite.

Keywords Begomovirus · Tomato leaf curl disease · Tobacco curly shoot virus · North-eastern India · Agroinoculation

Introduction

Begomoviruses (family *Geminiviridae*) contain circular single-stranded DNA genome, which is encapsidated in a geminate (18–22 nm) particle and is vectored exclusively by whitefly (*Bemisia tabaci*). Begomoviruses are originally known to have bipartite genome consisting of two genomic components designated as DNA-A and DNA-B, each of which has ca. 2.7 kb size [1]. DNA-A encodes genes responsible for encapsidation, virus replication, and regulation of gene expression, whereas DNA-B component encodes two proteins involved in efficient systemic spread in hosts and symptom modulation [2, 3]. Several monopartite begomoviruses, which have one genome component homologous to that of DNA-A of bipartite begomovirus, are also reported. Until recently, it was thought that monopartite begomoviruses were present only in the Old World; however, a monopartite begomovirus associated with tomato leaf curl disease was reported recently from New World countries like Ecuador and Peru [4]. The monopartite begomoviruses do not contain DNA-B, however, many of them have been shown to be associated with

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S. Shilpi · A. Kumar · A. Roy · B. Mandal (✉)
Advanced Centre for Plant Virology, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi 110012, India
e-mail: leafcurl@rediffmail.com

S. Biswas
ICAR Research Complex for NEH Region, Lembucherra, Tripura, India

a novel single-stranded DNA satellite referred as betasatellite, which are approximately half the size of DNA-A genome [5]. In a few instances, monopartite begomoviruses produce disease symptoms alone without the requirement of betasatellite, but in majority of the cases, monopartite begomoviruses require betasatellite for development of typical disease symptoms [6–10]. Association of a betasatellite with a particular begomovirus is not often very rigid. Some monopartite begomoviruses were shown to be associated with betasatellites, which were originally reported to be associated with other begomoviruses [11, 12].

Begomovirus have emerged as a major threat to cultivation of crops in many tropical and subtropical regions [13]. As many as 55 distinct begomovirus species and 26 tentative species have been identified to infect tomato worldwide [14]. Considerable diversity of begomoviruses infecting tomato is known in India [15, 16]. *Tomato leaf curl New Delhi virus* (ToLCNDV) [17], *Tomato leaf curl Gujarat virus* (ToLCGV) [18], and Tomato leaf curl Palampur virus (ToLCPV) [19] have bipartite genome, whereas *Tomato leaf curl Bangalore virus* (ToLCBV) [20], *Tomato leaf curl Joydebpur virus* (ToLCJV) [16], *Tomato leaf curl Karnataka virus* (ToLCKaV) [21], *Tomato leaf curl Kerala virus* (ToLCKeV) [22], Tomato leaf curl Patna virus (ToLCPaV) [23], *Tomato leaf curl Pune virus* (ToLCPuV), Tomato leaf curl Rajasthan virus (ToLCRaV), and Tomato leaf curl Ranchi virus (ToLCRnV) [24] have monopartite genome. During last one year, three other monopartite begomoviruses, cotton leaf curl Burewala virus (CLCuBV) [25], *Ageratum enation virus* (AEV) [26] and *Tobacco curly shoot virus* (TbCSV) [27] were reported to be associated with leaf curl disease of tomato in India. It is now evident that both mono- and bi-partitie begomoviruses are distributed across India. Majority of the begomoviruses on tomato in India were characterized from northern, southern, and western part of the country. Three species of begomovirus, ToLCJV, ToLCRnV, and ToLCPaV have been reported from West Bengal and Bihar, the two eastern states of India [16, 23, 24]. Information regarding tomato-infecting begomoviruses from the far north-eastern region of Assam, Arunachal Pradesh, Manipur, Meghalaya, Mizoram, and Tripura is not available. Like the other parts of India, leaf curl disease of tomato is a severe production constraint in north-eastern states too. Very recently, preliminary identification based on coat protein gene showed association of begomovirus with leaf curl disease of tomato in north-eastern part, which is one of the biodiversity hot-spot in India [28]. However, due to the lack of complete genome sequence, no conclusive evidence could be drawn regarding the exact identity of begomovirus species occurring in north-eastern region of India. In this study, based on complete genome sequence and agroinfection, we provide evidence that TbCSV is occurring in tomato in Tripura, a north-eastern state of India. Further, we show that TbCSV trans-replicates a non-cognate betasatellite resulting in increased virulence of the virus.

Materials and methods

Collection of virus samples, DNA extraction, and PCR amplification

Tomato plants showing leaf curl symptoms were collected (five samples) from fields of Agartala (23° 50′ N, 91° 25′ E), Tripura, a north-eastern state of India, in 2010. Total nucleic acids were extracted from those leaf samples (symptomatic and asymptomatic) obtained from field using cetyl trimethyl ammonium bromide method [29]. Initially, for detection of the virus, PCR was conducted for all the samples using universal degenerate primer pairs PAL1v1978/PAR1c496 (for DNA-A), and PBL1v2040/PCRc1 (for DNA-B), which are routinely used for begomovirus detection [30]. PCR was also carried out with another primer pair AV30F: ttggatccatggcgaagccgacca and AV31R: aagagctcttaatttgtgaccga and these were designed from the coat protein gene sequence of ToLCNDV [31]. To amplify the associated betasatellite, PCR was conducted using universal primers (β-01: ggtaccactacgctacgcagcagcc and β-02: ggtacctaccctcccaggggtacac) [7]. PCR was conducted in 20 μL reaction mixture containing 100 ng of total plant DNA, 2 μL of 10× reaction buffer, 1 μL of 10 mM dNTP, 10 μM of each primer, 0.5 μL (5 U μL^{-1}) of RealTaq DNA polymerase (Real Biotech Corporation, Banqiao city, Taiwan). The PCR was carried out in a Biometra T Personal Thermal Cycler (Biometra GmbH, Goettingen, Germany). Thermocyclic parameters for betasatellite, begomovirus, and ToLCNDV were followed as described in reference numbers 7, 30, and 31, respectively. The amplified fragments were resolved in 1 % agarose gel electrophoresis.

Rolling circle amplification

To isolate the complete genome of the begomovirus, rolling circle amplification (RCA) was carried out using phi-29 DNA polymerase (Fermentas, Glen Burnie, Maryland, USA) following the standard protocol [32]. Briefly, 50 ng of total DNA was mixed with 1× phi-29 enzyme buffer, 10 μM exo-resistant random primer, 2 mM dNTP, and denatured for 5 min at 95 °C. After cooling on ice for 2 min, 0.02 unit of pyrophosphatase and 5 unit of phi-29 DNA polymerase were added and incubated for 18 h at

30 °C, followed by heat inactivation of enzyme at 65 °C for 10 min.

Cloning, sequencing and phylogenetic analysis

RCA products were digested with different restriction endonucleases (*Bam*HI, *Eco*RI, *Hind*III, *Sal*1 and *Xba*I) using FastDigest kit (Fermentas, GlenBurnie,Maryland,USA). Except *Sal*I and *Eco*RI, all other restriction enzymes generated begomovirus-genome specific 2.7 kb fragments, which were cloned in pDrive vector (Quiagen, Valencia,CA, USA). The ligated products were used to transform DH5α strain of *Escherichia coli*. Recombinant clones were detected through restriction digestion with respective enzymes. The selected clones were sequenced at commercial facilities of Xcelris Genomics, Ahmedabad, India. The nucleotide sequence data of the clones were assembled in BioEdit sequence alignment editor [33] and open reading frames (ORFs) were identified by ORF Finder (www.ncbi.nlm.nih.gov). The sequences were initially compared using BLAST [34], and related sequences were retrieved from the sequence database for further comparison. The sequence identity matrix was generated using BioEdit sequence alignment editor. Multiple alignment was performed using Clustal W algorithm and phylogenetic relationships were determined by neighbor-joining method using the MEGA5 software [35]. The tree was bootstrapped with 1000 replication and finally a consensus tree was generated. The recombination breakpoint analysis was carried out using a combination of RDP, GENECONV, BOOTSCAN, MAXCHI, CHIMERA, SISCAN, and 3SEQ recombination detection methods implemented in RDP v.3.44 [36]. Default settings (P value cut-off = 0.05) and the standard Bonferroni correction were used. Only potential recombination events detected by three or more of the above mentioned methods were considered significant.

Construction of infectious clone

To study the infectivity of the virus, partial tandem repeats (PTR) construct was developed from a full-length clone generated with *Hind*III, pTo-Ag-1 in binary vector pCAMBIA 2300. Briefly, pTo-Ag-1 clone was digested with *Hind*III and *Xba*I to generate a 1.0 kb (containing origin of replication) and a 1.7 kb fragments. The *Hind*III-*Xba*I digested 1 kb fragment was purified, and cloned into the pCambia2300 vector within the same restriction site to give pC-T0.4mer. The full-length genome specific (1.0-mer) component was then released from pTo-Ag-1 clone with *Hind*III digestion and was cloned into *Hind*III-linearized pC-T0.4 mer to generate a partial dimeric construct, designated as pC-T1.4. The orientation of the construct was confirmed by restriction digestion with *Xba*I, which was expected to release 2.7 kb fragment.

To study the effect of betasatellite, co-inoculation was conducted with a dimeric construct of Croton yellow vein mosaic betasatellite (CroYVMB) (pC-β2.0) developed earlier in our lab (Roy et al., unpublished). CroYVMB genome was earlier cloned and sequenced (JX270685). The entire genome of CroYVMB was amplified with a mutated forward primer β-01* (5′ggtaccactacgctacgag3′) and β-02 universal primer using the previous CroYVMB clone (M4β) as template. This amplified product was then cloned in pGEMT easy vector through blunt end ligation to create pGEMT-M4β. The 1.3 kb betasatellite was released from pGEMT-M4β clone through digestion with *Sac*I and *Kpn*I and cloned into pCAMBIA 2300 vector in between the same restriction site, designated as pC-β1.0 mer. For inserting another copy of betasatellite, a full-length betasatellite (ca. 1.3 kb) was released as *Kpn*I fragment from M4β and was re-cloned in *Kpn*I-linearized pC-β1.0 mer. The resulting complete dimeric construct was then designated as pC-β2.0. The tandem orientation of the construct was verified by digestion with *Xba*I, which was expected to release a ca. 1.3 kb fragment. A CYVMV partial dimer construct (pC-C1.4) (unpublished), alone and in combination with CroYVMB construct were also used in agroinoculation for comparison.

Agroinoculation

The pC-T1.4, pC-C1.4, and pC-β2.0 were mobilized into the *Agrobacterium* strain EHA105. The agro-mobilized constructs were separately grown in Luria agar for 48 h at 28 °C and then harvested in 500 µl of B5 medium. *Agrobacterium* containing pC-T1.4 was prick inoculated to young seedlings (3–4 leaf stage) of *Nicotiana benthamiana* and tomato individually and along with pC-β2.0 to understand the interaction of TbCSV with a heterologous betasatellite. To compare the interaction, *Agrobacterium* containing pC-C1.4 was inoculated into these plants singly and in combination with pC-β2.0. The agroinoculated seedlings were maintained in an environment controlled plant growth room at 28–30 °C for 30 days and observations were recorded periodically. A mock inoculation with agrobacterium containing only pCAMBIA2300, which served as negative control, was performed in each case.

Detection of viral DNA

Total genomic DNA was extracted by CTAB method [37] from the newly emerged leaves of agroinoculated *N. benthamiana* and tomato plant showing typical symptoms. To detect the presence of TbCSV in the agroinoculated plants, a specific primer set (BM390F: 5′tccccaagttgcccaca3′ and

BM340R: 5′ttgtatatgtacaacaaactta3′), which is expected to produce ca. 1.0 kb amplicon, was used in PCR. To detect the betasatellite in the co-inoculated plants, universal betasatellite primers [7], which is expected to produce ca. 1.3 kb amplicon, were used.

Results

Disease occurrence

The occurrence of leaf curl disease in tomato was observed from October to February with a peak incidence from December to January in Tripura. In most cases, the disease appeared with upward curling of leaves or rolling of leaf margins, stunting of plant growth, and dull green leaf coloration with or without yellowing (Fig. 1a). The disease appeared in all most all tomato fields in the South, West, and Dhalai districts of Tripura with about 50–80 % disease incidence resulting in drastic yield loss.

Detection of the virus

Initially, PCR was conducted for all the five samples collected from Agartala with degenerate primer pairs for both DNA-A and DNA-B of begomovirus, but none of the samples gave any amplification. All the samples were further analyzed by PCR with a pair of CP primer to ToLCNDV and universal primer to betasatellite, which also did not yield any amplification with repeated attempts. However, an isolate (To-Ag-1) that was subjected for RCA, yielded a 2.7 kb fragment following restriction digestion with *Bam*HI, *Hind*III, and *Xba*I (Fig. 1b). The digestion of RCA product with *Eco*RI generated slightly shorter than 2.7 kb fragment, whereas no digested product was obtained with *Sal*I.

Genome organization and phylogenetic relationships

The partial sequence of clones obtained with *Hind*III, *Bam*HI, and *Xba*I showed identical sequences. Therefore, a full-length clone generated with *Hind*III, pTo-Ag-1 was completely sequenced, which contained 2,755 nucleotides [nt] (JN387045) and showed genome organization similar to other begomoviruses reported from Old World. The genome contained six open reading frames (ORFs), two of which AV1 (303–1073 nt) potentially encoding 29.86 kDa coat protein and AV2 (143–499 nt) encoding 13.7 kDa pre-coat protein, were on the viral sense strand and four ORFs AC1 (1522–2607 nt) encoding replication associated 40.38 kDa protein, AC2 (1215–1619 nt) encoding transcriptional activator 15.05 kDa protein, AC3 (1070–1474 nt) encoding replication enhancer 15.98 kDa protein, and AC4 (2157–2579 nt) encoding 16.09 kDa protein, were on the complementary strand. The intergenic region (IR) was 290 nt long containing a characteristic inverted repeat of 34 nt forming a stem-loop structure, which includes the highly conserved nonanucleotide sequence, TAATATT↓AC found in all geminiviruses. This intergenic region also contained iteron sequence CAATCGGG (nucleotides 2646–2653, repeated at 2618–2625) and GGGTCCT (nucleotides 2658–2664, repeated at 2623–2629).

Comparison of the sequence of To-Ag-1 (JN387045) to that of the other begomoviruses available in the database showed that the present virus isolate shared (88.4–94.7 %) sequence identity with the different isolates of TbCSV reported from China and India (Table 1). It shared highest sequence identity (94.7 %) with TbCSV isolates reported on pepper (GU001879) and tobacco (AJ420318) from china. To-Ag-1 shared 71.3–90.1 % sequence identity with the other tomato-infecting begomoviruses occurring in India (Table 2). Among the other tomato-infecting begomoviruses, it showed highest sequence identity (90.1 %)

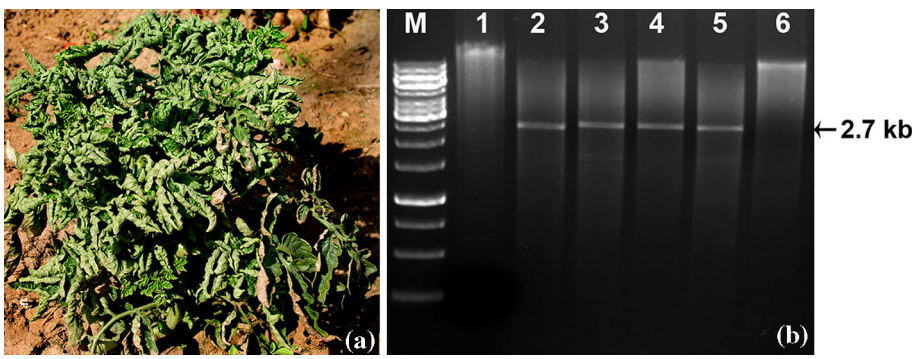

Fig. 1 Leaf curl disease affected tomato plants in Agartala, Tripura state during 2010 showing marginal rolling and curling of leaves (**a**) and amplification of complete genome of the begomovirus through rolling circle amplification (RCA) followed by restriction digestion with *Eco*RI, *Hind*III, *BamH*I, *Xba*I and *Sal*I (lane 2–6, respectively); lane M: 1 Kb DNA ladder, lane 1: RCA-undigested product (**b**)

Table 1 Percent sequence identities of To-Ag-1 isolate (JN387045) with other isolates of *Tobacco curly shoot virus* (TbCSV) infecting different hosts reported from China and India

Accession No.	TbCSV isolate	Host	Country	DNA-A	IR	AC1		AC2		AC3		AC4		AV1		AV2	
						Nt	AA	Nt	AA	Nt	AA	Nt	AA	Nt	AA	Nt	AA
GU001879	SC118	Pepper	China	94.7	84.4	95.4	95.5	97.0	94.7	96.5	93.2	***69.0***	***68.5***	96.8	96.8	93.8	91.5
AJ420318	Y35	Tobacco	China	94.7	84.4	95.6	95.8	96.7	94.0	96.2	93.2	***69.0***	***68.5***	96.8	97.2	93.5	90.6
AJ971266	Y282	Ageratum	China	94.6	84.4	95.3	95.2	97.0	94.7	96.5	93.2	98.8	97.1	96.8	97.2	93.2	89.8
JX457342	TCb1	Tomato	India	94.4	86.7	95.3	96.1	95.3	93.2	94.8	91.0	99.2	97.8	97.0	97.6	92.7	91.5
JQ733557	FB-01	Common bean	India	93.7	82.7	93.0	93.0	96.2	94.0	94.5	97.0	***66.1***	***61.4***	98.0	98.8	96.0	94.0
AF240675	Y1	Tobacco	China	93.2	83.1	94.2	89.1	95.3	91.7	92.5	91.0	***69.2***	***68.5***	95.7	96.8	96.6	94.0
AJ457986	Y41	Tomato	China	92.5	77.5	92.0	92.5	96.5	93.2	96.0	92.5	***66.4***	***63.5***	96.7	96.4	93.2	90.6
GU199583	YN20	Alternanthera	China	92.2	76.2	92.3	93.0	96.5	94.7	95.8	91.7	***67.3***	***63.5***	96.1	95.7	91.8	88.1
GU199584	YN18	Mirabilis	China	91.8	76.5	91.7	91.9	96.7	94.7	96.5	93.2	***66.4***	***62.8***	95.2	96.0	95.2	94.0
HQ407395	WSF1	Sunflower	India	88.4	72.0	86.6	78.4	90.3	83.5	91.1	87.3	***63.5***	***57.1***	95.7	96.8	92.7	90.6

Bolditalic values indicated <70 % sequence identities in AC4 gene and protein

Nt nucleotide sequence, *AA* amino acid sequence

with ToLCRnV followed by AEV (86.3 %). The ORF wise sequence identities both at nucleotide and protein level between To-Ag-1 and ten other isolates of TbCSV reported from China and India revealed a wide sequence diversity in AC4 gene (0.8–36.5 % for nucleotide and 2.2–42.9 % for amino acid) followed by in IR (13.3–28.0 %) (Table 1). Similarly, the present isolate showed maximum diversity with the other tomato-infecting begomoviruses in AC4 gene (46.7–66.5 %) (Table 2). Phylogenetic analysis of all the begomoviruses reported on tomato from India along with the TbCSV isolates reported on different crops from India and China showed that the present isolate belongs to the TbCSV cluster (Fig. 2).

Different methods used for recombination breakpoint analysis provided strong evidence for presence of recombination events in the sequence of To-Ag-1 isolate (Table 3). Multiple overlapping inter- and intra-specific recombination events were detected with different parental combinations. These recombination events had significant phylogenetic support as evidenced from the corresponding UPGMA trees developed by the RDP (supplementary Fig. 1a–e). The recombination events were distributed throughout the genome. Inter-specific recombination events in TbCSV involving different major and minor parents were predominantly distributed in the genome covering from the right hand side of the IR region to AC4 region: In IR-AV2, it was between ToLCKaV and ToLCRaV, in AV1 region it was between ToLCBaV and ToLCRaV, in AV1-AC3 region: ToLCBaV and ToLCKeV, in AC3-AC2 region: ToLCGuV and ToLCKeV. However, intra-specific recombination among different isolates of TbCSV took place in the AC1, AC4, and left hand side of the IR region. No recombination event was observed between any TbCSV isolate and other tomato-infecting begomovirus in India.

Infectivity of cloned DNA

The results of agroinoculation is summarized in Table 4. TbCSV alone produced marginal leaf rolling, vein thickening, and stunting symptoms in newly emerging leaves of *N. benthamiana*at 10–13 dpi. In tomato, TbCSV alone produced mild marginal leaf rolling symptoms in the newly developed leaves at 15–20 dpi. Co-inoculation of TbCSV with CroYVMB resulted in severe curling, marginal leaf rolling, vein thickening, and severe stunting symptoms in *N. benthamiana* (Fig. 3a), and curling, smalling of leaves and stunting in tomato (Fig. 3b). *N benthamiana* and tomato plants inoculated with CYVMV alone developed typical leaf curl symptoms, whereas co-inoculation with CroYVMB dramatically increased the severity of curling and stunting symptoms. Co-inoculation of CroYVMB either with TbCSV or CYVMV in both

Table 2 Percent sequence identities of To-Ag-1 isolate (JN387045) with other tomato-infecting begomoviruses reported from India

Virus	Accession No.	DNA-A	IR	AC1		AC2		AC3		AC4		AV1		AV2	
				Nt	AA	Nt	AA	Nt	AA	Nt	AA	Nt	AA	Nt	AA
TolCRnV	GQ994095	90.1	73.5	88.3	88.9	95.8	93.2	94.5	91	***63.1***	***57.1***	93.3	96.4	92.9	93.2
AEV	KC818421	86.3	69	80.5	83.3	92	85.8	91.6	91.7	***45.1***	***29.2***	96.1	96.4	95.5	94
ToLCKaV	U38239	84.4	66.7	86	85.8	87.9	79.8	87.4	86.5	***61.9***	***55***	86.2	94.1	93.5	94
ToLCJV	JN176565	84.1	75.7	86.1	85	84.6	76.1	81.2	76.8	***61.5***	***51.4***	88.4	96	87.7	87.6
ToLCGuV	AF413671	81.6	66.5	87.6	88	86.6	79.1	80	77.6	***63.3***	***57.1***	77.9	78.9	86.2	82.2
ToLCKeV	EU910141	80.6	63.6	79.4	81.1	88.3	80.5	87.1	84.3	***49.8***	***30***	85.3	94.1	92.9	90.6
CLCuBV	HM461863	79.5	56.6	76.4	80.1	76.5	65.6	82.4	76.1	***50.2***	***34.2***	92	95.7	90.7	91.5
ToLCPuV	AY754814	78.6	58.1	83.5	85.3	84.9	76.8	83.2	76.8	***58.3***	***47.8***	79.3	89	74.7	68.6
ToLCBaV	Z48182	77.6	57	81	83.1	85.9	79.1	85.6	80.5	***58.8***	***50***	78.8	88.6	72.2	68.6
ToLCRaV	DQ339117	77.1	66.5	74.2	76.7	72	58.2	71.5	66.1	***33.5***	***23.5***	86.5	94.9	94.3	91.5
ToLCPaV	EU862323	74.6	56	76	77.2	73	63.7	77.5	68.6	***49.5***	***32.1***	76.6	81.3	79.7	74.7
ToLCNDV	FJ468356	72.6	58.6	73.5	75.9	69	56.1	70.5	66.1	***34.5***	***27.1***	80.6	91	73.1	68.6
ToLCPV	KF663700	71.3	60	71	72.4	68.3	53.9	70.8	60.2	***33.5***	***25.7***	78.9	87.5	73.3	66.1

Bolditalic values indicated <70 % sequence identities in AC4 gene and protein

Nt nucleotide sequence, *AA* amino acid sequence

N. benthamiana and tomato resulted early onset of symptoms by 2–5 dpi (Table 4).

Detection of TbCSV in agroinoculated plants

Newly emerging symptomatic leaves of tomato and *N. benthamiana*, which were agroinoculated with TbCSV alone gave ca. 1.0 kb amplicon (Fig. 3c). *N. benthamiana* and tomato plants, which were co-inoculated with CroYVMB produce ca. 1.3 kb amplicon of betasatellite along with the TbCSV amplicon in the newly developed leaf tissues indicating replication and systemic distribution of both the viral genomic and satellite DNA (Fig. 3d). However, no amplification was observed in the plants inoculated either with CroYVMB alone or plants inoculated with binary vector.

Discussion

In this study, we demonstrated the occurrence of TbCSV, a monopartite begomovirus species in north-eastern region of India. Leaf curl disease of tomato is a major problem in tomato production in north-eastern part of India, however, identity of the virus associated with leaf curl disease of tomato in this region was lacking.

Degenerate primers are used generally for detecting begomovirus in suspected field samples [30]. However, in our experiment negative amplification with degenerate primers indicated the virus associated with the samples might have no sequence complementarities with the primers used. As ToLCNDV is known to be widely prevalent in the tomato growing region in India [15], the samples were further subjected to PCR using primers to ToLCNDV and betasatellites. Failure in PCR-based detection prompted to identify the virus through RCA approach [32]. Cloning and sequencing revealed the presence of TbCSV with the leaf curl disease affected tomato samples collected from Tripura. As limited samples were analyzed, it is difficult to assess the extent of prevalence of the specific begomovirus in this region. Various types of leaf curl symptoms were observed in tomato in Tripura, which may be associated with different begomoviruses. Hence, further investigation is necessary to determine the diversity of the begomovirus species occurring in tomato in north-eastern India.

The TbCSV was first identified in tobacco in China [38] and subsequently it was detected in different hosts: tomato (AJ457986), pepper (GU001879), *Alternenthera* sp. (GU199583), *Mirabilis jalapa* (GU199584), and ageratum (AJ971266). Recently, TbCSV was detected in different hosts in India too; sunflower (HQ407395), common bean (JQ733557) as well as on tomato (JX457342). This shows that TbCSV is prevalent in India. The sequence comparison among the isolates of TbCSV reported from China and India revealed interesting sequence divergence (<70 % sequence identity) in AC4 gene of the present isolate from the most of the isolates except a tomato isolate from India (JX457342) and an Ageratum isolate from China (AJ971266), which may be due to exchange of AC4 gene from these isolates, a situation also observed in other begomovirus [39].

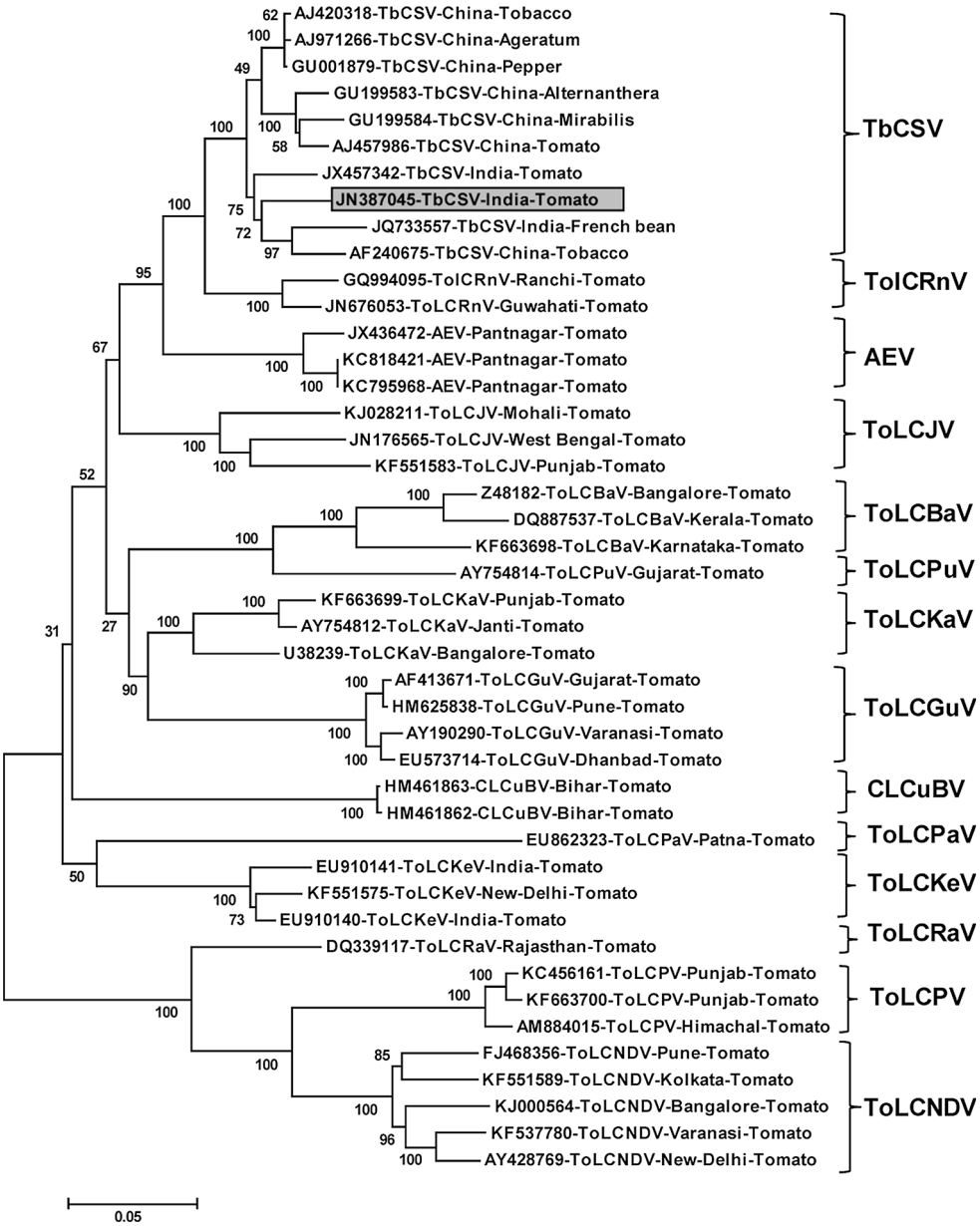

Fig. 2 Phylogenetic relationships among *Tobacco curly shoot virus* (TbCSV) isolates infecting different hosts in India and China with other begomoviruses infecting tomato in India. The dendrogram was constructed using Neighbor-Joining method in MEGA5. A bootstrap analysis with 1,000 replicates was performed. The present isolate of TbCSV was highlighted. The 14 begomovirus species infecting tomato in India representing the cluster is labeled

Phylogenetic analysis revealed that the present isolate grouped along with other TbCSV isolates reported from India and China. Interestingly, in the same cluster, another tomato-infecting begomovirus ToLCRnV was also present. Sequence identity revealed that ToLCRnV shared more than 89.0 % identity (present cut-off value for species identification in begomoviruses) with TbCSV, hence it might be an isolate of TbCSV, rather than a distinct species as claimed in the other study [24].

Different methods used for recombination breakpoint analysis using RDP software provided strong evidence for the presence of past recombination events in most of the genome sequence. Analysis of recombination showed TbCSV isolate on tomato from north-eastern region of India, had inter-specific recombination between different begomovirus species associated with tomato leaf curl disease in India. Intra-specific recombination was observed among different TbCSV isolates reported from China. Such inter- and intra-specific recombination is a predominant feature of begomovirus evolution [40] and has been implicated in the emergence of new begomovirus species and adaptation in new hosts in agricultural system [41]. Interestingly, in the recombination events, majority of recombination did not involve TbCSV isolates, only a portion of the genome showing intra-specific recombination between TbCSV isolates. Among the tomato-infecting begomoviruses, predominantly ToLCNDV and ToLCPV are present in northern India and ToLCJV and ToLCRnV

Table 3 Recombination breakpoint analysis of To-Ag-1 isolate (JN387045) of *Tobacco curly shoot virus* infecting tomato in north-eastern India

Break Point (Nucleotide coordinate)	Position in genome	Recombination events (avg *P* value) using diff. methods	Major parent	Minor parent
24–256	IR,AV2	RDP-38 (5.224×10^{-03}), GENECONV-28 (4.943×10^{-03}), Bootscan-48 (6.459×10^{-03})	ToLCKaV-Pun (KF663699)	ToLCRaV-Raj (DQ339117)
545–947	AV1	RDP-121 (7.958×10^{-04}), GENECONV-55 (3.774×10^{-03}), Bootscan-127 (2.153×10^{-03})	ToLCBaV-Har (AY456684)	ToLCRaV-Raj (DQ339117)
539–1123	AV1,AC3	RDP-121 (7.958×10^{-04}), Bootscan-127 (2.153×10^{-03}), SiScan-57 (6.041×10^{-04})	ToLCBaV-Har (AY456684)	ToLCKev (KF551575)
1205–1578	AC3,AC2	RDP-274 (8.525×10^{-03}), Bootscan-164 (1.188×10^{-02}), SiScan-243 (6.388×10^{-03})	ToLCGuV-Pun (HM625838)	ToLCKeV-Ind (EU910141)
2146–2706	AC1,AC4,IR	RDP-65 (6.347×10^{-03}), GENECONV-29 (6.721×10^{-03}), Bootscan-50 (3.378×10^{-03})	TbCSV[Y41]-China (AJ457986)	TbCSV[SC118] (GU001879)

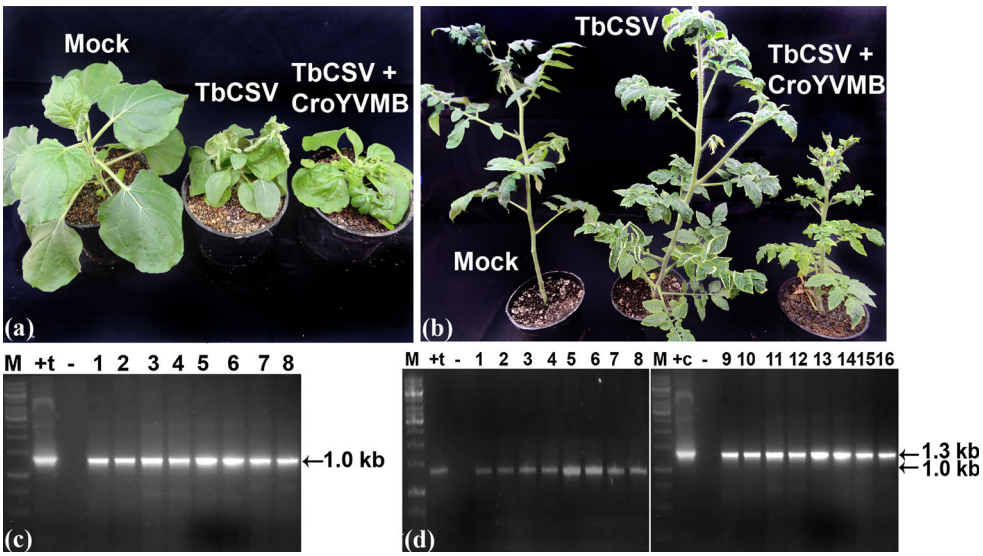

Fig. 3 Infectivity of *Tobacco curly shoot virus* (TbCSV) and Croton yellow vein mosaic betasatellite (CroYVMB) in *Nicotiana benthamiana* (**a**) and tomato (**b**) plants. (**c**) PCR detection of TbCSV in plants inoculated with the partial dimer of TbCSV alone using primers BM390F and BM340R (lanes 1–4: *N. benthamiana* and lanes 5–8: tomato). (d) PCR detection of TbCSV and CroYVMB in plants co-inoculated with partial dimer of TbCSV and dimer of CroYVMB (lanes 1–4: *N. benthamiana* and lanes 5–8: tomato) using primers BM390F and BM340R and using universal betasatellite primers (lanes 9–12: *N. benthamiana* and lanes 13–16: tomato). M: 1 Kb DNA ladder, +: positive control (t: TbCSV; c:CroYVMB); −: mock-inoculated plant with pCAMBIA2300

are present in eastern India. Recombination analysis revealed that none of the recombination event had either one of these predominant viruses as a parent.

TbCSV was originally described on tobacco, tomato and other crops in China [39, 42]. TbCSV was demonstrated to cause leaf curl alone in tobacco and tomato, and co-

Table 4 Agroinoculation of cloned DNAs of *Tobacco curly shoot virus* (TbCSV) isolate To-Ag-1, *Croton yellow vein mosaic virus* (CYVMV), and *Croton yellow vein mosaic betasatellite* (CroYVMB)

Construct[a]	Plant	Symptomatic/Inoculated plants		Dpi	Symptoms
		E1	E2		
TbCSV	NB	8/8	8/8	10–13	Marginal leaf rolling and vein thickening, stunting
	Tomato	6/10	7/10	15–20	Mild marginal rolling
CYVMV	NB	5/5	5/5	8–10	Leaf curling, rolling, vein thickening, stunting
	Tomato	4/5	3/5	10–12	Leaf curling, smalling of leaves, stunting
TbCSV + CroYVMB	NB	6/6	6/6	8–10	Severe curling, marginal leaf rolling, vein thickening and severe stunting
	Tomato	4/6	5/6	10–12	Curling, smalling of leaves and stunting of plants
CYVMV + CroYVMB	NB	5/5	5/5	7–8	Severe curling, leaf rolling, crinkling, vein thickening and severe stunting with deformed small leaves
	Tomato	4/5	5/5	8–10	Leaf curling, smalling of leaves and severe stunting of plants
pC2300	NB	0/4	0/4	NA	No symptoms
	Tomato	0/4	0/4	NA	No symptoms

NB Nicotiana benthamiana, *dpi* days post inoculation, *E* experiment number

[a] TbCSV: DNA-A partial dimer (pC-T1.4) was used, CYVMV: DNA-A partial dimer (pC-C1.4) was used, CroYVMB: complete dimer (pC-β2.0) was used. Constructs were mobilized into *Agrobacterium tumefaciens* EHA105 strain

inoculation with Tobacco curly shoot betasatellite (TbCSB) intensified the symptom in a host-dependent manner [6]. The TbCSB was found in the small proportion of field samples from southern China [6]. In our study, we failed to detect TbCSB in the field smples of tomato. As we have tested limited field samples, it is not possible to comment on whether TbCSV is not associated with TbCSB or other betasatelllites. Association of several betasatellite was detected with different begomoviruses in India [11, 43]. However, in case of begomovirus causing tomato leaf curl, it has been demonstrated that not all non-cognate betasatellite can *trans*-replicate by their helper virus [44]. The natural occurrence of any other betasatellite with TbCSV has neither been recorded, nor any interactions with other betasatellite have been demonstrated experimentally. In the present study, we have demonstrated co-inoculation of a non-cognate betasatellite, CroYVMB with TbCSV caused severe form of leaf curl in *N. benthamiana* and tomato. CroYVMB is known to induce yellow vein mosaic in *Croton bonplandianum* along with *Croton yellow vein mosaic virus* (CYVMV) [45]. CroYVMB also is widely prevalent with the other begomoviruses and crops in India [32, 43, 45, 46]. In the present study, CYVMV with CroYVMB-induced severe leaf curl disease in tomato and *N. benthamiana*. In tomato, CroVYMB has been demonstrated to induce severe leaf curl with CYVMV and other begomoviruses [45, 46]. Therefore, it is concluded that CroYVMB contributes severity of disease in association with either CYVMV or other begomoviruses.

To date, 14 begomovirus species are known to cause leaf curl in tomato in the different parts of India [15, 16]. However, prior to this study, no conclusive evidence of occurrence of any begomovirus species was known in any north-eastern states of India. Our study for the first time showed that a highly recombinant TbCSV isolate causing leaf curl disease in tomato in a north-eastern state of India. We also showed that this recombinant isolate has potentiality to *trans*-replicate a non-cognate betastellite and cause severe leaf curl disease in tomato. The management of tomato leaf curl disease through the use of resistant cultivar requires the knowledge of prevalence of begomovirus species in a region. For example, tomato cultivars, Nandi, Vybhav, and Sankranthi resistant to leaf curl disease in southern India are highly susceptible in northern India due to prevalence of different begomovirus species in northern and southern India [B. Mandal and V. Muniyappa, unpublished results]. The information generated in the present study will be useful for breeding for leaf curl resistant cultivar in tomato for north-eastern region of India.

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