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Molecular Evidence for Association of Tobacco Curly Shoot Virus and a Betasatellite with Curly Shoot Disease of Common Bean (*Phaseolus vulgaris* L.) from India

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Abstract

A new strain (FB01) of Tobacco curly shoot virus (TbCSV) showing curly shoot symptoms on common bean plants from Varanasi, Uttar Pradesh state of India was characterized. The analysis of the whole genome sequence and individual ORFs of this virus indicated that it is very closely related (sequence similarity of 89.1-94.5%) to the TbCSV infecting solanaceous and other weed crops in India and China. This was well supported by phylogenetic analysis with close clustering of the virus isolate with TbCSV. The absence of DNA-B and association of virus with betasatellite confirmed it as a monopartite begomovirus. The betasatellite identified here shared highest (53.9-93.9%) sequence identity with tomato leaf curl betasatellite. Further, putative recombination events were recognized within the virus sequence, suggesting that the virus is a recombinant and evolved from recombination of Tobacco curly shoot virus, Munbean yellow mosaic virus, Tomato leaf curl Jodhpur virus, Tobacco leaf curl Yunnan virus and Ageratum enation virus like ancestors. For betasatellite, the putative recombination events were recognized within the sequence, were interspecific. The new recombinant betasatellite was derived from recombination between Croton yellow vein mosaic betasatellite and Tomato yellow leaf curl China betasatellite, as the foremost parents in its evolution. The virus was transmitted by whiteflies as well as sap, and not by seed.

Keywords: Common bean; Tobacco curly shoot virus (TbCSV); Tomato leaf curl betasatellite (ToLCB); PCR; Whitefly; Phylogentic analyses; Recombination

Introduction

The begomoviruses belong to the family Geminiviridae, are apparently evolving as rapidly as some RNA viruses [1]. The Geminiviruses are divided into four genera, namely *Mastrevirus, Curtovirus, Topocuvirus* and *Begomovirus*, on the basis of virus insect vectors, host range and genome organization. The *Begomovirus* is the largest genus of this family and comprises whitefly transmitted geminiviruses, infecting dicotyledonous plants [2]. The genomes of Begomoviruses consist of single component (Monopartite) or two components (Bipartite), which are having approximate size of 2.6-2.8 kb each. The DNA-A in bipartite viruses and its homolog in monopartite viruses encodes pre-coat protein and coat protein in the sense strand, which are essential for transmission [3], and Replication-associated protein (Rep); the Replication Enhancer protein (REn) required for viral DNA replication; the Transcriptional Activator Protein (TrAP) required for gene expression control in the complementary strand. DNA-B encodes proteins required for intracellular movement (BC1, BV1) and transport of viral ssDNA in the host plant [4,5]. The two components share a region of high sequence homology that is known as CR, the place from where the replication of the viral DNA genomes initiates.

Most of the Begomoviruses originating from the Old World has been shown to be monopartite and known to associate with a class of ssDNA satellites, known as betasatellites and aphasatellites. Betasatellites are approximately half the size of their helper Begomoviruses, required to induce typical disease symptoms in their original hosts [6,7]. These satellites depend on their helper virus for replication, movement, encapsidation and vector transmission. Alpha-satellites are self replicating (Autonomous) circular ssDNA molecule, and are evolved from nanoviruses (*Nanoviridae*; family of circular ssDNA viruses) that became associated with Begomoviruses during mixed infections [8]. Alpha-satellites depend on the helper virus for movement, encapsidation and vector transmission, and play no role in symptom induction [6,7,9,10].

Grain legume crops across southern Asia suffer huge losses due to disease caused by Begomoviruses [11]. In southern Asia, four distinct begomoviruses associated with grain legumes are Mungbean yellow mosaic virus, Mungbean yellow mosaic India virus [12-15], Horsegram yellow mosaic virus [16] and Dolichos yellow mosaic virus [17]. They affect all major legume crops, including mungbean (Vigna radiata), blackgram (*Vigna mungo*), pigeonpea (*Cajanus cajan*), soybean (*Glycine max*), mothbean (*Vigna aconitifolia*), and common bean (*Phaseolus vulgaris*) [18].

The genus *Phaseolus* has over 50 species, and rajma or common bean (*Phaseolus vulgaris* L.) is one of them, accounting for 90% of cultivated

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species throughout the world. Globally, common bean is cultivated on about 28 million hectares per annum with a production of 19 million tonnes. Brazil is the leading producer of common bean. In India, both bushy and trailing types of common bean are grown in different part of the country, which is a key component of the cropping system due to its seeds as an importance source of rich protein (23%). Seeds are also rich in calcium, phosphorus and iron. The fresh pods and green leaves are used as vegetable in the diet, predominantly in vegetarian population of Uttar Pradesh state and eastern parts of India. The major limitation for cultivation of common bean (*Phaseolus vulgaris* L.) is Golden mosaic disease caused by whitefly-transmitted Geminivirus [11]. The random survey of different fields of common bean at Varanasi, India during 2010-2012 for incidence of viral diseases, revealed several farm fields of common bean showing predominantly, stunting, stem twisting, curly shoot, thickening of veins in the lower leaf surface and galling with dark green colour symptoms, along with whitefly *Bemisia tabaci*. These typical disease symptoms and occurrence of whitefly indicated the possibility of a Begomovirus infection. Therefore, the present study was taken up to characterize the new strain of Begomovirus associated with curly shoot disease of common bean in India.

Materials and Methods

Virus source, virus transmission and its maintenance

Leaf samples were collected from the common bean plants exhibiting stunting, stem twisting, curly shoot, thickening of lower leaf surface veins and galling with dark green colour symptoms, from the major common bean growing areas from Varanasi, Uttar Pradesh, India (Figure 1). From this infected leaf sample, the virus was transmitted to common bean cv. Arka komal using whitefly *B. tabaci*. In order to rule out the mixed infections to the least possible extent, repeated transmissions were carried out under controlled conditions and finally, the virus isolate was designated as-FB01 and used for all other studies. The culture of nonviruliferous whiteflies used for the transmission experiments were initially collected from egg plant (*Solanum melongena* L.), brought to the laboratory and allowed to feed and lay eggs on healthy cotton plants (*Gossypium hirsutum* L. cv. Laxmi) for sufficient period. After this, eggs were collected and virus-free stock of whiteflies was reared on healthy cotton plants in large wooden cages (45×45×30 cm), covered with insect proof cages galvanized with wire mesh (40-mesh size), and kept in the temperature (30°C) and Relative Humidity (RH) (60%) controlled glasshouse. Sample from non-symptomatic plant was also collected from the field.

DNA isolation

Total DNA was extracted from symptomatic and non-symptomatic plants maintained in glasshouse, and as well as field collected samples by Cetyl trimethyl ammonium bromide method [19]. The extracted DNA was diluted to required concentration with sterile distilled deionised water, before being subjected to PCR amplification and stored at -20°C.

Figure 1: Healthy bean plant (A) and virus infected bean plant showing curly shoot symptoms (B), under natural conditions observed during the sample collection.

ORFS	Start codon (nucleotide coordinates)	Stop codon (nucleotide coordinates)	Predicted size of ORFs (nt)	Predicted size of protein (no. of amino acids)	Predicted Mr (kDa)
AV1(CP)*	294	1064	771	256	28.16
V2	134	490	357	118	12.98
Rep	2598	1513	1086	361	39.71
TrAP	1610	1206	405	134	14.74
REn	1465	1061	405	134	14.74
C4	2441	2148	294	97	10.67

*Genes are indicated as Coat Protein (CP), Replication-associated protein (Rep), Transcriptional Activator Protein (TrAP), and Replication Enhancer (REn). The products encoded by ORFs V2 and C4 have yet to be named.

Table 1: Features of the begomovirus isolated from common bean.

PCR amplification, cloning and sequencing

Complete genome of virus isolate was amplified by PCR, as described by Venkataravanappa et al. [20]. For the confirmation of second component (DNA- B) and betasatellite in the sample, the universal degenerate primers specific to DNA-B [20,21] and betasatellite [22] were used. Amplified PCR products were purified from agarose gels and cloned into the plasmid vector pTZ57R/T, using T/A cloning kit (Fermentas Life Sciences, USA), according to the manufactures instructions. The complete nucleotide sequence of clones from each sample (three clones for each sample were sequenced) were determined by automated DNA sequencer, ABI PRISM 3730 (Applied Biosystems) from Anshul Biotechnologies DNA Sequencing facility, Hyderabad, Andhra Pradesh, India.

Comparison of DNA Sequences

The sequences obtained were verified for the presence of all Begomovirus specific ORFs (using NCBI ORF finder) and conserved nonanucleotide sequence. The sequence results were analysed using NCBI (www.ncbi.nlm.nih.gov) blast search, followed by sequence analysis using Bioedit Sequence Alignment Editor (version 5.0.9) [23], to determine percentage sequence identity/similarity with other species, which showed maximum identity in the blast search (Supplementary table 1). Full-length genome of selected Begomovirus species and betasatellites were aligned using Cluastal W [24], and phylogenetic trees were generated by MEGA 5.0 software [25], using the neighbour joining method with 1000 bootstrapped replications, to estimate evolutionary distances between all pairs of sequences simultaneously.

Detection of recombination events

The phylogenic evidence for recombination was detected by alignment of selected Begomoviruses sequences reported from India, which are available in the database along with bean isolate using Splits-Tree version 4.3 with neighbour-Net method [26]. The method depicts the conflicting phylogenetic signals caused by recombination as cycles, within unrooted bifurcating trees. Recombination break points analyses was carried out using Recombination Detection Program (RDP), GENECOV, Bootscan, Max Chi, Chimara, Si Scan, 3Seq integrated in RDP 3 [27]. Default RDP settings with 0.05 *P-value* cut off throughout and standard Bonferroni correction were used.

Virus transmission experiments

Vector transmission: The virus transmission protocols were carried out similar to those described by Venkataravanappa et al. [20]. Time required for optimum virus acquisition, inoculation and incubation

were determined by inoculating one week old healthy common bean cv. Arka komal seedlings with the virus isolate. Minimum number of *B. tabaci* adults required to transmit TbCSV was also determined.

Mechanical transmission: The infected common bean leaves (cv Arka komal) were harvested 10 days after whitefly inoculation with the virus isolate and macerated in a pestle and mortar by adding ice cold 0.05 M phosphate buffer, pH 7.5 containing 1 percent of 2-mercaptoethanol. The resultant pulp was squeezed between two folds of sterile absorbent cotton. Celite (6000 mesh) was added to the inoculum at 0.025 g per ml as abrasive, and one week old seedlings of common bean cv. Arka komal were inoculated by the unidirectional rubbing of forefinger dipped in inoculum. After 15 minutes, the excess inoculum was washed with a jet of water using the squeeze bottle. The plants were maintained in the separate compartment of the glasshouse for symptom production, which was free of insects. The experiment was repeated thrice and each time, 25 plants were inoculated.

Seed transmission: The matured seeds were collected from plants showing distinct curly shoot symptoms and non symptomatic healthy common bean cv. Arka komal. The seeds were treated with 2% (v/v) sodium hypochlorite for 2 min, rinsed with water several times. Three sets of 25 seeds, each from healthy and diseased plants were sown in soil, sand and compost (2:1:2 w/w) mixture in separate earthen pots. After recording germination percentage, the earthen pots with seedlings were kept in glasshouse for 1 month for symptoms development. The seedlings were sprayed with imidicloprid (0.05%) at 10 days interval to avoid chances of insect transmission, and the presence of virus in the seedlings was confirmed by PCR.

Results

Disease transmission by whitefly

All whitefly inoculated common bean seedlings showed symptoms those observed on the field infected plants, after every repeated inoculations (sub culturing) to healthy plants. The transmission tests were highly successful as the infection rate was 100% on tested common bean susceptible seedlings (Figure 2). These samples were used along with the field samples for all further experiments.

Genome amplification and sequencing

The complete genome of the virus was amplified by using three sets of primers from field infected and glasshouse inoculated samples, and attempts to amplify DNA-B components were unsuccessful. However, the positive amplification of betasatellite component by PCR with a universal abutting primer pair beta0l/beta02 in both samples, confirmed that the Begomovirus infecting common bean (*P. vulagaris*) is monopartite. Samples from non-symptomatic plants and healthy plant from the glasshouse failed to amplify for all three genome components, and served as a negative control. The amplified fragments were cloned and three clones in each case were sequenced. The alignment of sequences from multiple clones of field sample as well as glasshouse sample were identical, and all further analyses were done with the representative sequence of the virus isolate-FB01.

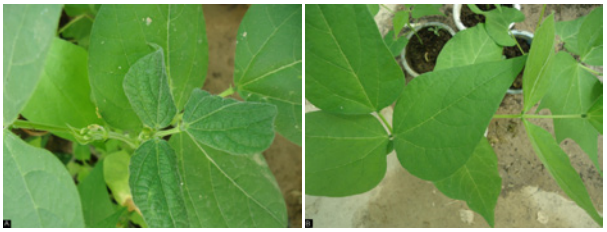

Figure 2: Common bean (cv. Arka komal) plant showing curly shoot symptoms (A), 17 days after whitefly (*B. tabaci*) inoculation with TbCSV-FB01. Bean plant without symptoms (B) inoculated with whiteflies, given acquisition access on healthy plant used as negative control in the experiment to rule out the whitefly contamination with viruses.

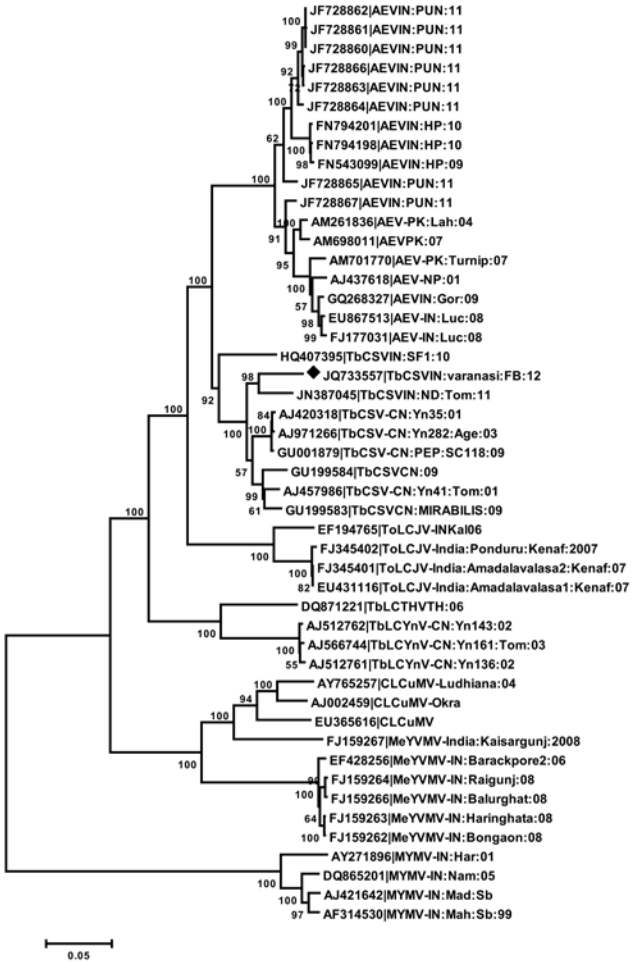

Figure 3: Phylogenetic trees constructed from aligned complete nucleotide sequence of genome component (homologous to DNA-A component of bipartite begomoviruses) of TbCSV-FB01 with other begomovirus sequences retrieved from NCBI (www.ncbi.nih.gov), using Neighbor-joining algorithm. Horizontal distances are proportional to sequence distances, vertical distances are arbitrary. The trees are unrooted. A bootstrap analysis with 1000 replicates was performed, and the bootstrap percent values more than 50 are numbered along branches.

Genome organization, sequence and phylogenetic analysis

The complete genome (homologous of DNA-A) of the virus isolate-FB01 infecting common bean was determined to be 2746 nts (JQ733557). The genome organization is typical of other Old world monopartite Begomoviruses, comprising two Open Reading Frames (ORFs) [AV1 (CP), AV2] in virion-sense strand and four ORFs [AC1 (Rep), AC2, AC3, AC4] in complementary-sense strand, separated by an Intergenic Region (IR) (Table 1). Comparisons of this virus sequence with other reported Begomoviruses sequences revealed the present isolate infecting common bean have highest sequence identity (89.1-94.5%) with Tobacco curly shoot virus (TbCSV), found in India and China infecting solanaceous and other weed crops, while it shares

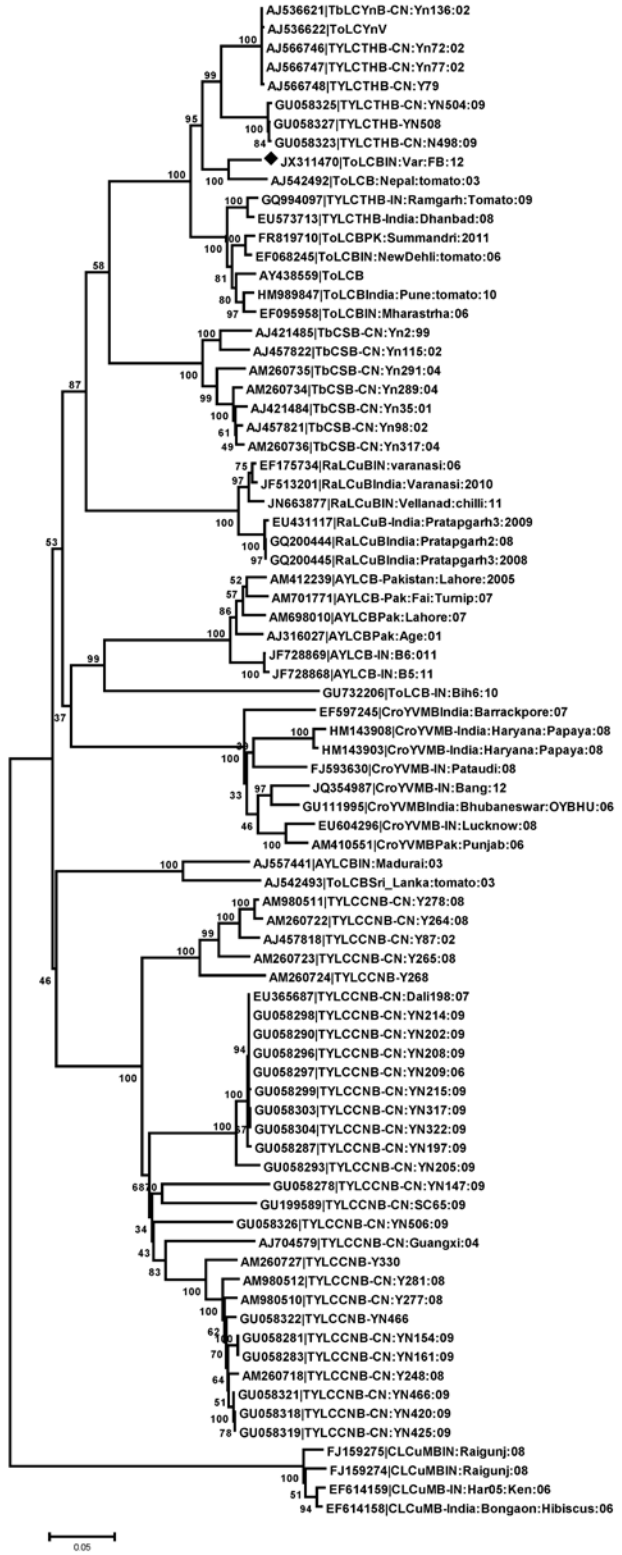

Figure 4: Phylogenetic trees constructed from aligned complete nucleotide sequence of betasatellite associated with TbCSV-FB01, with other betasatellite sequences retrieved from NCBI (www.ncbi.nih.gov) using Neighbor-joining algorithm. Horizontal distances are proportional to sequence distances, vertical distances are arbitrary. The trees are unrooted. A bootstrap analysis with 1000 replicates was performed, and the bootstrap percent values more than 50 are numbered along branches.

less than 83% identity with rest of the Begomoviruses infecting pulses, tomato, tobacco, mesta and cotton. These results suggest that virus isolate-FB01 is an isolate of TbCSV (Table 2), based on the current criteria for classification of Begomoviruses [2]. This was well supported by a phylogenetic analysis showing close clustering of virus isolate-FB01 with TbCSV infecting tomato in India, for which a full-length sequence is available in the databases (Figure 3).

Further, the percent amino acid identities of FB01 isolate with other Begomoviruses sequences revealed that the ORFAV2, AV1, AC1, AC2, AC3 and AC4 showed maximum identity with isolate of TbCSV infecting different crop plants with exception of ORF AC4, which is having maximum identity for both TbCSV and TbLCTHV (Table 2). The Intergenic Region (IR) is ~281 nts in length, and is similar to that of TbCSV infecting tomato plants and shared sequence identities, ranged from 73.1-95.3% with TbCSV, and only 13.1-78.3% with IRs of other Begomoviruses (Table 2). The IR contains a predicted stem-loop sequence with conserved nonanucleotide sequence (TAATATTAC) in the loop, which is found in the majority of the Geminiviruses characterized to date, and marks the origin of virion-strand DNA replication [28]. Within the intergenic region, incomplete two direct repeats (GGGTCC nt positions 2614-2619 and 2649-2654) of an iteron sequences were identified, adjacent to the TATA box of the Rep promoter and are probable Rep binding motifs, which binds in a sequence-specific fashion to iterated DNA motifs (iterons), functioning as essential elements for virus-specific replication.

Genome organization of betasatellite and sequence affinities to other beta satellites

The complete nucleotide sequence of betasatellite from virus isolate-FB01 was determined to be 1352 bp in length (JX311470). The sequence contain all the features of other betasatellites [7], a region of sequence rich in adenine, a single predicted gene in the complimentary sense (βC 1), with the capacity to encode a 118 amino acids, with a predicted molecular weight of 12.98 kDa and a region of sequence conserved across all betasatellites (known as the satellite conserved region). The satellite conserved region is approximately 142 bp and contains at its 3' end, a predicted hairpin structure having a loop, with the sequence TAATATTAC, similar to the origin of replication of Geminiviruses.

Pair wise sequence comparisons with other closely related sequences in the databases (Supplementary table 2) suggested that the satellite showed highest level of identity (53.9-93.9%) to the isolates of tomato leaf curl associated betasatellite (ToCLB), for which sequences are available in the database (Table 3). Based on the recently proposed species demarcation threshold of 78% for betasatellites [29], the results suggest that betasatellite identified here is an isolate of ToCLB. This was well supported by a phylogenetic analyses showing close clustering of betasatellite, associated with TbSCV infecting common bean with ToCLB infecting tomato in Nepal, for which a full-length sequence is available in the databases (Figure 3).

Recombination analysis

The phenomenon of mixed infections between viruses causing yellow mosaic of bean, tomato and ageratum providing the prerequisite for the process, where natural recombination might have contributed to the emergence of novel begomoviruses [30,31]. Initially, neighbour-net analysis was carried to detect the phylogenetic conflict by using sequences of Begomoviruses infecting pulses, tomato, tobacco, cotton and mesta, along with isolate (TbSCV) with Splits-Tree version 4.11.3 (Figure 4). Such networks are capable of graphically displaying patterns

Begomovirus#	Genome	IR	Gene (percentage amino acid sequence identity)					
			AV2	CP(AV1)	Rep (C1)	TrAP (C2)	REn (C3)	C4
TbCSV (8)*	89.1-94.5	73.1-95.3	92.3-96.6	96.0-98.8	77.3-91.4	91.7-94.0	87.3-97.0	61.4-89.6
TbLCYnV (3)	78.1-78.3	69.2-70.3	93.2-94.9	80.0-81.2	75.0-78.2	61.9-62.9	67.1-67.9	41.2-42.2
ToLCJV (4)	83.2-83.0	52.6-64.6	38.7-86.7	94.9-95.7	83.1-84.2	77.6-78.3	76.8-79.1	61.8-65.9
AEV (18)	85.0-86.3	31.1-70.7	45.2-95.6	96.8-97.6	76.5-79.3	81.1-88.8	86.5-9.25	38.1-42.2
CLCuMV (3)	72.9-73.0	53.3-54.4	65.0-66.5	90.6-91.4	74.4-76.0	62.6-64.0	69.4-70.1	44.0-45.0
MeYVMV (6)	71.7-72.0	51.0-53.7	64.4-67.7	79.2-90.6	73.9-76.3	56.6-59.3	60.2-67.9	41.1-42.0
MYMV (4)	62.9-63.2	13.1-35.5	35.5-37.2	73.9-75.0	70.5-71.5	41.0-42.5	41.0-42.5	47.4-55.5
TbLCTHV (1)	83.1	78.3	92.3	81.2	90.0	62.9	67.9	89.6

*Numbers of sequences from the databases used in the comparisons.
#The species are indicated as Tobacco curly shoot virus (TbSCV), Tobacco leaf curl Yunnan virus (TbLCYnV), Tomato leaf curl Joydebpur virus (ToLCJV), Ageratum enation virus (AEV), Cotton leaf curl Multan virus (CLCuMV), Mesta yellow vein mosaic virus (MeYVMV), Mungbean yellow mosaic virus (MYMV), Tobacco leaf curl Thailand virus (TbLCTHV). For each column, the highest value is underlined.

Table 2: Pairwise comparisons of percent nucleotide identities between the genomic components and amino acid sequence identities of encoded genes of TbCSV-FB01, infecting common bean with selected begomoviruses from the databases.

Begomoviruses	Complete sequence of DNAβ (percentage NSI)	Percentage amino acid sequence identity of βC1 gene
ToLCB (7)*	53.9-93.9	57.6-96.6
TbCSB (7)	73.2-74.1	61.0-64.4
TYLCTHB (10)	84.9-87.2	84.7-90.6
TbLCYnB (2)	85.3-86.5	87.7-90.6
CroYVMB (8)	59.7-62.4	51.6-57.6
AYLCB (8)	55.0-57.0	52.8-63.5
TYLCCNB (29)	57.0-59.6	39.8-66.1
CLCuMB (4)	50.3-50.6	28.6-29.6
RaLCuB (6)	65.3-67.0	34.7-72.8

*Numbers of sequences from the databases used in the comparisons.
#The species are indicated as Tomato leaf curl betasatellite (ToLCB), Tobacco curly shoot betasatellite (TbCSB), Tomato yellow leaf curl Thailand betasatellite (TYLCTHB), Tobacco leaf curl Yunnan betasatellite (TbLCYnB), Croton yellow vein mosaic betasatellite (CroYVMB), Ageratum yellow vein Sri Lanka betasatellite (AYLCB), Tomato yellow leaf curl China betasatellite (TYLCCNB), Cotton leaf curl Multan betasatellite (CLCuMB), Radish leaf curl betasatellite (RaLCuB). For each column, the highest value is underlined.

Table 3: Percentages of nucleotide or amino acid sequence identities of betasatellite associated TbCSV-FB01 infecting common bean with betasatellites, associated with other begomoviruses.

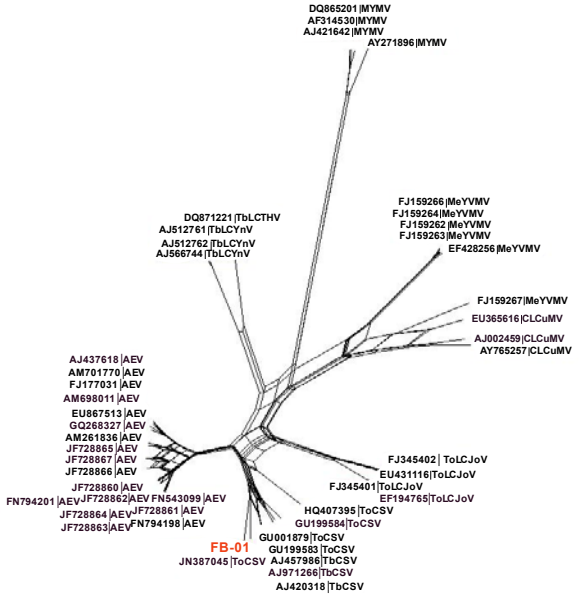

Figure 5: Neighbor-Net generated for the complete genome of TbCSV-FB01 with other begomoviruses has shown significant signals for phylogenetic conflict, indicating as recombinant virus.

of non-tree-like evolution, such as those expected in the presence of recombination (Figure 5). Based on this information, the sequences for break point analysis were subjected to know the recombination sites in virus isolate-FB01, using RDP3 with default settings [27]. Analyses showed that isolate is a recombinant and the DNA fragments might probably descended from TbCSV, MYMV, ToLCJoV, TbLCYnV and AEV like ancestors (Figure 6, Table 4). However, it is a derivative of known virus. Further, the analyses of satellite molecules showed betasatellite possess a hybrid genome of CroYVMB and TYLCCNB.

Mechanical transmission of TbCSV

Total one hundred of common bean cv. Arka komal was inoculated mechanically at two leaf stage. Out of these 80 plants expressed curly shoot, veins thickening and galling with dark green colour symptoms, with a minimum incubation period of 15-17 days. Later, the infected leaves become downward curled resulting in curly shoot appearance, similar to those observed on the field infected plants (Figure 7).

Virus-vector relationship

The relationship of virus-vector was characterized. Transmission efficiency of 100% was achieved on susceptible common bean cv. Arka komal plants. A minimum of eight whiteflies per plants was found to be effective for disease transmission (20%), with a minimum incubation period of 10-15 days to produce typical symptoms, under controlled conditions (Table 5).

The adult whiteflies required a minimum of 8 hrs acquisition access period to acquire the virus from infected common bean cv. Arka komal plant, and effectively vectored with transmission efficiency of 20%. However, there is increase in the transmission efficiency with the increase in acquisition access period, from 8hr to 24 hr (Table 6). The control plants inoculated with non-viruliferous whiteflies did not show any symptoms, ruling out the contamination of whitefly culture. The minimum IAP was found to be 8 hrs with transmission efficiency of 20%, and there is increase in transmission efficiency, with the increase in inoculation access period from 8 hr to 24 hr (Table 6).

Seed transmission

Seed transmission of virus was studied by planting 100 seeds, each collected from infected and healthy common bean cv. Arka komal plants grown in controlled condition. The result revealed that the virus was not seed borne in nature. None of the plants, emerged from seeds collected from diseased plants, produced symptoms, even up to 40 days

Component	Break point begin-end	Major Parent	Minor parent	RDP	GENECOV	Max Chi	Chimera	Si Scan	3Seq
Homologous DNA-A	20-76 2314-19 129-1161 531-1158 1543-2313	TbCSV-[IN:SF1:10] [HQ407395] MYMV-[IN:Har:01] [AY271896] ToLCJoV-[India:Pon:Kenaf:07] [FJ345402] TbLCYnV-[CN:Yn161:Tom:03] [AJ566744] AEV-[IN:HP:09] [FN543099]	TbLCTHV-[TH:06] [DQ871221] AEV-[PK:Turnip:07] [AM701770] TbCSV-[IN:ND:Tom:11] [JN387045] AEV-[PK:Turnip:07] [AM701770] TbLCYnV-[CN:Yn136:02] [AJ512761]	2.826×10^{-5} 1.496×10^{-6} NS 2.497×10^{-17} NS	4.817×10^{-5} NS 4.03×10^{-2} $9.751X10^{-24}$ 3.833×10^{-3}	8.374×10^{-3} NS NS 9.369×10^{-2} 1.224×10^{-2}	NS NS NS 9.875×10^{-9} NS	NS 1.898×10^{-3} NS 8.197×10^{-37} 9.819×10^{-9}	1.588×10^{-2} 8.054×10^{-5} 1.34×10^{-3} 2.467×10^{-9} 1.812×10^{-2}
Betasatellite	119-307	CroYVMB-[IN:Luc:08] EU604296	TYLCCNB-[CN:Y281:08] AM980512	NS	NS	4.309×10^{-2}	9.999×10^{-1}	NS	NS

NS-Non significance

Table 4: Breakpoint analysis of TbCSV-FB01and associated betasatellite, with their putative parental sequences.

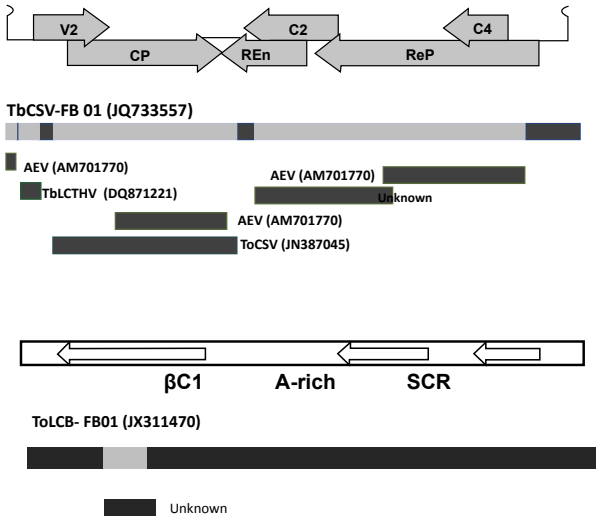

Figure 6: Analysis of recombination for complete genome of TbCSV-FB01 and associated betasatellite isolated from common bean plant. The Begomoviruses and betasatllites acronyms given are Tobacco curly shoot virus (TbCSV), Ageratum enation virus (AEV), Tobacco leaf curl Thailand virus (TbLCTHV) and Tomato leaf curl betasateliite (ToLCB). Sequence of indeterminate origin is indicated as "unknown". The box below at the top of the diagram indicates the approximate position recombination is occurring in the genome of the begomoviruses.

after emergence. But, germination of seed from diseased plants was lower (75%) compared to seeds from healthy plants (90%).

Discussion

Geminiviruses are considered to be the most important viral pathogens in various food crops in the tropics and sub-tropics [32]. In India, Begomoviruses impose particularly serious constraints on the production of common bean. Despite concerted efforts to control certain geminiviruses and their vectors, there is appearance of frequent disease epidemics caused by newly emerging or re-emerging Geminiviruses, even in regions where such diseases were not prevalent earlier [33]. Over the past decade, epidemics caused by Begomoviruses have been attributed to various factors like change in climate, as well as occurrence of highly virulant whitefly vector biotype complexes which might likely enabled, both transmission of indigenous begomoviruses to new cultivated hosts, and the emergence of new recombinant virus variants [34]. The Begomoviruses are known to induce a range of symptoms such as leaf curling, yellow vein and leaf distortion in the plants they infect and cause diseases frequently [35,36]. The pulses are highly susceptible to yellow mosaic diseases caused by four different Begmoviruses [11]. In the present study, for the first time, Tobacco curly shoot virus causing curly shoot disease in common bean was characterized based on molecular characteristics, phylogenetic relationship and transmission studies from India, which is provisionally designated as Tobacco curly shoot virus [IN: Varanasi: common bean], based on the guidelines proposed by ICTV Geminivirus Study Group [2].

The TbCSV was first identified in tobacco in China [37], and subsequently in pepper [38], and ornamental plants [39]. No reports are available pertaining to TbCSV infecting beans in India. However, only two virus sequences isolated from tomato (GenBank Acc.No.JN387045) and sunflower (GenBank Acc.No. HQ407395) from north India are available in the databases. The nucleotide identities and phylogenetic relationship indicated the virus is very closely associated with TbCSV infecting solanaceous crops, sunflower and ageratum weeds in India and china. The Begomoviruses originating from the same geographical area, even though infecting different host plants, are more likely to be closely related than viruses infecting the same host and with different geographical areas [40]. The TbCSV not only infects cultivated crops (tobacco, tomato, pepper & sunflower), but it also infects other weed plants such as ageratum (GenBank Acc. No. AJ971266). The weeds or wild, uncultivated plant species are commonly infected with viruses, and may act as sinks for diverse virus disease complexes [41], which spread to other cultivated plants subsequently [42]. In North eastern parts of India, especially Varanasi region, the spread of TbCSV in beans may be attributed firstly to growing of it in the adjacent fields of tomato. Secondly, large scale growing of tobacco in the adjacent state Bihar infected with both leaf curl and curly shoot virus. Thirdly, movement of viruliferous whiteflies between tobacco, tomato and bean fields during hot and dry season.

The betasatellite closely related to ToLCB is associated with the virus in the current study. The betasatellite have single Open Reading Frame (ORF) in the C1 gene. Start position on the C1 ORF was similar to other beta molecules, which potentially encodes a protein of 118 amino acids, which is extremely conserved in position and length [43]. The βC1 ORF has the capacity to encode a 12.98 kDa protein, comprising 118 amino acids fully functional in their respective hosts [6,43,44]. In India, both monopartite and bipartite Begomoviruses are causing many diseases in crop plants, and have been found with associated betasatellite [45]. The phylogenetic analysis of full-length sequence of betasatellite showed close clustering of the betasatellite associated with the ToLCB, indicating the possible spread of the betasatellite across geographical regions from solanaceous crops to legumes. Available evidence indicates that betasatellite does not contain iteron sequences of their helper viruses, yet they still depend on DNA-A for their replication [5,6,46,47].

The DNA-A component alone is infective in TbCSV and betasatellite is not necessary for infection, but intensification of symptoms in a host-dependent manner has been well proved through agro-inoculation

Figure 7: Common bean (cv. Arka komal) plant showing curly shoot symptoms (A), 16 days after mechanical sap inoculation with TbCSV-FB01 isolate. Bean plant without symptoms (B) inoculated without virus, used as negative control in the experiment.

Number of whiteflies Per Plant	No. of plants Infected/ Inoculated	Transmission (%)
0	0/10	0
1	0/10	0
2	0/10	0
4	0/10	0
6	0/10	0
8	2/10	20
10	4/10	40
12	4/10	60
14	8/10	80
20	10/10	100

Table 5: Transmission efficiency of TbSCV–FB01 by *Bemisia tabaci.*

Determination of minimum AAP*				Determination of minimum IAP#			
AAP*	IAP#	Transmission		AAP*	IAP#	Transmission	
		(plants infected/ plants inoculated)	Percentage of plants infected			(plants infected/ plants inoculated)	Percentage of plants infected
0 min	12 hrs	0/10	0	12hr	0 min	0/10	0
5 min		0/10	0		5 min	0/10	0
10 min		0/10	0		10 min	0/10	0
15 min		0/10	0		15 min	0/10	0
20min		0/10	0		20min	0/10	0
30 min		0/10	0		30 min	0/10	0
1 hrs		0/10	0		1 hr	0/10	0
4 hrs		0/10	0		4 hrs	0/10	0
8 hrs		2/10	20		8 hrs	2/10	20
10hrs		5/10	50		10hrs	5/10	50
12 hrs		8/10	80		12 hrs	8/10	80
16 hrs		9/10	90		16 hrs	9/10	90
24 hrs		10/10	100		24 hrs	10/10	100

*Acquisition access period
#Inoculation access period.

Table 6: Determination of minimum acquisition access period and minimum inoculation access period for insect transmission of TbSCV-FB01, to common bean plants.

[48,49]. In the present, the betasatellite associated with TbCSV infecting common bean showed more identity with ToLCB, rather than TbCSB. Further, phylogeny showed close clustering of TbCSB, characterized with ToLCB reported from Nepal than India. This indicates the trans-replication of betasatellite in common bean plants in the presence of TbCSV. Similarly, previous report showed that trans-replication of betasatellites in plants inoculated with TYLCCV and TbCSV has been well proved through agro-inoculation [50]. Further, yellow vein mosaic disease of pumpkin was caused by Tomato leaf curl Palampur virus, is associated with Pepper leaf curl betasatellite [51], and in cotton, single betasatellite molecule will support for pathogenicity of multiple viruses [10].

Recombination plays major role for the emergence of new viruses [30]. However, results obtained from comparisons of different parts of the viral genome of TbCSV revealed substantial differences in their place in the tree (data not shown). These results suggested that TbCSV possess a hybrid genome of TbCSV, MYMV, ToLCJoV, TbLCYnV and AEV, and betasatellite from CroYVMB and TYLCCNB like ancestors. These data provide evidence for the significant contribution of recombination, for emergence of novel Begomovirus, adding to the genetic diversification and its population structures in India.

Many reports are available worldwide; the Begomoviruses are mechanically transmissible only to *N. benthamiana* plants. However, a few reports such as Bean dwarf mosaic virus [52], Bean golden mosaic virus [53], Pepper golden mosaic virus [54] and Watermelon curly mottle virus (WCMoV) [55] were shown to be mechanically transmissible to their original, and/or other host plants. In bean plants, the Bean dwarf mosaic virus was sap-transmitted up to 100%, the mechanical transmission rate of ToLCNDV in oriental melon and potato is more than 93% [56,57]. The virus isolate in the present study was readily sap transmissible to common bean with 80% transmission efficiency. Similarly, the Begomovirus in the present study was successfully transmitted by whitefly, in accordance with other Begomoviruses known to date [58].

This is the first report of Begomovirus associated with betasatellite causing curly shoot disease of common bean. Further, the betasatellite reported here is distinct from other known Geminivirus components. In conclusion, the virus associated with curly shoot disease of common bean is a newly emerged variant of TbCSV moved to economically important new host and posing severe constraint on grain legumes production in India.

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