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 analyses 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; Phylogenetic 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 whitely 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. Alphasatellites 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
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 important 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 whitely-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 predominately, stunting, stem twisting, curly shoot, thickening of veins in the lower leaf surface and galling with dark green colour symptoms, along with whitely *Bemisia tabaci*. These typical disease symptoms and occurrence of whitely 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 whitely *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 vector transmission was 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.) in a glasshouse. The culture of nonviruliferous whiteflies used for vector transmission was reproduced 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 nonnucleotide 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 phylogenetic 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 whitely 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 (2:1:1 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 whitely 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 beta01/beta02 in both sets of field samples for all further experiments.

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**Figure 2:** Common bean (cv. Arka komal) plant showing curly shoot symptoms (A), 17 days after whitely 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 whitely 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.

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.

**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 (HQ733557). 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.
Effective for disease transmission (20%), with a minimum incubation efficiency of 100% was achieved on susceptible common bean cv. Arka komal similar to those observed on field infected plants (Figure 7). Leaves become downward curled resulting in curly shoot appearance, with a minimum incubation period of 15-17 days. Later, the infected shoot, veins thickening and galling with dark green colour symptoms, mechanically at two leaf stage. Out of these 80 plants expressed curly

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.
Curly shoot virus causing curly shoot disease in common bean was Begmoviruses [11]. In the present study, for the first time, Tobacco plants they infect and cause diseases frequently [35,36]. The pulses are symptoms such as leaf curling, yellow vein and leaf distortion in the variants [34]. The Begomoviruses are known to induce a range of to new cultivated hosts, and the emergence of new recombinant virus occurrence of highly virulant whitefly vector biotype complexes which have been attributed to various factors like change in climate, as well as earlier [33]. Over the past decade, epidemics caused by Begomoviruses Geminiviruses, even in regions where such diseases were not prevalent lower (75%) compared to seeds from healthy plants (90%).

**Table 4:** Breakpoint analysis of TbCSV-FB01 and associated betasatellite, with their putative parental sequences.

| Component | Break point begin-end | Major Parent | Minor parent | RDP | GENECOV | Max Chi | Chimeras | Si Scan | Seq |
|-----------|-----------------------|--------------|--------------|-----|---------|---------|----------|--------|-----|
| Homologous DNA-A | 20-78 129-1161 531-1158 1543-2313 | TbCSV-[IN:SF:1:10] [HQ407385] | MYMV-[IN:Har:01] [AY271896] | 2.62×10^-6 | 4.817×10^-4 | 3.74×10^-2 | 9.5×10^-1 | NS | NS |
| | | ToLCV-[IN:Lon:07] [KX11470] | AEV-[PK:Tumei:07] [AM701770] | | | | | | |
| | | AEV-[PK:Tumei:07] [AM701770] | ToLCV-[IN:Yn:1996:02] [AJ851276] | | | | | | |
| Betasatellite | 119-307 | CroVVMB-[IN:Luc:08] [EU604296] | TYLCCNV-[CN:Y281:08] [AT980512] | 8.39×10^-2 | 9.99×10^-1 | NS | NS | NS | NS |

**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 virulent 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 Begomoviruses [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 curl 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. AY971266). 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 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. AY971266). 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.

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The DNA-A component alone is infectious 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
The disease of pumpkin was caused by Tomato leaf curl Palampur virus, is well proved through agro-inoculation [50]. Further, yellow vein mosaic betasatellites in plants inoculated with TYLCCV and TbCSV has been identified. Similarly, previous report showed that trans-replication of betasatellite in common bean plants in the presence of ToLCB reported from Nepal than India. This indicates the trans-replication of betasatellite in plants inoculated with 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 (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 | 0/10 0 |
| 10 min 0/10 0 | 0/10 0 |
| 15 min 0/10 0 | 0/10 0 |
| 20 min 0/10 0 | 0/10 0 |
| 30 min 0/10 0 | 0/10 0 |
| 1 hr 0/10 0 | 0/10 0 |
| 4 hrs 0/10 0 | 0/10 0 |
| 8 hrs 2/10 20 | 2/10 20 |
| 10 hrs 5/10 50 | 5/10 50 |
| 12 hrs 8/10 80 | 8/10 80 |
| 16 hrs 9/10 90 | 9/10 90 |
| 24 hrs 10/10 100 | 10/10 100 |

*Acquisition access period
#Inoculation access period.

Reference:

Briddon RW, Bull SE, Amin I, Mansoor S, Bedford ID, et al. (2003) Diversity of DNA components required for induction of cotton leaf curl disease. Virology 312: 106-121.

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