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 from 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, Topoavirus 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 begomovirus 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 (Caianus 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 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 whitely *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 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 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 finally, the virus isolate was designated as-FB01 and used for repeated transmissions were carried out under controlled conditions.

**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 water, before being subjected to PCR amplification and stored at -20°C.

**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 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 Clustal W [24], and phylogenetic trees were generated by MEGA 5.0 software [25], using the neighbour joining method with 1000 bootstraped 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
**P. vulgaris** is monopartite. Samples from non-symptomatic plants confirmed that the Begomovirus infecting common bean PCR with a universal abutting primer pair beta0l/beta02 in both. However, the positive amplification of betasatellite component by and attempts to amplify DNA-B components were unsuccessful. 

Sets of primers from field infected and glasshouse inoculated samples, 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.

**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 cultivating) 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. vulgaris*) 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.

**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 (2:1:2 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.
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 ORFAY2, AV1, AC1, AC2, AC3 and AC4 showed maximum identity with isolate of TbCSV in infecting different crop plants with exception of ORF AC4, which is having maximum identity for both TbCSV and TbLCTHV (Table 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).

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 tomato, tobacco, cotton, mesta, and along with isolate (TbSCV) with Splits-Tree version 4.11.3 (Figure 4). Such networks are capable of graphically displaying patterns
efficiency of 100% was achieved on susceptible common bean cv. Arka komal. This is similar to those observed on the field infected plants (Figure 7).

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 10-15 days to produce typical symptoms, under controlled conditions (Table 5).

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).

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 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 might likely enabled, both transmission of indigenous begomoviruses 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 frequent disease epidemics caused by newly emerging or re-emerging on the production of common bean. Despite concerted efforts to impose particularly serious constraints pathogens in various food crops in the tropics and sub-tropics [32].

**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 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. A1971266). The weeds or wild, uncultivated plant species are commonly infected with viruses, and may act as sinks for diver 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 the TbCSV was first identified in tobacco in China [37], and many diseases in crop plants, and have been found with associated betasatellite [45]. The phylogenetic analysis of full-length sequence of the TbCSV was first identified in tobacco in China [37], and many diseases in crop plants, and have been found with associated betasatellite [45]. The phylogenetic analysis of full-length sequence of the TbCSV was first identified in tobacco in China [37], and many diseases in crop plants, and have been found with associated betasatellite [45].

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

<table>
<thead>
<tr>
<th>Component</th>
<th>Break point begin-end</th>
<th>Major Parent</th>
<th>Minor parent</th>
<th>RDP</th>
<th>GENECOV</th>
<th>Max Chi</th>
<th>Chimeras</th>
<th>Si Scan</th>
<th>3Seq</th>
</tr>
</thead>
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<tr>
<td>Homologous DNA-A</td>
<td>20-76</td>
<td>TbCSV-[IN:SF:1:10]</td>
<td>[HQ077385]</td>
<td>2.826×10^-6</td>
<td>4.817×10^-6</td>
<td>8.374×10^-10</td>
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<td>NS</td>
<td>NS</td>
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<td></td>
<td>129-1161</td>
<td>MYMV-[IN:Har:01]</td>
<td>[AY271896]</td>
<td>4.196×10^-8</td>
<td>4.03×10^-2</td>
<td>9.369×10^-3</td>
<td>NS</td>
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<td>NS</td>
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<tr>
<td></td>
<td>531-1158</td>
<td>ToLCoV-[India:Pon:Kenaf:07]</td>
<td>[FJ349402]</td>
<td>2.497×10^-3</td>
<td>7.95×10^-4</td>
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<td>NS</td>
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<td>NS</td>
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<td></td>
<td>1543-2313</td>
<td>TblCYNV-[CN:Yn161:Tom:03]</td>
<td>[AJ566744]</td>
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<td></td>
<td>AEV-[IN:HP:09]</td>
<td>[FN543099]</td>
<td>1.184×10^-7</td>
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<td>Betasatellite</td>
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<td>[EU804926]</td>
<td>4.309×10^-2</td>
<td>9.999×10^-1</td>
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<td>NS</td>
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<tr>
<td></td>
<td></td>
<td>TLYCCNB-[CN:Y281:08]</td>
<td>[AM980312]</td>
<td>1.898×10^-3</td>
<td>2.467×10^-3</td>
<td>1.812×10^-3</td>
<td>NS</td>
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<td>NS</td>
</tr>
</tbody>
</table>

NS-Non significance

**Figure 6:** Analysis of recombination for complete genome of TbCSV-FB01 and associated betasatellite isolated from common bean plant. The Begomoviruses and betasatellites acronyms given are Tobacco curly shoot virus (TbCSV), Ageratum enation virus (AEV), Tobacco leaf curl Thailand virus (TbLCTHV) and Tomato leaf curl betasatellite (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.

The DNA-A component alone is infective in TbCSV and betasatellite dependence manner has been well proved through agro-inoculation.
well proved through agro-inoculation [42]. Further, yellow vein mosaic betasatellites in plants inoculated with TYLCCV and TbCSV has been reported. Similarly, previous report showed that trans-replication of betasatellite in common bean plants in the presence of ToLCNDV was more effective than that of the virus alone [48,49].

In the present, the betasatellite associated with TbCSV infecting common bean showed more identity with ToLCB, rather than TbCSB. 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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