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Begomovirus causing leaf curl disease in tomato (*Lycopersicon esculentum* L.) in sub-Himalayan West Bengal, India.

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Abstract
Tomato (*Lycopersicon esculentum* L.) is an extensively cultivated vegetable crop in India. In the year 2009, a survey was conducted to find leaf curls of tomato in different locations of sub-Himalayan West Bengal, India. During the survey a severe leaf curl disease was observed. The characteristic disease symptoms (puckered leaves) and presence of whitefly (*Bemisia tabaci*) population indicated the possibility of begomovirus infection. Total DNA was extracted from the infected samples and PCR was carried out using begomovirus specific primers. An amplicon of expected size (~1280 bp) was found when PALIc1960 and PARIv722 were used as primers in agarose gel electrophoresis. The PCR Amplicons of two samples (collected from two different places of present study area) were cloned and sequenced (GenBank accession nos. HM856626 and HM856627). The sequence data analysis of partial coat protein gene (AV1), full replication enhancer protein gene (AC3) and partial transcription activator protein gene (AC2) of 831nt revealed highest 98% similarities with several isolates of *Tobacco curly shoot virus* (TbCSV) at both nucleotide and amino acid levels. The phylogenetic analysis also showed close relationship of the present isolates with different variants of TbCSV. Based on highest sequence similarities and closest relationships with TbCSV the viruses (present in infected tomato plants) were considered as *Begomovirus*. Transmission of the virus in tomato could not be done by sap transmission procedure. In experimental insect transmission tests, test plants showed symptoms very much like the natural symptoms. Artificial transmission was confirmed by comparing the PCR Amplicons raised from the experimentally infected plants.

Key words: Tomato, *Begomovirus*, *Bemisia tabaci*.

Introduction

Tomato leaf curl diseases (TLCDs) occur in many tomato producing regions of the world. The disease is characterized by severe leaf curling, shrinking of tomato leaves and stunted plant growth. TLCD is caused by geminiviruses (genus *Begomovirus*, family *Geminiviridae*) and is transmitted by whitefly. Geminiviruses are a major constraint for the successful cultivation of tomato in tropic and subtropics (Czosnek, 1988; Hong and Harrison, 1995). Several geminivirus species infecting tomatoes from old world have been characterized (Padidam *et al.*, 1995).

Vasudeva and Samraj (1948) for the first time reported the occurrence of tomato leaf curl disease in India. Presently TLCD is a serious problem for tomato-growing regions in India. On the basis of biological and molecular characteristics attempts have been made to characterize the causal agent(s) of the disease. A number of species or strains of tomato leaf curl geminiviruses have been reported to cause TLCDs in India (Vasudeva and Samraj, 1948; Sastry and Singh, 1973; Muniyappa and Saikia, 1983; Saikia and Muniyappa, 1989; Harrison *et al.*, 1991; Reddy *et al.*, 2005; Kirthi *et al.*, 2002; Paximadis *et al.*, 2001; Ramappa *et al.*, 1998). Reddy *et al.* (2005) reported *Tomato leaf curl New Delhi virus* (ToLCNDV) from Assam and West Bengal (Kolkata). Beside *Tomato leaf curl virus*, TLCDs of tomato are also caused by *Tobacco curly shoot virus* (TbCSV) and *Tobacco leaf curl Yunnan virus* (TbLCYNV) in China (Li *et al.*, 2004).

In tropical and subtropical climate zones, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) is an important insect pest. *B. tabaci* provokes direct feeding damage but also causes considerable indirect damage as a vector of numerous geminiviruses (Credi *et al.*, 1989) such as *Tomato*

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yellow leaf curl virus, a threatening virus of tomato (Markhan *et al.*, 1995; Moriones and Navas-Castillo, 2000; Pico *et al.*, 1996).

The present study deals with the begomovirus, a causal pathogen of tomato leaf curl disease.

MATERIALS AND METHODS

Field observation and virus source

A survey of tomato growing fields of sub-Himalayan West Bengal was conducted during winter season of 2009 and 2010 for observation of viral disease problems of tomato plants. The naturally infected plants showing symptoms of tomato leaf curl diseases (TLCDs) were collected from the fields and were tested by polymerase chain reaction (PCR) using universal begomovirus specific primer pair. Viruses were maintained in tomato plants grown in separate net houses in experimental garden, Department of Botany, University of North Bengal, Siliguri.

Transmission by whitefly and symptom development

Virus-free white flies were used as vectors in transmission experiment and insect transmission was done following the technique as described by Ghanem *et al.* (2001). About twenty insects were allowed to feed on infected tomato plants in an insect proof cage for 24 hours (acquisition access period). After 24 hours all the 20 insects were transferred to healthy plants, duly covered by a fine mosquito net. The insects were allowed to feed for 72 hours on healthy tomato plants. After 72 hours all the insects were removed carefully from the plant by shaking the plant and the insect-free plant was left for symptom development up to 60 days. Symptoms started appearing after 20 days but severe symptom development was found after 30 days. However, the presence of virus was further confirmed by PCR amplification of the viral coat protein (partial) from the artificially infected (insect-transmitted) leaves of tomato.

Extraction of total DNA of virus infected plants

Total DNA were extracted from the Infected and healthy plants. Cetyl trimethyl ammonium bromide (CTAB) method of Dellaporta *et al.* (1983) modified by Sharma *et al.* (2003) was followed for extraction of DNA. All extracted DNAs were diluted 10-fold in sterile distilled deionised water just before PCR amplification.

Polymerase chain reaction (PCR)

The forward and reverse Rojas universal primers (Rojas *et al.*, 1993) PALIc1960 (5'ACNGGNAARACNATGTGGGC3') and PARIv722 (5'GGNAARATHTGGATGGA3') were used for amplification of position "722" within the CP gene through the AC2 and AC3 gene to position ~1960 of DNA-A segment. Amplification of the DNA was performed in a volume of 25µl of reaction mixture containing 2µl DNA template, 2.5µl 10x Taq DNA buffer B (Genie, Bangalore), 1.5µl 1.5mM $MgCl_2$ (Genie, Bangalore), 0.5µl 2.5mM dNTPmix (Genie, Bangalore), 0.5µl 1 pmol each forward and reverse primers (Sigma, USA) and 1µl 0.5U/µl Taq DNA polymerase (Genie, Bangalore). The amplification was carried out using a Gene Amp 2400 thermal cycler PCR system (Perkin Elmer). Amplification programme consisted of one initial cycle of denaturation at 94ÚC for 1 min, annealing at 52ÚC for 1min 30sec and extension at 72ÚC for 2min. After the initial cycle, 40 cycles of PCR were performed of denaturation (94ÚC for 50sec), annealing (at 52ÚC for 45sec) and extension (at 72ÚC for 1min 30sec). After that amplification programme was continued for 10 minutes at 72ÚC. Amplification products were electrophoresed at 5V/cm through 1.5% (w/v) agarose gels in 1X Tris Acetic acid EDTA (TAE) electrophoresis buffer, and visualized under UV transilluminator following ethidium bromide staining of the gel (30min in 1µg/ml ethidium bromide).

DNA sequencing and phylogenetic analysis

The amplified products of expected size were either directly sent for sequencing or cloned into the pGEM T-Easy vector (Promega, Madison, USA). The sequencing was done from DNA sequencing service, Genie, Bangalore. The sequences were submitted to GenBank with proper annotations. The accessions of the GenBank have also been received. The sequences were compared to the equivalent sequences from a range of other geminiviruses present in GenBank and have been

mentioned in details in elsewhere in the present article. Multiple sequence alignment was carried out using the software clustalW in MEGA *version* 4 (Tamura *et al.*, 2007). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1973). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004).

Results and Discussion

Field observation and virus isolation

Infected tomato plants were observed in ten different places of sub-Himalayan West Bengal during December 2009 to February 2010. Considerable damages were found to occur in two widely cultivated tomato varieties (Pusa Ruby and Rocky variety). Out of ten infected plants two tomato plants (from Siliguri and Haldibari) with TLCDs (Figure 1) showed PCR positive results. PCR positive plants were selected for further analysis.

Figure1: Tomato plants showing TLCDs in field from Siliguri (a) and Haldibari (b). Healthy tomato plant in field (c)

Polymerase chain reaction (PCR)

Whole DNA of infected samples were extracted and specific genes of viruses were amplified in PCR. For PCR experiments specific primers were used and molecular weight of the amplicons were determined by using standard DNA-molecular weight markers (ladders) on agarose gel. The size of the amplified DNAs (by primers PALIc1960 and PARIv722) were ~1280bp which matched with the molecular weight as suggested by Reddy *et al.* (2005). The DNA samples showed presence of virus, were subjected to cloning and finally clones were sent for sequencing. The results of some amplified samples along with DNA-molecular weight markers have been presented in Figure 2. Lanes designated as M contained DNA ladders, Lanes (L1 and L2) contained PCR product of healthy plants (control) and Lanes (L3-L6) contained PCR amplified products.

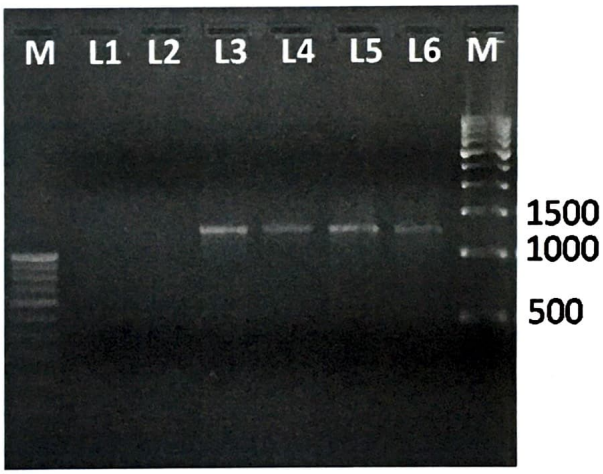

Figure 2: 1.5% agarose gel electrophoresis showing the PCR products of primer PALIc1960/PARIv722 of tomato samples from Siliguri (L3) and Haldibari (L4). L5 and L6 represent the PCR product from insect transmitted tomato samples. L1 and L2 are control. M: DNA Ladder 500 and 1000bp.

Sequence analysis

Sequence data revealed that the PCR product of Siliguri (SILIGURI-1) consisted of 831 nucleotides spanning 3'-terminal 375 nucleotides of CP gene (125aa), the entire 405 nucleotides of AC3 coding region (135aa) and the 3'-terminal 315 nucleotides of AC2 gene (105aa) and the PCR products of HALDI-1 consisted of 741 nucleotides. BLAST

analysis revealed that the new viruses are closely related to *Tobacco curly shoot virus*. The highest percent sequence identities (presented in table-1) for nucleotide sequence of SILIGURI-1 sample was 98% with *Tobacco curly shoot virus*, China (Accession no. GU001879). Similarly, HALDI-1 also showed 98% sequence identities with another *Tobacco curly shoot virus*, China (Accession no. AF240675). The amino acid sequence of complete AC3 gene showed 99% sequence similarity with *Tobacco curly shoot virus* (Figure 3). The Accession numbers for the samples SILIGURI-1 and HALDI-1 are respectively HM856626 and HM856627.

Phylogenetic analysis

In phylogenetic tree (Figure 4), the isolates from Haldibari and Siliguri (HM856626 and HM856627) formed cluster with three *Tobacco curly shoot virus* (Accession nos. GU001879, AJ971266 and GU199583) reported from China with bootstrap value ranging from 83% to 96%. BLAST analysis and Phylogenetic analysis revealed that the two viruses are closely related to *Tobacco curly shoot virus*.

Tomato leaf curl samples were collected from several places of sub-Himalayan West Bengal. DNA extracts of field samples were amplified using begomovirus specific primer pairs. The amplified sequences were compared with the near-similar sequences present in the GenBank. Similar studies were also done by Reddy *et al.* (2005) but they included only three main locations (Kolkata, Maligaon and Patna) in eastern India. From our study it is evident that TLCDs were produced by the begomoviruses. Reddy *et al.* (2005) reported two other begomoviruses from tomato plants of Patna and Kolkata. However, those two viruses (PepLCV and ToLCGV) were not reported from the present tomato samples studied. Presence of *Tobacco curly shoot virus* (TbCSV) and *Tobacco leaf curl virus* (TbLCV) in tomato causing leaf curl diseases have also been reported by Li *et al.* (2004) from China.

In the family *Geminiviridae* CP genes are most

Table 1: The percent identities between the partial AV1 gene, complete AC3 gene and partial AC2 gene regions of isolate SILIGURI-1 DNA associated with the tomato disease and those of 10 most closely related geminiviruses.

Accession No. of geminiviruses	Locus name	Percent identities [with Partial AV1, complete AC3 and Partial AC2 of isolate SILIGURI-1 DNA (Accession No. HM856626)]
GU001879	*Tobacco curly shoot virus*-[SC118], complete genome	98
AJ971266	*Tobacco curly shoot virus*-[Y282] complete genome, isolate Y282	98
GU199583	*Tobacco curly shoot virus* clone 20-9, complete genome	98
AJ437618	Ageratum enation virus complete genome	97
AY738103	*Papaya leaf curl virus* from India	95
AF188481	*Tomato leaf curl Bangladesh virus* complete genome	94
EU194914	*Euphorbia leaf curl virus* isolate Pusa Bihar, complete genome	94
EF175733	*Radish leaf curl virus* segment A, complete sequence	94
FN543112	*Croton yellow vein virus*, complete genome, clone 1	93
GQ183868	*Sunn hemp leaf distortion virus* [India: Barrackpore:2008] segment DNA A, complete sequence	92

(a)

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HM856626 1    NSVMFFLVRDRRPVDKPQDFGEVFNMFDNEPSTATVKNVHRDRYQVLRKWHATVTGGQYA 60
              NSVMFFLVRDRRPVDKPQDFGEVFNMFDNEPSTATVKNVHRDRYQVLRKWHATVTGGQYA
ADB19845 133  NSVMFFLVRDRRPVDKPQDFGEVFNMFDNEPSTATVKNVHRDRYQVLRKWHATVTGGQYA 192

HM856626 61   SKEQALVKKFVRVNNYVVYNQQEAGKYENHSENALMLYMACTHASNPVYATLKIRIYFYD 120
              SKEQALVKKFVRVNNYVVYNQQEAGKYENHSENALMLYMACTHASNPVYATLKIRIYFYD
ADB19845 193  SKEQALVKKFVRVNNYVVYNQQEAGKYENHSENALMLYMACTHASNPVYATLKIRIYFYD 252

HM856626 121  SVTN                                                        124
              SVTN
ADB19845 253  SVTN                                                        256
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(b)

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HM856626 1    MDSRTGELITAAQAENGVYIWEIQNPLYFKITEHQNRPFLMKEDIITIQIQFNYNLRKAL 60
              MDSRTGE+ITAAQAENGVYIWEIQNPLYFKI EHQNRPFLMKEDIITIQIQFNYNLRKAL
ADB19846 1    MDSRTGEVITAAQAENGVYIWEIQNPLYFKIIEHQNRPFLMKEDIITIQIQFNYNLRKAL 60

HM856626 61   GVHKCFLVYRIWMTSQPQTGRFLRVFKTQVFKYLNNLGIISINNVIRAVDRVLWDVLEHI 120
              GVHKCFLVYRIWMTSQPQTGRFLRVFKTQVFKYLNNLGIISINNVIRAVD VLWDVLEHI
ADB19846 61   GVHKCFLVYRIWMTSQPQTGRFLRVFKTQVFKYLNNLGIISINNVIRAVDHVLWDVLEHI 120

HM856626 121  VYVDQSYSIKFNIY                                              134
              VYVDQSYSIKFNIY
ADB19846 121  VYVDQSYSIKFNIY                                              134
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(c)

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HM856626 6    CGCSYFIALACHDHGFTHRGTHHCSSSREWRVYLGDSKSPLFQDNRAPEPSISHERRHNH 65
              CGCSYFIALACHDHGFTHRG+HHCSSSREWRVYLGDSKSPLFQDNRAPEPSISHERRHNH
ADB19847 36   CGCSYFIALACHDHGFTHRGSHHCSSSREWRVYLGDSKSPLFQDNRAPEPSISHERRHNH 95

HM856626 66   NPNTVQLQPAESSGSAQVFSSLPNLDDFTASDWSFLKGL                     104
              +PNTVQLQPAESSGSAQVFSSLPNLDDFTASDWSFLKGL
ADB19847 96   HPNTVQLQPAESSGSAQVFSSLPNLDDFTASDWSFLKGL                     134
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Figure 3: The difference of amino acid sequences between TbCSV isolate SILIGURI-1(Accession no. HM856626) partial gene and *Tobacco curly shoot virus*, China (Accession no. GU001879). The region corresponding to the partial AV1 gene (a), complete AC3 gene (b) and partial AC2 gene (c) is indicated on the alignment. Amino acids are presented with the single-letter code. Identical amino acids were shown with black letters and non identical amino acids are shown with red letters. The consensus amino acid sequence between the two sequences is shown under the alignment.

conserved (Wyatt and Brown, 1996). Identification of virus and their geographic and vector relationship can be correlated with CP sequences (Brown *et al.*, 2001). CP gene partial sequence provides provisional virus identification. For establishing new begomovirus species and for definite classification complete sequence of DNA-A is necessary (Faquet and Stanley, 2005). Many viruses and viral variants were found in *Begomovirus*, and begomoviruses are classified on the basis of genome sequences, especially DNA-A sequence (Harrison *et al.*, 1993). According to Padidam *et al.* (1995) two species of a virus should have less than 90% nucleotide sequence identity and two strains of a virus must have sequence identity greater than 90%. The nucleotide sequences of isolate SILIGURI-I and HALDI-1 showed highest 98% sequence similarity with Chinese *Tobacco curly shoot virus* (TbCSV). The nucleotide sequence of the SILIGURI-1 isolate comprises partial coat protein gene (AV1), replication enhancer protein gene (AC3) and partial transcription activator protein gene (AC2) as reported by Yan *et al.* (2002). In phylogenetic tree the isolate made a cluster with TbCSV (Chinese isolate). In the present study, the causal pathogen was provisionally determined to be a *Begomovirus* through cloning, nucleotide sequence analysis and phylogenetic analysis. This is the first record of a *Begomovirus* infecting tomatoes of sub-Himalaya West Bengal, India. The

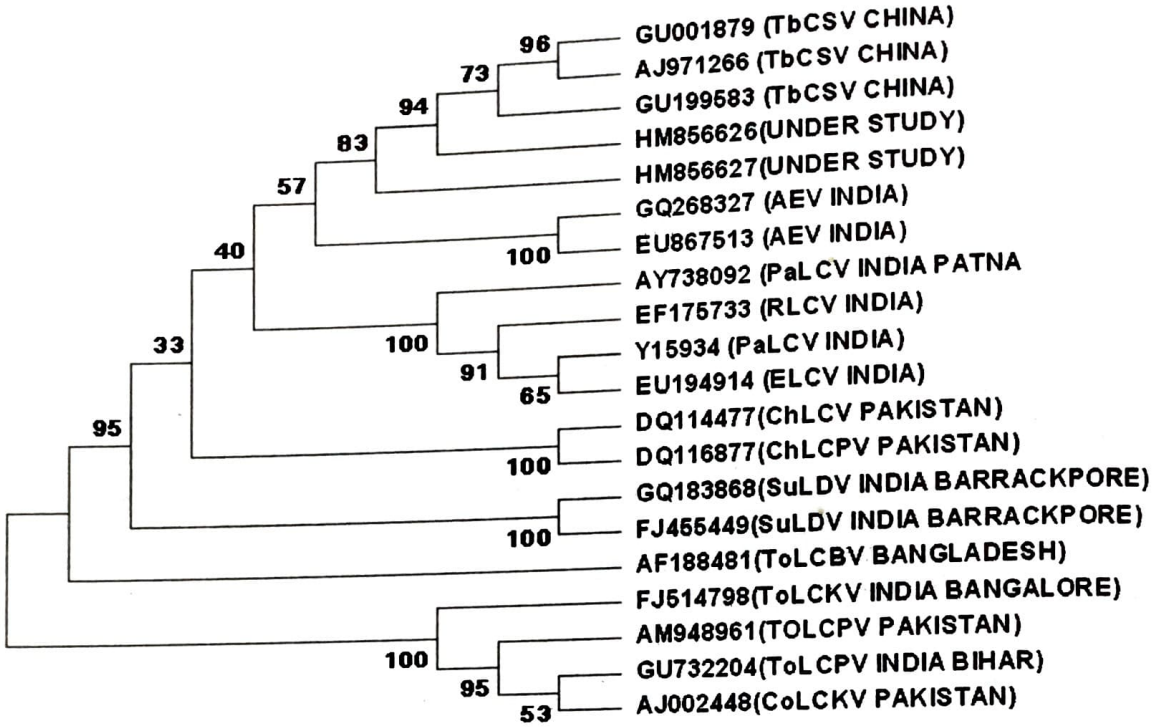

Figure 4: Most parsimonious tree showing the relationship of Partial coat protein (AV1), replication enhancer protein (AC3) and partial transcription activator protein (AC2) gene (HM856626 and HM856627) of isolate SILIGURI-1and HALDI-1 with published begomovirus sequences from GenBank. Numbers at nodes indicate the bootstrap percentage scores out of 1000 replicates.

sequence information in this study would be helpful in understanding and management of the new pathogen of TLCDs.

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