

COMPARISON AND MOLECULAR PROFILING OF BEGOMOVIRUS INFECTING CHILLI (*Capsicum annum*) IN GANGETIC ALLUVIAL ZONE OF WEST BENGAL

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ABSTRACT

In our present investigation the leaf curl disease on chilli was observed up to an extent of 18-85% at different regions Gangetic alluvial zone of West Bengal. Symptoms like upward leaf-curling, distortion, vein yellowing, leaf yellowing and/or stunted growth with bushy appearance of chilli plants observed in the fields. The disease was successfully transmitted by whitefly (*Bemisia tabaci*), on experimental plants. The total DNA was extracted from infected leaf samples and PCR was carried out using a pair of begomovirus-specific degenerate primers (SPG1/SPG2). The replication-associated protein gene fragment (Rep; AC1) of 920 bp were amplified from naturally begomovirus-infected and experimentally vector transmitted plants. After sequencing, Phylogenetic analysis of partial nucleotide sequences of DNA-A revealed that Bongaon-24parganas-WB begomovirus isolate (Accession no. LT622253) made separate cluster and showed 91% nucleotide sequence identity with Pepper leaf curl Bangladesh virus (Accession no. AF314531) and Dhatrigram-Burdwan-WB begomovirus isolate (Accession no. LT622254) is clustered with Pepper leaf curl Bangladesh virus India (Accession no. HM007111) with 95% nucleotide sequence identity. Moreover, these studies supply precise information about the frequency and distribution of distinct begomovirus isolates in chilli in Gangetic alluvial West Bengal.

INTRODUCTION

India is the largest producer, consumer and exporter of chilli (*Capsicum* spp.) and contributes 25% of total world production. Amongst the foliar diseases, virus diseases are very important and becoming the most limiting factors for better pepper production which affect the crop both qualitative and quantitatively (Hernandez, 1995; Singh *et al.*, 1996; George *et al.*, 1995). Out of the 22 viruses infecting chilli, Chilli leaf curl virus (ChiLCV) is one of the major limiting constrain for chilli production in the Indian subcontinent and is consistently caused by begomoviruses (Briddon *et al.*, 2003; Shih *et al.*, 2003; Senanayake *et al.*, 2006). Leaf curl disease of chilli showed typical symptoms of begomoviruses. The genus Begomovirus is the largest genus of family Geminiviridae and contains approximately 300 species (Brown *et al.*, 2011), only infects dicotyledonous plants and is transmitted by the phloem feeding whitefly, *Bemisia tabaci* Genn. (Kalaria *et al.*, 2013). Begomovirus is characterized structurally by twinned (geminata) quasi-icosahedral capsids (Lazarowitz, 1992) of approximately 18*30 nm in size (Padidam *et al.*, 1995). Most viruses belonging to the Begomovirus genus possess a bipartite genome, with two circular ssDNA molecules, DNA-A (2.6-2.8 kb) and DNA-B (2.5-2.8 kb) but single genome (DNA-A) monopartite viruses are being reported like recently a new monopartite Begomovirus species, Chilli leaf curl Vellanad virus and associated beta-satellites infecting chilli in the Vellanad region of Kerala, India reported (Kumar *et al.*, 2012). Begomoviruses can broadly be divided into two groups, those originating from new world (NW) and those originating from the old world (OW) (Padidam *et al.*, 1999; Paximadis *et al.*,

1999). Begomoviruses present in both worlds differ from each other in many aspects, like all NW begomoviruses are bipartite, while in OW majority of begomoviruses are monopartite and most of these viruses are associated with satellite molecules and few of them are bipartite. Chilli leaf curl etiology was established during the 1960s in India (Mishra *et al.*, 1963; Dhanraj *et al.*, 1968). In 2004, epidemics of Chilli leaf curl disease occurred in Jodhpur, the main chilli-growing area in Rajasthan, India and then Senanayake *et al.* (2006) have reported first time chilli leaf curl virus on chilli crop from Jodhpur (Rajasthan) with closest sequence identity (96.1%) with an isolate of ChiLCV from potato in Pakistan and showed sequence diversity up to 12.3% among the ChiLCV isolates reported from India and Pakistan. In India, Tomato Leaf Curl New Delhi Virus (ToLCNDV) was recently shown to be associated with chilli leaf curl disease occurring in Lucknow (Khan *et al.*, 2006). Tomato leaf curl Joydebpur virus (reported earlier from tomato in Joydebpur, Bangladesh) has also been found to be associated with chilli leaf curl disease in Punjab (Shih *et al.*, 2006). An isolate from Kalyani (West Bengal) shared maximum nucleotide identity with Pepper leaf curl Bangladesh virus (96.3%) while 3 isolates from Rajasthan showed nucleotide identity of 88.9 to 91.1% with Chilli leaf curl virus-Multan, and 8 isolates (2 each from Delhi, Haryana, Punjab and Uttar Pradesh) shared nucleotide identity of 92.4 to 98.5% with Tomato leaf curl New Delhi virus (Reddy and Reddy, 2010). Recently identification of Tomato Leaf Curl Virus (ToLCV) strain causing leaf curl disease in Tomato and Chilli in Maharastra reported by Chavan *et al.*, 2013 results in 90-100% yield loss.

The rapid evolution of virus variants, the massive increase of

vector populations (with the appearance of whitefly 'B' biotype) and the introduction of modern human agricultural practices have contributed to a rapid and worldwide spread of the virus (Varma and Malathi, 2003).

However, attempt was taken in the present investigation to study the incidence, transmission, detection, characterization and sequence variations of the ssDNA virus (Begomovirus) isolates from different locations of West Bengal (Gangetic alluvial zone) and phylogenetic relationship with other related begomoviruses for further analysis of the viral genome of the begomoviruses infecting chilli in that region.

MATERIALS AND METHODS

Survey of disease incidence and sample collection

Samples showing begomovirus infection symptoms (upward leaf-curling, distortion, vein yellowing, stunted growth & sometimes with bushy appearance of plants) were collected from North 24-parganas (Bongaon), Burdwan (Dhatrigran), Kalyani (Nadia) districts of West Bengal, similarly healthy leaf samples also collected from above mentioned regions. Both the infected and healthy leaf samples were brought in cool boxes, kept in -20 °C for further experiments. In addition to that single severely infected/ stunted chilli plant is uprooted with soil from each of these locations and maintained in glasshouse at optimum growth condition in Directorate of Research farm, BCKV, Kalyani, West Bengal.

The survey was conducted during July- August, 2015 when the crop was at 2-3 month during. The disease diagnosis in the field was based on typical chilli leaf curl symptoms. The percent disease incidence was recorded at randomly in different locations in the field by counting total no. of plants and no. of plants showing chilli leaf curl symptoms using the formula (Sankara and Acharyya, 2012) given below-

$$\text{Percent disease incidence (PDI)} = \frac{\text{Number of diseased plants}}{\text{Total number of plants observed}} \times 100$$

Artificial transmission by whitefly

Transfer of begomovirus from the pre-collected infected chilli plants was carried out during September, 2015 using whitefly vector (*Bemisia tabaci*). Newly emerged whitefly adults are collected carefully from chilli fields of Gayeshpur, BCKV, Nadia using a whitefly aspirator made with tube (Bib size) and released in some cotton plants inside a wooden-net rearing cage (45 x 45 x 30 cm) for rearing and maintenance of the whiteflies.

Whiteflies were released on infected chilli plants of different region, maintained at glass house, for 24 hours virus acquisition, then the viruliferous whiteflies were removed and allowed to feed on 18-20 days old healthy chilli seedlings (var: Bullet), at the rate of 10 whiteflies per seedling for 24 hours inoculation feeding. The inoculated seedlings of chilli were sprayed with insecticides to kill the white flies and kept in the insect proof condition for symptoms development up to 40 days (Govindappa *et al.*, 2011). Inoculated chilli seedlings showing chilli leaf curl symptoms were subjected for PCR for confirmation of begomovirus infection.

DNA isolation and PCR detection of virus

Total DNA were extracted from 100 mg of infected and healthy

plants using Cetyl trimethyl ammonium bromide (CTAB) method (Dellaporta *et al.*, 1983 and Sharma *et al.*, 2003). Presence of begomovirus was confirmed by PCR amplification with a begomovirus specific degenerate primer pairs SPG1/SPG2(5'-CCCCCKGTGCGWRAATCCAT-3'/5'-AT CCVAAWY WT YCAGGGAGCTAA-3' (Lotrakul *et al.*, 1998), which amplified a partial segment of replication associated protein (AC1) gene. The PCR amplification was carried out in a thermal cycler (Mastercycler, Eppendorf, AG22331, Germany). The PCR reaction mixture contained 2 µl DNA sample, 1 µl of each primers (Conc. 10 mM), 1 µl dNTPs (10mM), 1.5 µl Mgcl₂ (25 mM), 2.5 µl 10x reaction buffer, 0.5 µl Taq polymerase and 15.5 µl sterile HPLC-H₂O, that will give rise to a volume of 25 µl PCR reaction. The conditions for PCR reaction were, an initial denaturation at 94°C for 5.00 minute followed by 34 cycles of denaturation at 94°C for 30 sec., annealing at 59°C for 30 sec and extension for 1 minute at 72°C. Then final extension at 72°C for 7 minute was included. Amplified PCR product were separated by electrophoresis on 1.5% agarose gel and DNA fragments were visualized using ethidium bromide stain, here 1 kb DNA ladder (Fermentas, Life Science) is used and documented by Gel documentation system (Vilber-Lormout, France). The expected amplicon of 920 bp were obtained from the DNA of all diseased leaf samples chosen. PCR product is further amplified up to 200 µL and run on 1% agarose gel and finally the desired band (920 bp) were purified using gel extraction kit (Gene JET Gel Extraction kit, Thermo Scientific, Lithuania). The amplified products were stored in -4°C for further use.

Sequence and Phylogenetic analysis

The complete nucleotide sequences were subjected for similarity search using BLASTn search program. The sequence was submitted to GenBank with legal annotations. The accessions of GenBank were also received (Accession numbers - LT622253 and LT622254). The sequences were compared with equivalent sequences from a range of other geminiviruses present in GenBank. Multiple sequence alignment was carried out using the software ClustalW and ClustalX version 2.0 (Larkin *et al.*, 2007). The pair-wise alignments and Percent Identity Matrix of nucleotide and amino acid sequences were performed using Clustal Omega programme from EMBL Sequence Database online. Phylogenetic trees were constructed on matrices of aligned sequences with 1000 bootstrap replicates by using MEGA version 7.0 at its default settings using best fit model.

RESULTS AND DISCUSSION

Disease symptoms and incidence- All the locations surveyed had a varying degree of leaf curl incidence with mild to severe upward leaf curl symptoms with interveinal & marginal chlorosis (Fig. 1a-e) resemble the begomovirus infection on chilli occurring in India and outside of India. The severely infected plants produced no fruit; however, plants with mild infection produced fruits with reduced sizes. The disease incidence ranged from 18-85%. In-some places chilli plants with mixed infection of mites along with begomovirus was noticed.

PCR detection of the virus

One set of universal degenerate primers (SPG1/SPG2) were



Figure 1: a-e

Table 1: List of begomoviruses compared with the Bongaon-North 24parganas-West Bengal, Dhatrigram-Burdwan-West Bengal begomovirus isolates in the present study, their origin and NCBI gene accession numbers

Virus strains	Acronyms	Origin	Gene Accession No.
Pepper leaf curl Bangladesh virus segment A, complete sequence	PepLCBV-BB	Bangladesh	AF314531
Pepper leaf curl Bangladesh virus India [India/Mograhat/2007] clone	PepLCBV-IM	India	HM007111
pChMogK4 segment DNA-A, complete sequence			
Tomato leaf curl Liwa virus isolate Tom43, complete genome	ToLCLV-OB	Oman	KF111685
Tomato leaf curl Liwa virus isolate Tom44, complete genome	ToLCLV-OB	Oman	KF111686
Tomato leaf curl Liwa virus isolate Tom42, complete genome	ToLCLV-OB	Oman	KF111684
Pepper leaf curl Lahore virus isolate Nagpur segment DNA-A, complete sequence	PepLCLV-IN	India	JN663864
Tomato leaf curl Liwa virus isolate LW1, complete genome	ToLCLV-O	Oman	HF912280
Tomato leaf curl Joydebpur virus clone Moh9, complete genome	ToLCJV-IM	India	KJ028211
Pepper leaf curl Bangladesh virus-India isolate Chhapra segment DNA-A, complete sequence	PepLCBV-IC	India	JN663853
Chilli leaf curl virus isolate Sh1, complete genome	ChiLCV-O	Oman	JN604491
Papaya leaf curl virus av2 gene, av1 gene, ac5 gene, ac3- truncated gene, ac2 gene, ac1 gene and ac4- truncated gene, clone MI68	PapLCV-Pk	Pakistan	FM955602
Chilli leaf curl virus-[Pakistan] segment DNA A, complete sequence	ChiLCV -Pk	Pakistan	DQ114477
Chili leaf curl Pakistan virus isolate Khanewal 2 clone PC5 segment A, complete sequence	ChiLCPV-Pk	Pakistan	DQ116879
Tomato leaf curl virus [India:Kalyani-B.C.K.V.:Ridgegourd:2013] partial AC1 gene for replication initiation protein, isolate kly-bck-ridg-9	ToLCV-IK	India	HF679119
Pepper leaf curl virus isolate ANG_CREP replication initiation protein gene, partial cds	PepLCV-I	India	JN887127
Pepper leaf curl virus isolate CAK226 replication initiation protein gene, partial cds	PepLCV-I	India	JN887125
Pepper leaf curl virus isolate CESC_CREP replication initiation protein gene, partial cds	PepLCV-I	India	JN887128

used for amplification and confirmation of the partial replication associated protein gene (AC1) with expected DNA fragment of 920 bp (Fig. 2) from the virus infected samples of Bongaon-North 24-parganas and Dhatrigram-Burdwan chilli leaf curl &

Nadia samples.

The samples were considered to contain begomovirus when the molecular weight of the amplified products matched with standard reported molecular weights. The Bongaon- North 24

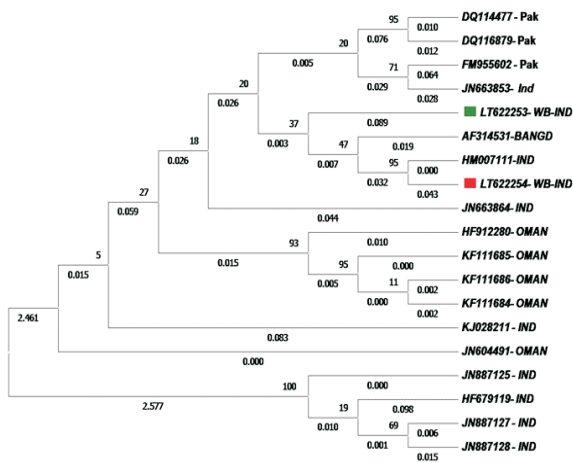


Figure 4 : Phylogenetic relationship of partial nucleotide sequences of DNA-A between begomoviruses infecting chilli of Bongaon-24parganas-WB, Dhatrigram-Bardwan-WB and other related begomoviruses. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura M., 1980). The tree with the highest log likelihood (-3504.78) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.5562)]. Evolutionary analyses were conducted in MEGA7 (Kumar S., Stecher G., and Tamura K., 2016).

parganas (LT622253) and dhatrigram-Burdwan, West Bengal isolate (LT622254) DNA-A containing replication associated protein (AC1) gene partial and showed highest nucleotide sequence identity (nsi) with Pepper leaf curl Bangladesh virus segment A, complete sequence (91%) [Acc. No.- AF314531] and Pepper leaf curl Bangladesh virus India [India/Mograhat/2007] clone pChMogK4 segment DNA-A, complete sequence (95%) [Acc. No.- HM007111] respectively. The nucleotide sequence of Bongaon-North 24 parganas-West Bengal and Dhatrigram-Burdwan-West Bengal begomovirus isolate was compared with 17 previously reported begomovirus isolates infecting chilli, peeper, tomato, cucurbit, legume and black-nightshade (Table – 1) and respective begomovirus accessions available from the GenBank (EMBL). For LT622253 (North24-parganas) and LT622254 (Burdwan), out of 17 accessions, 7 begomovirus belongs to PepLCV isolates, 6 to ToLCLV, 3 to ChiLCV and 1 to PapLCV.

Multiple sequence alignment was carried out using ClustalW and ClustalX version 2.0 (Larkin *et al.*, 2007). Bongaon-N24P-WB begomovirus isolate gave maximum nucleotide sequence identity with one isolate from Bogra, Bangladesh [Acc. No.- AF314531] 91%, whereas Dhatrigram-Bardwan-WB begomovirus isolate gave maximum nucleotide sequence identity with two GenBank accession within the range of 91% to 95, reported from mograhat, India [HM007111] 95% and bogra, Bangladesh [AF314531] 91%. The pairwise sequence comparison for Bongaon-N24P-WB begomovirus isolate and Dhatrigram-Burdwan-WB begomovirus isolate registered a maximum of 89.69% pairwise alignment with AF314531 (Table - 2) and 91.19% with HM007111 begomovirus isolate respectively (Table- 2).

A phylogenetic tree was drawn using the MEGA version 7.0 to analyze the phylogenetic relationship of the sequenced genome of begomoviruses under study with a number of selected begomovirus DNA sequence retrieved from GenBank. Constructed phylogenetic tree showed LT622253 (North24-parganas) and LT622254 (Burdwan) isolate has many main and sub-clusters. The LT622253 (North24-parganas) and LT622254 (Burdwan) chilli begomovirus isolate, India was segregated from other chilli infecting begomoviruses (Fig. 4). Phylogenetic tree clearly indicates that Bongaon- North 24 pargana-WB (LT622253) chilli begomovirus isolate clustered with Pepper leaf curl Bangladesh virus segment A, complete sequence [Acc. No.-AF314531] (Fig. 4) and dhatrigram-Burdwan-WB (LT622254) chilli begomovirus isolate is clustered and most closely related to Pepper leaf curl Bangladesh virus India [India/Mograhat/2007] clone pChMogK4 segment DNA-A, complete sequence [HM007111] (Fig. 4). Both the isolates (LT622253 & LT622254) also showed evolutionary relationship with Pakistan, Oman, and Bangladesh begomovirus isolates.

The PCR detection of begomovirus using degenerate primers confirmed the association of begomoviruses with chilli growing in West Bengal, which showed mild to severe upward leaf curl symptoms with interveinal and marginal chlorosis, stunting and less or no fruit on infected plant. The variation of symptoms between different chilli growing areas indicated mixed infections with different begomoviruses and mites.

The PCR technique using degenerate primer pair (SPG1 and SPG2) allowed rapid, sensitive, and accurate detection of begomoviruses in chilli with minimal sample preparation, thus it could be very effective method for depicting the current situation of begomovirus in a region. Phylogenetic tree analysis of Bongaon-North 24 parganas (LT622253) and dhatrigram-bardwan-West Bengal (LT622254) begomovirus isolate showed closer relationship with Pepper leaf curl Bangladesh virus isolates. If the identities of two different sequences are > 91%, they are considered to be the variant of same virus (Zerbini *et al.*, 2017, Brown *et al.*, 2015). Thus LT622253 and LT622254 can be considered as the variant of Pepper leaf curl Bangladesh virus isolates (AF314531 and HM007111). This provides evidence for the possible risk of trans-boundary movement of the virus through plant materials and insect vectors.

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