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RESEARCH ARTICLE

A new monopartite Begomovirus associated with betasatellite molecule causing leaf curl disease of chilli in India.

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Abstract

Leaf curl disease in chilli is one of the major constraints to the production of chilli (*Capsicum annum* L.) in the Indian subcontinent which is caused by begomovirus and associated satellite. The full-length genome of begomovirus and its cognate betasatellite DNA component associated with chilli leaf curl disease (ChiLCD) originating from Bijnour, Uttar Pradesh (U.P.), region of India were cloned and sequenced. The pairwise nucleotide sequence identity shared <91% identity with DNA-A segment of Tomato leaf curl New Delhi virus (ToLCNDV), while betasatellite DNA shared highest nucleotide identity (>90%) with Tomato leaf curl Bangladesh betasatellite (ToLCBDB). According to new species demarcation rules, we hereby, report identification of a new begomovirus species infecting chilli plant in natural conditions, and we propose to name as, *Chilli leaf curl Bijnour virus*. Head to tail tandem repeat dimers of DNA-A and betasatellite DNA were prepared and agroinoculated into chilli plant, which later showed slight curling and mosaic development in leaves, thus fulfilling the Koch's postulates.

Introduction:-

Chilli is an important commodity that is used as a vegetable, spice, medicinal herb and a source of red pigment. In India, generally three varieties of chilli are grown in fields namely *Capsicum annum*, *Capsicum frutescens* and *Capsicum chinense*, although most of the cultivar varieties are *C. annum*. The chilli leaf curl disease (ChiLCD) is a major limiting factor for chilli production in the Indian subcontinent and is invariably caused by begomoviruses (Briddon et al., 2003; Senanayake et al., 2006; Shih et al., 2003). The symptoms include upward curling of leaves, yellowing and stunted growth of the plant etc. The Begomovirus belongs to family *Geminiviridae* (largest known family of a single-stranded DNA virus), which is characterized by its twinned icosahedral particle nature, approximately 18*30nm in size (Padidam et al., 1995). The viruses in this family are divided into seven genera on the basis of the host range, insect vector and genome arrangement (Varsani et al., 2014). Begomoviruses are transmitted exclusively by the whitefly *Bemisia tabaci* (Order: Hemiptera), and are often associated with satellite molecules, viz. alphasatellite and betasatellite DNA. Interestingly, the symptoms and pattern of tomato leaf curl disease (ToLCD) and ChiLCD are often similar. In India chilli has been reported to be infected with begomoviruses such as ToLCNDV, Chilli leaf curl virus, Tomato leaf curl Joydebpur virus etc. (Senanayake et al., 2006; Chattopadhyay et al., 2008), which eventually have both monopartite and bipartite genome. An experimental demonstration in 2008 has fulfilled Kochs' postulates for ChiLCD, to be caused by a complex consisting of monopartite chilli leaf curl virus and a betasatellite DNA component (Chattopadhyay et al., 2008).

Materials and methods:-

Collection of diseased leaf sample, confirmation of begomovirus complex, cloning and sequencing:-

Twenty-five symptomatic leaf samples from different chilli plants and five different fields showing typical yellow leaf curl symptoms were collected from the chilli fields of Bijnour, India (29°22′00″ N, 78°07′59″ E) during the year 2012.

DNA isolation and PCR:-

Total nucleic acid was isolated from 500 mg of leaves following the method described by Dellaporta (1983) and the final pellet was dissolved in 100 µl of TE buffer. Presence of begomovirus was confirmed by PCR amplification with a primer pair for begomovirus P1/P2 (5' GGYCACGAHTTAATKAGRGA 3'/ 5'-GTTGATYATGTATTGTWYVATGTG 3') (Khan et al., unpublished), which amplify part of pre-coat protein and coat protein gene segment. The conditions for PCR reaction were, initial denaturation at 94^0 C for 3 min., followed by 30 cycles of reaction comprising of: denaturation at 94^0C/30sec, annealing at 48^0C/2min., and an extension of 72^0C/1min with a final extension of 72^0C/5min. The expected amplicon of 850 bp were obtained from the DNA of all diseased leaf samples chosen, and further the sequence analysis showed sequence identity of 98-99% with each other. One sample was randomly chosen for further analysis, and the DNA isolated from that sample was subjected to Rolling circle Amplification (RCA) using Phi DNA amplification kit, strictly following manufacturers recommendation (Templi Phi kit, GE Healthcare, USA). The phi amplified product was linearized with *Sac*I for 25 min., run on a 1% agarose gel, and finally the desired band (*ca*. 2.7 kb) was purified using gel extraction kit (Gene JET Gel Extraction kit, Thermo Scientific, Lithuania). The amplified fragment was cloned at the respective site of pBluescript SK^+ (Stratagene), and the competent cells of *Escherichia coli* (DH5-α) were transformed with this ligated product. However, repetitive attempts to get satellite molecules and DNA-B failed, so universal primers were employed to amplify them (Briddon et al., 2002; Bull et al., 2003; Rojas et al., 1993). An amplicon of size ~1.3 kb was obtained with betasatellite specific primers, which was cloned into pGEMT easy vector (Promega, USA) and transformed into the competent cells of *E. coli* (DH5-α). The potential DNA clones (three from each DNA-A and betasatellite) were sequenced with automated sequencer (3730 XL DNA Analyzer, Applied Biosystems, USA) by primer walking. Notably, no alphasatellite DNA and DNA-B segment was observed upon PCR amplification with their respective specific primers.

Sequence analysis:-

The complete nucleotide sequences were subjected for similarity search using BLASTn search program (http://www.ncbi.nlm.nih.gov/BLAST/). The pair wise sequence comparison was done with the help of PASC (http://www.ncbi.nlm.nih.gov/sutils/pasc/viridty.cgi). Phylogenetic tree was drawn with the help of MEGA software version 4.0 (Tamura et al., 2007). While predicting ORFs, the Expasy proteomic server tool was used to translate the set of protein encoding genes.

Infectivity testing:-

Standard procedure was followed to develop head to tail tandem repeat dimers of monopartite begomovirus (DNA-A) and betasatellite DNA (Pratap et al., 2015). The infectivity of monopartite begomovirus under study was investigated with the help of *Agrobacterium tumefaciens* (strain LBA4404) mediated inoculation of the components (monopartite begomovirus whole genome and betasatellite DNA), either separately or in the combination of both in the leaves of *C. annum* under standard conditions.

Results and discussion:-

Bioinformatic analysis:-

The complete nucleotide sequence of monopartite DNA and betasatellite DNA was deposited in the GenBank and assigned accession numbers KC465466 (2766 nt) and KF188707 (1362 nt). The virus isolated and cloned from chilli plant had the typical Old World begomovirus genome organization with six ORFs, two in sense and four in the complementary sense strand separated by an intergenic region (IR). The IR typically contains a nona-nucleotide sequence, TAATATTAC motif which is common to all geminiviruses. The pairwise sequence comparison with the help of PASC programme (BLAST based Alignment) for Geminiviridae registered a maximum of 87.19% pairwise alignment with ToLCNDV-[Pk: Khn: 06] (Acc. No.DQ116880) and 87.04% with ToLCNDV-[IN: Tmk: Chi: 08](Acc. No. AM849548). The global alignment shows maximum percentage pairwise alignment of 89.47% (Table1) with ToLCNDV-[IN: Tmk: Chi: 08]AM849548.

Phylogenetic analysis:-

A phylogenetic tree was drawn to analyze the phylogenetic relationship of the full-length genome of begomovirus under study with a number of selected begomovirus DNA sequence retrieved from GenBank. To select, closely related begomovirus and begomovirus infecting chilli and tomato plants from Indian subcontinent were considered, with Bhendi yellow Vein Haryana virus (Acc. No. FJ561298) as an outgroup. The begomovirus under study did not cluster with any of the begomovirus considered for analysis (Fig.1A). Also, the sequence obtained from different colonies shared approximately 100% similarity with each other, thereby, ruling out the possibility of mixed infection.

According to the new guidelines for begomovirus classification system, genome-wide pairwise identities of 91 % and 94 % are proposed as the demarcation threshold for begomoviruses belonging to different species and strains respectively (Brown 2015). In the light of above rule, the begomovirus under study is thus considered to be a new species of begomovirus infecting chilli plants in natural conditions, for this we propose to name as Chilli leaf curl Bijnour virus (ChiLCBjV) with an isolate descriptor ChiLCBjV[India:Bijnour:2013]. Interestingly, nucleotide sequence identity of begomovirus under study with other reported ChiLCD associated begomovirus from India (Chattopadhyay et al., 2008; Kumar et al., 2011) found to be 73.3% and 77.8%.

We have also used Sequence Demarcation Tool (SDT), a computer program that produces pairwise identity plots and colour-coded distance matrices to aid the classification of sequences according to ICTV approved taxonomic demarcation criteria (Muhira et al., 2014). A color-coded pairwise identity matrix was generated from several related begomovirus, which demonstrated that the begomovirus under study shared less than 91% pairwise identity with ToLCNDV-[IN: Tmk: Chi: 08] (Acc. No. AM849548).

The betasatellite DNA molecule under study shared highest nucleotide sequence identity (>90%) with Tomato leaf curl Bangladesh betasatellite DNA (HM007107). The Geminivirus betasatellite DNA typically contains three common features which is also present in the chilli betasatellite DNA under study: a single ORF βC1, which is conserved in position and sequence, a region rich in adenine residues (A-rich) and a sequence conserved among all betasatellites that contains, at its 3' end, a predicted hairpin structure having similarity to the origin of virion-strand DNA rep. of geminiviruses. The ORF βC1 of the ChiLCV under study encodes 118 amino acid and the A-rich region is approximately 280 bp in length with 60% adenine content. The dendrogram drawn showed clustering of betasatellite DNA under study with Tomato leaf curl Bangladesh betasatellite (ToLCBDB) (Fig.1B). After considering the current demarcation value for betasatellite DNA (78%) (Briddon et al., 2008), the chilli betasatellite is thus an isolate of ToLCBDB, for which the descriptor ToLCBDB-[In: Bijnour: 2013] was proposed.

Infectivity testing:-

Slight vein thickening was observed when the leaves of *C. annum* was inoculated with monopartite begomovirus DNA alone (Fig. 2C), but the vein thickening, stunting and yellowing of the leaves was observed when both components (monopartite begomovirus DNA and betasatellite DNA) were inoculated simultaneously (Fig. 2A). Inoculation of betasatellite DNA alone gave only yellowing of leaf (Fig. 2B). We have used six leaf stage of *C. annum* for the inoculation studies, and the presence of begomovirus was confirmed after 28 days post inoculation (d.p.i.), by PCR methods using different primer combinations for betasatellite DNA and monopartite DNA-A component (Briddon et al., 2002; Kumar et al., 2011). PCR amplification of expected size appeared on the gel (data not shown). A mock inoculation of empty vector in chilli leaves gave no phenotypic change (Fig. 2D). It is concluded that this new begomovirus alone is capable of infecting chilli plant; however betasatellite DNA is necessary to induce symptoms which are analogous to that of symptoms under field conditions.

The ChiLCD is the most worrisome disease of chilli across India, and the extent of disease is spreading every year. To the best of our knowledge, the monopartite begomovirus under study is a new species of begomovirus infecting chilli under field condition. However, further studies on the geography and distribution of ChiLCD will develop a clear picture of such begomovirus disease. New concepts which are based on gene silencing could help more in the understanding of this disease.

Legends of Figures

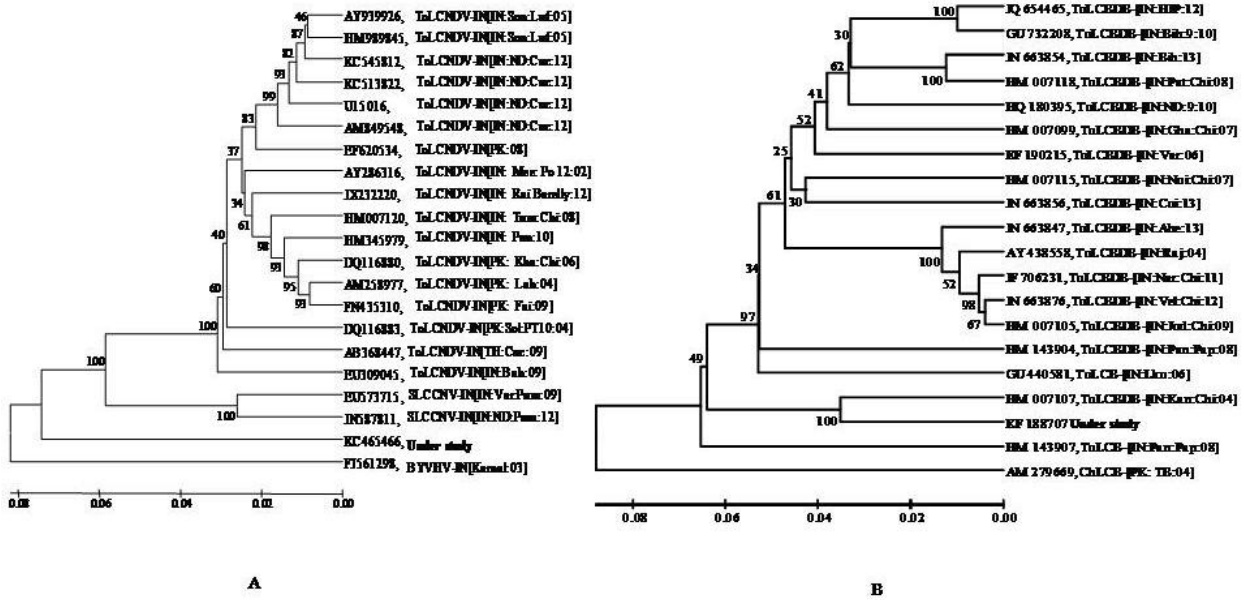

Figure1. Phylogenetic trees of (A) the monopartite begomovirus under study (Acc. No.KC465466) and (B) the betasatellite under study (Acc. No. KF188707) with some well-characterized begomoviruses and associated beta satellites constructed with the help of MEGA4 software. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The sequences were obtained from GenBank. For DNA-A, Bhendi yellow Vein Haryana virus (Acc. No. FJ561298) and for betasatellite DNA, chilli leaf curl virus (Acc. No. AM279669) were used as out-group. Abbreviations used ToLCNDV: Tomato leaf curl New Delhi virus; SLCCNV: Squash leaf curl China virus; ToLCBDB: Tomato leaf curl Bangladesh betasatellite; ToLCB: Tomato leaf curl betasatellite; ChLCB: Chilli leaf curl betasatellite.

Figure 2. A: Induction of symptoms in *C. annum* after agroinoculation of DNA-A and betasatellite DNA combined together. B: Yellowing of leaf when inoculated only with betasatellite DNA. C: Symptoms induced after agroinoculation of DNA-A alone. D: Mock inoculation by empty vector in the leaves of chilli plant.

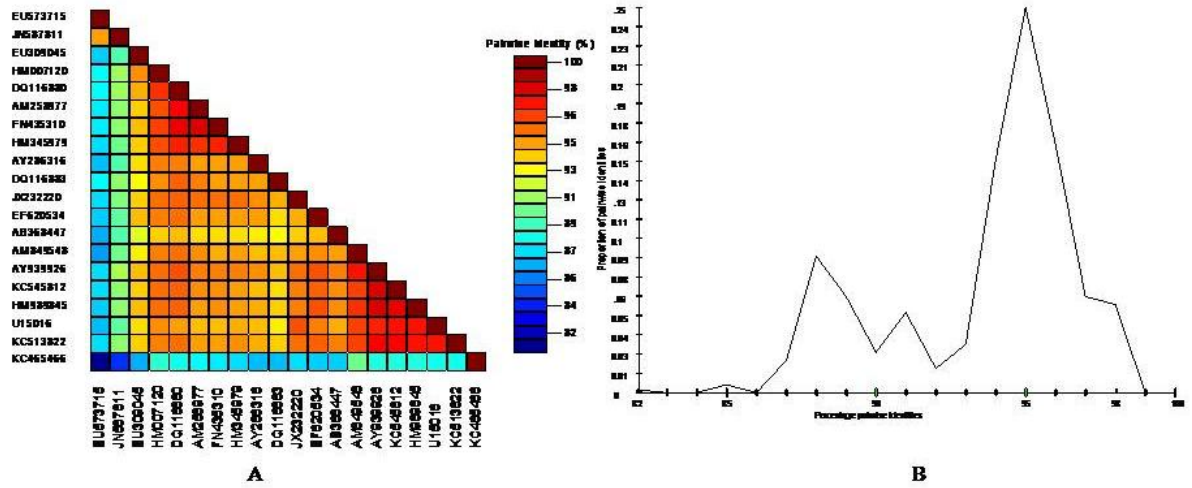

Figure 3. (A) Colour-coded pairwise identity matrix generated from begomovirus genomes. Each coloured cell represents a percentage identity score between two sequences (one indicated horizontally to the left and the other vertically at the bottom). A coloured key indicates the correspondence between pairwise identities and the colours displayed in the matrix. (B) Pairwise identity frequency distribution plot. The horizontal axis indicates percentage pairwise identities, and the vertical axis indicates proportions of these identities within the distribution.

Table1: BLAST based alignment and global alignments between KC465466 and related begomoviruses (PASC based alignments)

Virus#	BLAST based alignment (%)	Global alignment (%)
DQ116880	87.19	87.95
AM849548	87.04	89.47
AJ620187	86.7	87.56
AF448058	86.59	87.46
DQ169056	86.53	87.46
U15015	86.21	87.16
AF448059	86.13	87.01
DQ116883	85.88	87.72
DQ116885	85.54	86.31
EF043230	85.26	86.99
AY428769	84.81	86.17
AY286316	84.78	86.82

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