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MOLECULAR EVIDENCE OF ASSOCIATION OF *CHILLI LEAF CURL VIRUS* WITH LEAF CURL DISEASE OF PAPAYA IN PUNJAB

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ABSTRACT
Papaya leaf curl disease (PaLCuD) appears to be one of the most serious and widespread disease of Papaya. The infected plant show leaf curling and bears only few fruits with distorted shape. The present investigation was carried out at PAU, Ludhiana to determine the molecular association of *Begomovirus* with the papaya leaf curl disease in Punjab. Association of *Begomovirus* with leaf curl disease of Papaya (*Carica papaya* L.) was detected by polymerase chain reaction using *Begomovirus* coat protein gene specific primers and conûrmed by highest sequence similarities and close phylogenetic relationships. The complete nucleotide sequence of cloned *Begomovirus* sequence showed the highest nucleotide sequence identity (97%) to an isolate of *Chilli leaf curl virus* from Ludhiana (ChiLCV), conûrming it as an isolate of ChiLCV.

INTRODUCTION

Papaya (*Carica papaya* L.) is cultivated commercially throughout the tropical and subtropical regions of the world. The limiting factor of successful papaya cultivation is its susceptibility to viral diseases like leaf curl, ring spot and mosaic. Among these, leaf curl disease is one of the most serious threats to papaya cultivation worldwide (Singh, 2006). The viruses known to be associated with the leaf curl disease of papaya are: *Papaya leaf curl virus* (Nadeem *et al.,* 1997, Singh *et al.,* 2006) *Papaya leaf curl China virus, Papaya leaf curl Guangdong virus* (Wang *et al.,* 2004) and *Papaya leaf curl Taiwan virus* (Chang *et al.,* 2003). These viruses are members of the *Begomovirus* group of the family *Geminiviridae*. A typical leaf curl disease was observed on papaya grown in and around Ludhiana (India) during September 2013. Naturally infected papaya plants by *Papaya leaf curl virus* shows severe downward leaf curling, swelling of veins, twisting and reduction of petioles and stunted growth of the whole plant which bore a few small, distorted fruit (Summanwar and Ram, 1993).

Gemini viruses, according to the International Committee on Taxonomy of Viruses (ICTV) are subdivided into three subgroups based on the insect vector, host range and genome structure (Francki *et al.,* 1991). Subgroup I include viruses with mono partite genomes transmitted by leafhoppers to monocotyledonous plants and the type member is *Maize streak virus* (MSV). The viruses transmitted by leafhoppers to dicotyledonous plants belong to Subgroup II having *Beet curly top virus* (BCTV) as type member. Subgroup III belongs viruses having bipartite genomes transmitted by whiteflies to dicotyledonous plants and type member is *Bean golden mosaic virus* (BGMV) (Mayo and Martelli, 1993). Based on these characters, *Geminivirus* are classified in to seven genera namely, *Becurtovirus, Mastreovirus, Curtovirus, Turncurtovirus, Eragrovirus, Topocuvirus* and *Begomovirus* (Varsani *et al.,* 2014). The majority of Gemini viruses fall into the genus *Begomovirus* transmitted by *Bemisia tabaci*, infecting only dicotyledonous plants. Begomoviruses described to-date fall into a number of genome categories, the main division being monopartite and bipartite depending on whether they have one or two circular ss DNA components, each of these DNA strand being approximately 2.5-3 kb nucleotides in size. In the Old World, the majority of begomovir uses have monopartite genomes and majority of them posses ssDNA satellites known as beta satellites and satellite like components known as alpha satellites (Briddon and Stanley, 2006). The family comprises viruses with circular single stranded DNA genomes encapsidated in geminate quasi-isometric virion particles of ~20-30 nm in size which are transmitted through white ûy (*Bemisia tabaci*) (Usha rani *et al.,* 2013). During the present investigation, an attempt has been made to identify and characterize the virus associated with leaf curl disease of papaya in Punjab. This paper describes the molecular evidence of association of *Chilli leaf curl virus* with Papaya leaf curl disease in Punjab during the year 2014-15 at Punjab Agricultural University, Ludhiana.

MATERIALS AND METHODS

Leaves of papaya plants exhibiting severe leaf curling, vein thickening and reduction in leaf size were collected from

kitchen gardens and orchards growing papaya in the area of Ludhiana in during 2013-14 (Fig. 1) and brought to the Virology Laboratory of Department of Vegetable Science, PAU, Ludhiana for further studies.

Total DNA was extracted from the 100 mg sample of infected papaya leaf using the method given by Ghosh *et al.* (2009) with slight modification. The extracted DNA was used as template for Polymerase Chain Reaction (PCR). The PCR was performed using *Begomovirus* coat protein (CP) speciûc primers, AV494/AC1048 in a 25μl reaction mixture containing: template DNA 2 μl (50ng/μl), dNTPs 0.5 μl (0.2mM), primers 1 μl (each 20pmol), Taq DNA polymerase 0.2 μl (1U), reaction buffer 5 μl (1X), $MgCl_2$ 1.5 μl (1.5 mM) and Sterile water 13.8 μl. PCR amplifications was accomplished in a programmable DNA thermolcycler. The PCR conditions were: Initial denaturation at 94ºC for 3 min followed by 35 cycles consisting of 94ºC for 1 min, 60ºC for 1 min, 72ºC for 1 min and final extension at 72ºC for 10 min (Wyatt and Brown, 1996). The amplified product was visualized on agarose gel electrophoresis in 0.8% agarose gels. For cloning and sequencing, the amplified products of expected size were eluted from agarose gel by using Nucleo spin Gel and PCR cleanup kit (Macherey-Nagel GmbH & Co. Germany). The eluted fragment of desired size was ligated into cloning vector with 10 μl of ligation reaction. The ligation reaction included 5μl ligation buffer (2x), 1 μl pGEM-T-Easy vector (50ng/μl), 1 μl T4 DNA ligase, 2 μl Purified PCR product (35ng/μl) and 1 μl Nuclease-free water. The competent cells of *E. coli* strain DH5á (100 μl) were transformed with the 5 μl ligation mixture. The transformed cells were cultured on LB Agar-Amp/X-gal plates at 37ºC for 8-10 hr which resulted into appearance of blue and white bacterial colonies. The confirmation of recombinant plasmid was done through colony PCR using universal M13 primers. Once positive colony was confirmed, the plasmids of transformed bacterial cells were isolated. Isolated plasmids were further confirmed by using gene specific AV/AC primers. The samples were out-sourced for sequencing to Xcelris Lab Ltd. (Ahmedabad). The bidirectional sequencing was done using M13 primers in ABI 3730Xl DNA Analyser. The sequences (both forward and reverse) were assembled using Bioedit Sequence Alignment Editor software (version 5.09) (Hall, 1999). The similarity of genomic sequences was initially analyzed by using the BLAST program available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). The representative sequences were downloaded from GenBank database so that the comparisons could be made between the sequences found in this study with similar sequences from different hosts and geographical regions. The Clustal W algorithm (Thompson *et al.*, 1994) available in MegAlign (Lasergene, DNASTAR, Madison, WI, USA) was used to calculate pairwise nucleotide and protein identities. Phylogenetic analysis was carried out using the neighbor-joining algorithm and the Kimura two-parameter model available in Mega 6 (Tamura *et al.*, 2013) and one thousand bootstrap iterations.

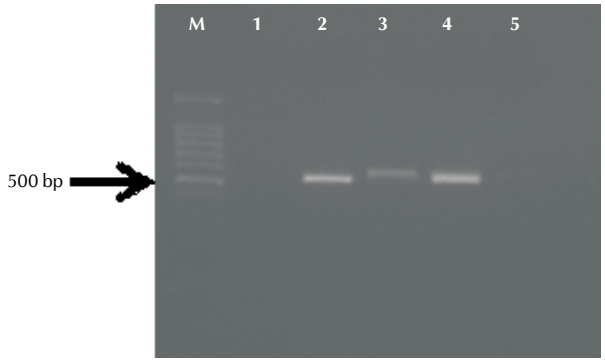
RESULTS AND DISCUSSION

The DNA from both infected and healthy samples was used as template for amplification of core coat protein region of begomovirus. The DNA sample from infected plants yielded a band of ~576 bp by AV494/AC1048 primers (Fig. 2). No amplification was observed in samples from healthy plants as well in water. The initial analysis of obtained sequences for nucleotide similarity by BLASTn (NCBI) indicated the sequences in question were of begomovirus. These sequences were closely related to isolates of *Chilli leaf curl virus* (ChiLCV) (91-97% nucleotide sequence identity to all ChiLCV sequences available in the databases) with the highest to a ChiLCV isolate of Papaya originating from New Delhi, India (HM140365.1). The retrieved sequences were submitted to the GenBank data base (Accession No. KR074211 and KR074212). The top hits of blast along with sequences in question were aligned by Clustal W algorithm showed that sequence in question shares maximum 90.0 per cent nucleotide similarity with ChiLCV isolate of Papaya originating from Panipat, India (HM134223.1).

A phylogenetic analysis, based upon an alignment of the nucleotide sequences of the core CP region identified here with previously reported begomovirus sequences from chilli, papaya and other crop from the database is shown in Figure 3. In the tree the begomovirus isolates from Papaya segregate with the previously characterized begomovirus isolates. The obtained sequences of *Begomovirus,* cloned from the symptomatic papaya sample falls in the same clade as *Sunflower leaf curl virus* and *Chilli leaf curl virus* (Fig. 3).

Papaya has affected by several *Begomoviruses* that causes papaya leaf curl disease in all over the world. The core CP gene has been used and illustrated to be useful in begomovirus classification (Harrison, 2002). According to Chowda *et al.,* (2005) papaya is a favourable alternative host for *Begomoviruses* of tomato, chilli and cotton. PaLCuV causing leaf curl disease of papaya has also been found in other hosts like *Gossypium* spp. (Mansoor *et al.,*2003), *Nicotiana tabacum* (Kumar *et al.,* 2009), *Rhynchosia capitata* (Ilyas *et al.,* 2010) and *Aster alpinus* (Srivastava *et al.,* 2013). According to Singh *et al.* (1978) Chilli, *Datura stramonium*, Holly-hock, *Petunia*, *Zinnia* and some of weed species act as reservoir of the PaLCuV. Singh *et al.* (2010) has mentioned *Carica cauliflora* is also a susceptible host for the PaLCuV.

Figure 1: Infected papaya plant with severe leaf curling



Ladder; 1 = healthy papaya sample; 2,4 = infected papaya sample,3 positive control and 5 = nuclease free water

Figure 2: 1% Agarose gel image showing amplified core CP gene of ~576 bp from DNA sample of infected papaya leaves. M = 100 bp

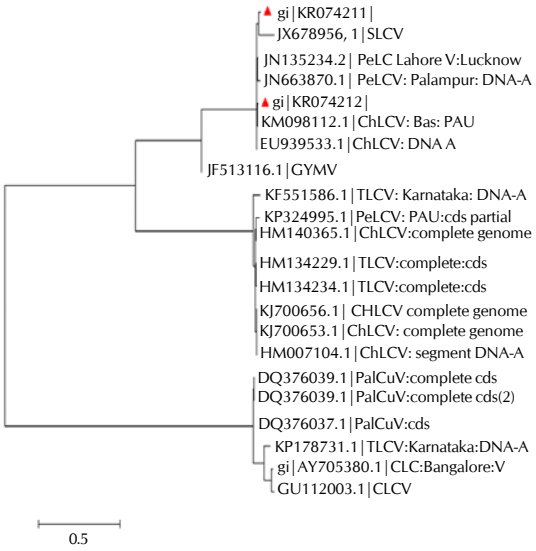


Figure 3: Phylogenetic dendrogram based on an alignment of partial coat protein sequences of cloned *Begomoviruses* in this study (labeled with red diamond) with selected sequences of other *Begomoviruses* obtained from GenBank data base

During the present findings, the virus characterized in Papaya is the variant of *Chilli leaf curl virus* with 97 per cent nucleotide identity. This finding is in the agreement with Brown *et al.* (2011) who proposed that if nucleotide sequence identity of newly isolated *Geminivirus* is >94 per cent to the already reported *Geminivirus* sequence then it will be the variant of that strain. Likewise, Nadeem *et al.*(1997) detected PaLCuV from Pakistan causing papaya leaf curl disease in papaya. Similarly, Wang *et al.* (2004) also reported *Pepper leaf curl virus Malaysia* (97.7 per cent amino acid sequence identity) causing papaya leaf curl disease in China. Raj *et al.*(2008) and Reddy *et al.* (2009) observed association of *Tomato leaf curl New Delhi virus* with the leaf curl disease in Papaya. Shahid *et al.*(2013) reported *Begomovirus* infecting papaya with 99 per cent nucleotide sequence identity to *Ageratum yellow vein virus* (AYVV) from Nepal.

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