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DETECTION AND CHARACTERISATION OF TOBACCO LEAF CURL VIRUS ISOLATES INFECTING FCV TOBACCO IN INDIA

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Tobacco leaf curl disease is widespread in several states in India causing economic losses to farmers. In the present study an attempt has been made to identify and characterize 11 tobacco leaf curl virus isolates collected from FCV tobacco growing region of Andhra Pradesh and Karnataka based on virus coat protein gene (cp). PCR primers specific to coat protein gene (cp) of tobacco leaf curl virus were designed based on the sequences of tobacco leaf curl virus-Karnataka1 (TbLCV-Kar1) and tobacco leaf curl virus-Karnataka2 (TbLCV-Kar2) genes available in the NCBI data base and used to amplify the DNA isolated from the leaf curl infected plants. In all the samples, amplicon of 725bp was observed confirming the presence of leaf curl virus in all the collected samples. Sequencing of the resultant amplicons from selected isolates and analysis using NCBI-BLAST for their similarities in the existing database revealed more than 70% similarity with Tomato leaf curl Karnataka virus, Sunflower leaf curl virus isolate ToLCKV, Croton yellow vein mosaic virus and Papaya leaf curl virus from *Carica papaya* indicating that the presence of leaf curl virus. The study also identified the existence of sequence differences in leafcurl isolates. In order to facilitate for further studies, ~725 bp purified PCR amplicon was ligated in to the pDRIVE commercial cloning vector in *E. Coli.* The presence of the insert in the vector was confirmed through the plasmid isolation and restriction analysis with ECoRI enzyme. Thus, the designed primers in the study can be used in identification and confirmation of leaf curl in the tobacco samples.

INTRODUCTION

Tobacco leaf curl disease (TbLCD) is known for its devastating effects in tobacco production zones of India. Different types of viruses cause different leaf curl symptoms. With mild types, the leaves show slight wrinkles and drooping. In case of severe types, the leaves and stem curl and leaves become thick and shrinkled. The affected plants remain stunted, their leaves develop vein thickening, curling and results in complete distortion of the morphology and make it unfit for curing and causes serious loss to the farmers. It accounts for more than 70% crop loss in severe cases. The disease incidence was reported highest in Andhra Pradesh followed by other tobacco growing states, Gujarat, Karanatka, Bihar and West Bengal (Valand *et al.,* 1992). The causative agent of Tobacco leaf curl is a Begomovirus belongs to Geminiviridae family. Begomoviruses generally transmitted by vectors (whitefly) and cause significant economic losses to many crops, including cotton, cucurbits, okra, tomato and tobacco (Moffat, 1999: Briddon *et al.,* 2000; Qing *et al.,* 2010). These viruses have either monopartite genome having one circular ssDNA designated as DNA-A (2.7Kb size), or bipartite genomes designated as DNA-A and DNA-B. Four genes including coat protein (CP) gene will be encoded by DNA-A and DNA-B aids in encoding two genes required for movement of virus, host range and pathogenicity.

Tobacco crop, majority of the times is infected with complex of viruses showing multifarious symptoms. Detection of the virus infecting the crop at such times becomes difficult. Molecular characterization using virus specific primers targeting the various genomic regions is being attempted by many researchers in India and abroad for virus identification. Further, no source of resistance to this disease is available either in the germplasm or in the wild *Nicotiana* species for

Key words: Leaf curl virus, FCV tobacco, DNA

its effective control in tobacco cultivation. Expression of truncated defective transdominant viral coat protein gene has proved more promising in developing resistance to begomoviruses. In this connection, molecular characterisation for leaf curl coat protein gene assumes significance for the detection of virus and developing host plant resistance to leaf curl virus. Hence, in the present study an effort has been made to study the leaf curl viruses coat protein gene in the infected tobacco plants from different Flue-cured Virginia (FCV) tobacco growing regions of India by designing and amplifying the coat protein specific primers.

MATERIALS AND METHODS

The FCV plant samples infected with Tobacco Leaf Curl Virus were collected from the farmers' fields of East Godavari (2 villages), West Godavari (2 villages) and Nellore districts (2 villages) of Andhra Pradesh, Khammam districts (2 villages) of Telengana and Hunsur (one village), Hosur (one village) and Solapur (one village) areas of Karnataka Karnataka light Soils.

DNA extraction

Total DNA was extracted from leaf samples as described by Dellaporta *et al.,* (1983) with a few modifications. Five mg of leaf tissue was ground with 1 ml of extraction buffer (50 mM EDTA, 100 mM Tris-HCl, 500 mM NaCl, 10 mM b-mercaptoethanol), vortexed and allowed to stand at 65 C for 10 min. After adding potassium acetate (5 M, pH 8), the mixture was incubated on ice for 10 min and clarified by centrifugation at 15000rpm for 20 min at 4 C. An equal volume of isopropanol was added to the supernatant, and the solution was incubated for 10 min at -20 C and centrifuged for 10 min at 15000rpm. The pellet was resuspended in sterile water and was further purified with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). After precipitation of the supernatant with three volumes of absolute alcohol for 30 min at -20 C and centrifugation for 10 min at 15000rpm, DNA was washed with 70% ethanol and resuspended in TE buffer. quantified on 0.8% agarose gels. The stock DNA was diluted, to make a working solution of 30 ng/µl for PCR analysis.

PCR amplification, Sequencing & Cloning

PCR primers specific to coat protein gene (cp) of tobacco leaf curl virus were designed based on the sequences of tobacco leaf curl virus-Karnataka1 (TbLCV-Kar1) and tobacco leaf curl virus-Karnataka2 (TbLCV-Kar2) genes available in the NCBI data base. Primers (TLCV-F and TLCV-R) designed in such a way that they annealed to conserved nucleotide sequence within both the above Karnataka, India genome types and amplify 725 base pairs out of 771 bp of coat protein gene. The sense primer (TLCV-F) sequence was: 5'-GAAGCGACCAGCAGATATAATCA -3' and the complementary TLCV-R primer sequence: 5'-AAAGTAGCATACACGGGGTTAGAG -3'. The isolated DNA was used for PCR amplification as described by Williams *et al.* (1990) with minor modifications. Amplifications were carried out in a 25 ml reaction mixture containing 30 ng template DNA, 0.5 units of Taq polymerase, 0.2 mM dNTPs and 30 ng of each primer using Thermal cycler (Eppendorf, Germany). The presumed viral DNA was amplified using the following conditions: 3 min of denaturation at 95 C followed by 35 cycles at 95 C for 50 s, 55 C for 50 s and 72 C for 1 min with a final extension step of 72 C for 10 min. Amplified fragments were subjected to electrophoresis in 1% agarose gels having 0.5 ìg ml^{-1} ethidium bromide. The gel images are captured using Gel Doc-2000 (Biorad, Australia). The PCR amplicons of selective samples from East Godavari, Nellore and Khammam districts were column purified using the Qiaquick kit (Qiagen, Hilden, Germany) and sequenced using Sangers method. The sequences analysis was carried out using NCBI tools.

Simultaneously the PCR amplified partial coat protein gene was cloned in the pDrive Cloning Vector (Qiagen) as per the instructions of kit and the positive clones were selected using blue white screening. Plasmid Isolation was done using Qiagen mini prep kit and the presence of insert was confirmed by restriction digestion with EcoRI.

RESULTS AND DISCUSSION

Tobacco leaf curl infected plants were collected from the farmer's fields of of Andhra Pradesh and Karnataka. The infected plants in the field

conditions exhibiting the symptoms of leaf curling, crumpling, twisting of stem with stunted growth were selected for analysis (Fig.1). A total of 11 samples were collected from different regions and genomic DNA was extracted.

The leaf curl disease in tobacco has been found to be caused by various begomoviruses. In order to confirm the disease symptoms at molecular level, leaf curl virus specific genomic sequences especially Coat Protein (CP) region was targeted for PCR amplification. The CP specific primers were designed using the conserved regions of 725bp from tobacco leaf curl virus-Karnataka1 (TbLCV-Kar1) and tobacco leaf curl virus-Karnataka2 (TbLCV-Kar2) genes available in the NCBI data base. The PCR amplification with CP specific primers in the infected samples resulted in the amplification of around 725 bp fragment in the isolates (Fig.2). The amplified fragment of the partial coat protein region confirms the presence

Fig. 1: Tobacco plant infected with Leaf Curl

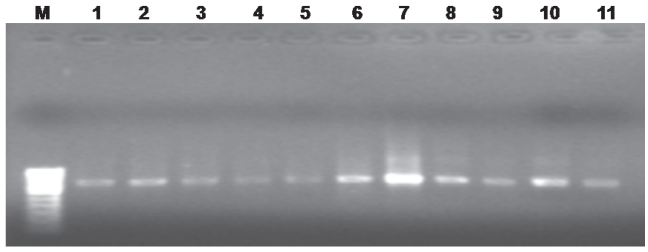

Fig. 2: PCR amplification of genomic DNA with coat protein gene specific primers in the tobacco plants infected with Leaf Curl. Lanes: M: Marker, Lanes 1-2 East Godavari samples, 3-4 West Godavari, 5-6 Nellore , 7-8 Khammam, 9 Hunsur, 10 Hosur and 11 Solapur

of leaf curl causing begomovirus in the infected plants. For further authentication the PCR amplicons of selective samples from East Godavari, Nellore and Khammam districts were column purified and sequenced using Sangers method. The sequence analysis using NCBI tools revealed that the amplified fragments belong to the leaf curl virus and have shown more than 70% similarity with TobCLV Kar-1 isolate and more than 88% similarity with Tomato leaf curl Karnataka virus, Sunflower leaf curl virus isolate ToLCKV, Croton yellow vein mosaic virus and Papaya leaf curl virus from *Carica papaya* (Table1). This further strengthens the fact that amplified PCR fragment is from a begomovirus and infected plant harbours the same. Further, the East Godavari isolate showed 40% and 70% similarity with Nellore and Khammam isolates and Nellore found 42% similar with Khamman isolate indicating isolate differences. A begomovirus specific degenerate Deng primers developed by Deng et al., (1994) in the conserved segment of DNA-A component has been used to detect the presence of virus in different plants. In the present study, the CP specific region pertained to tobacco leaf curl was targeted for amplification. Previously, Singh *et al.*, (2011) also used CP specific primers to characterize the tobacco Leaf curl Pusa virus. Recently Gurudevi *et al.*, 2018 also used CP specific primers in a survey of Tobacco leaf curl virus in Karnataka region.

Cloning of Partial CP gene sequence: In order to facilitate for further studies, the ~725 bp purified PCR amplicon was ligated in to the pDRIVE

Table 1: Sequence analysis of partial Coat Protein genes amplified from the different regions.

S.No	Name	Sequence length (bp)	Homology Hits	Query coverage	Per - Identity
1	East Godavari-1, Andhra Pradesh	712	Croton yellow vein mosaic virus	93%	97.01%
			Papaya leaf curl virus from Carica papaya	93%	96.86%
2	Nellore, Andhra Pradesh	715	Sunflower leaf curl virus isolate ToLCKV	92%	95.03%
			Tomato leaf curl Karnataka virus,	92%	94.88%
3	Khammam,Telangana	579	Tomato leaf curl Karnataka virus	88%	93.18%
			Sunflower leaf curl virus isolate	88%	93.18%

commercial cloning vector in *E. coli.* The positive clones containing the insert was selected using the blue white screening (Fig.3). To assure the presence of insert, plasmid isolation was carried out in the positive clones and restriction analysis was done with ECoRI enzyme. The release of 715 bp fragment from the vector confirmed the presence of partial sequence of Coat Protein gene in the vector. Thus the information generated and cloned partial CP gene sequence in the study can be used for molecular analysis, interaction and expression studies to divulge more about Tobacco Leaf Curl virus.

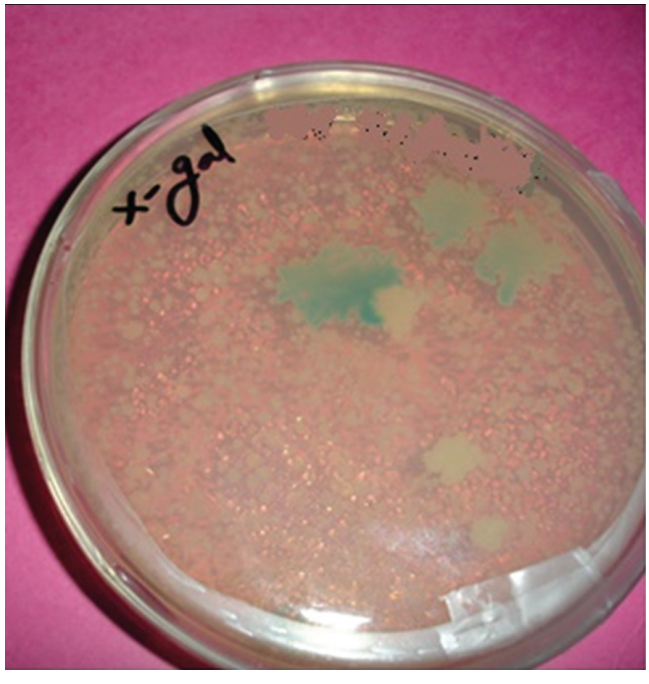

Fig. 3: Blue White Screening for selection of positive clones containing partial Coat protein gene sequence

The leaf curl virus coat protein specific primers designed in the present study could able to amplify the target sequence of specific length indicating that these primers can be employed in detecting the presence of leaf curl virus in tobacco samples. The study revealed that variation existed in leaf curl isolates

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