Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2017; 5(5): 733-736

Received: 05-07-2017
Accepted: 06-08-2017

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Diagnosis of banana bunchy top virus in Red banana

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Abstract
Banana Bunchy Top Disease (BBTD) caused by the Banana Bunchy Top Virus (BBTV), is the most serious virus disease of banana and plantain worldwide. Detection of the BBTV infection in the planting material could help in the effective management of the disease in banana. A polymerase chain reaction (PCR) assay was used to detect the of banana bunchy top virus at early stages of infection in banana suckers before the expression of symptoms. In the present study, primers were designed for amplification of the BBTV coat protein (CP) gene and the gene of 513 bp size was successfully amplified through polymerase chain reaction (PCR). The amplified PCR product was cloned and sequenced. The homology search of the sequence using NCBI blast showed similarity (99%) to ten CP gene sequences and are 98% similar to four CP gene sequences**.** 40 randomly selected banana suckers were subjected for the detection of BBTV through PCR. Out of 40, size specific amplification of coat protein gene was found, which indicates infection of planting material with BBTV. The PCR result was confirmed and validated through Dot blot hybridization analysis by using sequences of BBTV CP gene as probe.

Keywords: red banana, BBTV viral Coat protein gene, PCR, Dot blot hybridization, cloning

1. Introduction
Banana (*Musa spp*.) is one of the most important fruit crops in the world [3]. In India, banana is cultivated in Karnataka, Tamil Nadu, Gujarat, Maharastra, Andhra Pradesh, Assam and Kerala. The main varieties of banana are Dwarf Cavendish, Bhusaval Keli, Basrai, Poovan, Harichhal, Nendran, Safed velchi, Robusta and Grand naine. Among all the banana growing states, Maharastra has the highest productivity of 58.60 metric tons against India's average production of 32.50 t/ha. In Tamil Nadu, banana is cultivated in more than 81,498 ha with an annual production of 34.62 tons with a productivity 42.48 t/ha contributing to about 19.00 per cent of national production [19].

Banana bunchy top disease, caused by BBTV is the devastating viral disease of banana. BBTV was first recorded during 1889 in Fiji. BBTV is transmitted in persistent, circulative manner by the black banana aphid (*Pentalonia nigronervosa*) [10]. The infected plants with advanced symptoms have a rosette appearance with narrow, upright and progressively shorter leaves, giving rise to the common name "bunchy top " (Fig. 1).

Molecular investigations on BBTV genome revealed that BBTV has isometric virions [12], 18-20 nm in diameter, and a multicomponent genome consists of at least six circular single stranded DNA (ssDNA) component (BBTV DNA-1 to 6) ranging in size from 1108 bp to 1111 bp [2, 18]. BBTV virions have a single coat protein of approximately 20 kDa [13]. Several approaches have been attempted to manage bunchy top disease in banana and none of the strategies were able to give complete protection [5].

Successful control of bunchy top disease depends on the availability of reliable BBTV detection methods which will assist to select disease free plantlets. Symptoms of bunchy top disease are not visible at the time of planting. Hence, development of a detection kit for quick detection of BBTV is essential for diagnosis of the disease at the time of planting. In this context, nucleic acid based polymerase chain reaction [11] and hybridization methods have the advantage of amplifying the target nucleic acid present even at very low level and it has become an attractive technique for the diagnosis of plant viral diseases. The objective of this study was PCR analysis to confirm the presence of BBTV in Red banana plant samples at early stage (suckers) of plant growth and detection of viral load in infected Red banana samples and confirmation of PCR results by dot blot.

2. Materials and Methods

2.1 Collection of Plant Sample

Red banana suckers were collected from Thadiyankudisai (Lower Palani Reds) and the leaf samples were collected from the plants at early stage of planting viz., planting materials suckers and subjected to PCR and dot blot hybridization (Fig. 1).

2.2 Total Genomic DNA extraction from banana leaf midribs

Fresh young emerging green leaves with midribs were collected from the field trial plants and genomic DNA was isolated by CTAB (Cetyl Trimethyl Ammonium Bromide) protocol with slight modification [9]. Prior to extraction, 100 to 300 mg of young leaves were cut into bits and transferred to a zip lock bag (7 x 9 cm). Extraction buffer 1 ml (0.2 M EDTA, 1.4 M NaCl, 1 M, CTAB 2%) was added immediately. The samples were kept at room temperature and squeezed by rolling a glass rod over the sample to extract the cell contents. About 500 µl of the cell extract was transferred into an eppendorf tube, and then 33 µl of 20 % SDS was added into the tube and mixed well. The tube was incubated at 65º C (heating blocks) for 10-12 min and then centrifuged at 12,000 rpm for 10 minutes. Immediately 450 µl of the supernatant was transferred to a new tube and added with 450 µl of ice cold IPA (Isopropyl alcohol). Tube content then gently mixed and immediately incubated on ice for 20 min. The tube was centrifuged at 12,000 rpm for 15 min and supernatant was discarded without disturbing the pellet. The pellet was washed with 500 µl of 70% ice cold ethanol and centrifuged for 10 min at 12,000 rpm. The supernatant was discarded and the pellet was air dried for 5 min and suspended in 40 µl of 0.1X TE buffer (1mM Tris HCl pH 8.0 and 0.1mM EDTA pH 8.0) and incubated at 65^0C for 3 min (to suspend the pellet well) and stored at -20ºC for further use. The isolated DNA was checked for its purity on 0.8% agarose gel and quantified by ND 1000 spectrophotometer (Nanodrop technologies, Inc., USA).

2.3 Multiple sequence alignment and primer designing

ClustalW [14] program was used for multiple sequence alignment of retrieved DNA 3 component (coat protein gene) of BBTV genome sequences of different [17]. Conserved blocks in the DNA 3 component of BBTV genome sequence were identified after a sequence alignment. Primer 3 software was used for designing primers (Table 1). Primer quality parameters like GC percentage, melting temperature and product size were taken into consideration and the worthiness of the designed primer was analyzed using Fast PCR software.

2.4 Cloning and Sequencing of BBTV CP Gene

2.4.1 Cloning of BBTV Coat protein Gene

PCR amplification of '*CP*' gene was done using the designed forward primer (ATGGCTAGGTATCCGAAG) and reverse primer (TCAAACATGATATGTAATTC). The PCR amplified product was resolved by 0.8% agarose gel electrophoresis [15]. DNA band of expected size was excised from the gel and the DNA fragment was eluted using gel extraction kit by following manufacturer's instructions (gel elution kit, Fermentas-give precise description of kit). The eluted DNA fragment was ligated into a T-tailed vector pTZ57R/T by T/A cloning method (catalog # K1214; MBI Fermentas). Ligation reaction was set up by mixing 25 ng of vector DNA, 75 ng of amplified product, 1 U of T4 DNA ligase and 1 µl of 10X buffer in a 10 µl reaction. The reaction mix was incubated at 16°C for 16 h and the ligated product transformed into competent *E. coli* (DH5α) cells.

2.4.2 Screening of recombinant colonies and Colony PCR analysis

The white colonies were selected and screened for the recombinant plasmid by colony PCR analysis and restriction digestion. Colony PCR analysis was done to identify the recombinant clones by using the '*CP*' gene-specific primers [8]. Amplification reactions were performed as described above. The product was used for electrophoretic analysis on 0.8% agarose gel.

2.5 PCR screening of BBTV infection in Red banana

Genomic DNA isolated from leaf samples of the Red banana were used as a template. PCR amplification was performed to amplify the full length CP gene of BBTV. PCR reactions were performed in a final volume of 20 µl (2 µl diluted total genomic DNA, 2.0 µl of 10X PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 15 mM $MgCl_2$), 0.5 µl of 100 mM dNTPs, 0.5 µl of 150 ng of respective forward and reverse primers, 0.5 µl of 3U *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India) and 14 µl of sterilized double distilled water). Amplification was performed in a Programmable Thermal Cycler (PTC-100^{TM}, MJ Research, Inc., Watertown, USA). The amplification conditions were as follows: Initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min annealing at 57°C for 1 min, extention at 72°C for 1 min and final extension for 10 min at 72 °C. After amplification, 8 µl of the product was used for electrophoretic analysis on 1 % agarose gels.

2.6 Sequencing of the coat protein

The isolated recombinant plasmid was sequenced. The obtained nucleotide sequences were analyzed through NCBI-BLAST search. Multiple sequence alignment was performed using ClustalW program to analyze the similarity between the cloned insert and the gene sequence 513 base pair retrieved from the database.

2.7 Dot blot analysis

To confirm the virus infection and viral load at vegetative growth stages, the dot blot hybridization were done with infected and healthy banana samples and positive control. Dot blot hybridization was performed as described by [9] with required modifications.

2.7.1 Dot blot

Equal amounts of genomic DNA (10 µg/lane) were spotted as a dot on positively charged nylon membrane (Biorad). The membrane was then immersed in 0.25 N HCl for 15 minutes for partial depurination of DNA followed by denaturation by incubating in 0.4 M sodium hydroxide for 20 minutes at room temperature. After soaking the membrane in 10 X SSC for 30 minutes, the membrane was carefully shade dried.

2.7.2 Preparation of radioactive probes

About 25 ng of PCR amplified coat protein gene DNA fragments were denatured with 0.5 µg of random hexanucleotide primers in a boiling water bath for 5 minutes and cReded immediately on ice. To the mixture (25 µl), other reaction components are added (25 µM each of which contain 0.33 mM dATP, 0.33 mM dTTP, 0.33 mM dGTP, 50 µCi of α [^{32}P] dCTP (BRIT, CCMB campus, Hyderabad) and 10 units

of klenow fragment of DNA polymerase I). The reaction was incubated at room temperature for three hours.

2.7.3 Dot blot hybridization

Nylon membrane carrying DNA from healthy and infected samples were pre-hybridized (PH) in PH-solution (5XSSC, 5X Denhardt's solution, 1 mg/ml denatured salmon sperm DNA) at 62 ºC for 4 hours. The pre-hybridized filters were then hybridized with radioactive probe in hybridization solution (pre-hybridization solution with radiolabelled probes at a concentration of 1X 10^6 cpm/ml.) at 62ºC for 18 hours. After hybridization, nylon membrane was washed sequentially with 2X SSC + 0.1% SDS for 15 minutes at room temperature (twice), and 0.1X SSC + 0.1% SDS for 15 minutes at 62 ºC. Washed membranes were dried at room temperature and exposed for autoradiography.

3. Results

3.1 Cloning and sequencing of the CP Gene of BBTV

The CP gene-specific primers of BBTV were designed using the conserved region in the CP gene to amplify the size of 513 bp. The primer set was used for the complete amplification of the CP gene in the BBTV infected Red banana plants. The PCR products were resolved on 0.8 % agarose gel and an expected size of 513 bp was observed (Fig 2). The full length CP gene (513 bp) was then eluted from the 0.8 % agarose gel and cloned into the T/A cloning vector, pTZ57R/T. The ligated product was transformed into the *E. coli* strain (DH5α) and was selected on ampicillin containing medium. The recombinant colonies were identified by colony PCR using the same CP gene specific primer and positive clones was selected and further confirmed by releasing the cloned fragment using restriction enzymes, *Kpn*I and *Hin*dIII. The selected three recombinant clones were sequenced. Sequencing resulted in a length of 513 bp and the homology search of the sequence using NCBI blast [1] showed similarity (99%) to the reported ten CP gene sequences and are 98% similar to four CP gene sequences**.** The identity search was made with BLASTX algorithm using the same nucleotide sequence.

3.2 PCR screening of Red banana planting materials

Total genomic DNA was isolated from all the 40 Red banana suckers were collected from Horticulture Research Station for preliminary screening. PCR was done with 40 DNA of using forward and reverse primer of BBTV CP gene revealed that 5 plants DNA produced band at specific size (513 bp) remaining 35 plants DNA did not produce any band. (Fig 3).

3.3 Dot blot hybridization analysis

BBTV infection was further confirmed by Dot blot method using the CP gene of BBTV as a probe. The Hybridization of BBTV CP gene exhibited similar patterns (Fig 4) like PCR i.e tissue cultured banana plant (negative control) and uninfected plants selected based upon the bands exhibiting in the PCR did not produce any signals (Block dots) whereas infected banana plant (positive control) and screened plants which shows BBTV positive in PCR produced intense hybridization signal on the membrane.

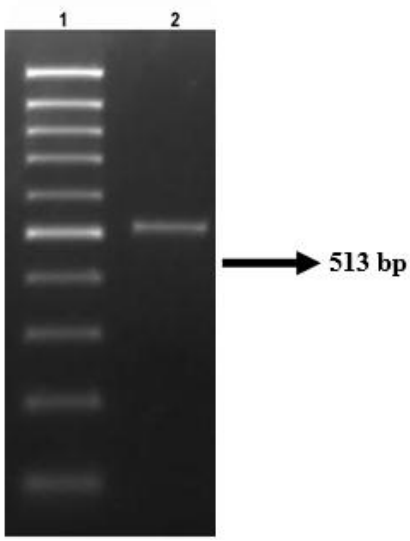
4. Discussion

Assortment of disease free planting material is one of the very important preventive measures in banana farming. Visual identification of BBTV infection in the planting material is very difficult as the development of the symptoms is not visible at the early stage of infection. Several reports are available for the molecular detection of the plant virus. Multiple sequence alignment of the CP gene sequences using Clustal W software revealed that the sequences encoding for CP gene from different isolates were sharing significant level of similarity among them [16]. The leaves of the Red banana contain high amounts of secondary metabolites which make it difficult to isolate good quality DNA and interfere with the PCR amplification**.** DNA isolated using the modified CTAB method contained low level of phenolic contamination.

In order to clone the CP gene, PCR was carried out with CP gene specific primer and the DNA isolated from BBTV infected Red banana plant. The sequence of the coat protein gene about 513 bp successfully amplified by the forward and reverse primers designed by the PRIMER 3 tool showed that these primers can be used as molecular key to diagnose the BBTV infection in the early stage (suckers) before planting and it helps to eliminate the affected plants also disease management.

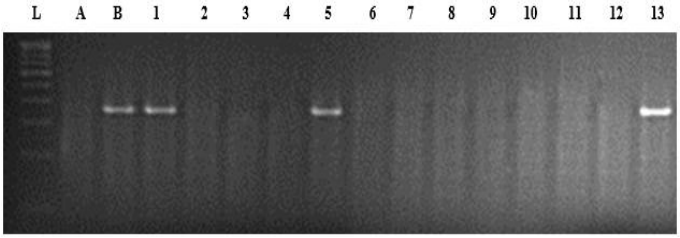
The coat protein gene was successfully cloned in the T-tailed vector pTZ57R/T and cloned DNA was sequenced [7, 6]. Homology search analysis using nucleotide and protein sequences by NCBI blast revealed a 99% similarity to most of the BBTV CP sequences available in the NCBI database [4].

Fig 1: External appearance of BBTV infected Red banana plant



Lane 1. 100 bp ladder, 2. Coat protein gene

Fig 2: PCR amplification of BBTV '*CP*' gene for cloning



Lane 1. 100 bp ladder, lane, Lane A. Tissue culture banana sample (Negative control), Lane B. BBTV Infected plant (Positive control), Serial 1 to 13. Testing plants

Fig 3: PCR amplification of BBTV *'CP'* gene in testing plants

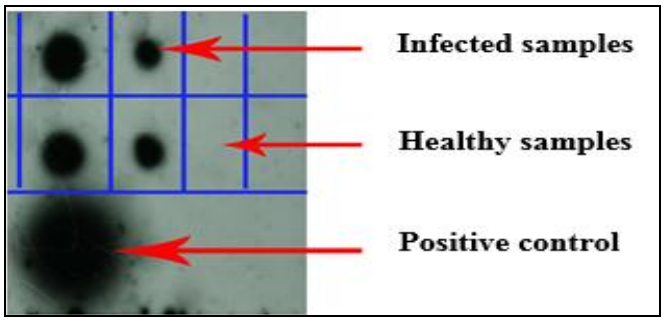


Fig 4: Dot blot hybridization analysis of Red banana using BBTV *CP* gene as a probe

Table 1: Primers sequences for amplifying Coat protein gene

Primer name	Sequence	Product size
CP gene forward primer	5'-ATGGCTAGGTATCCGAAG-3'	513 bp
CP gene reverse primer	5'-TCAAACATGATATGTAATTC-3'	

5. Conclusion

PCR-based detection technique had the advantage of amplifying the target nucleic acid present even at very low level, contain least amounts of secondary metabolites and it had become an attractive technique for the diagnosis of many plant viral diseases and the dot blot analysis was also performed to detect virus load. The PCR confirmed presence of BBTV coat protein gene plants were used to dot blot analysis to validate the PCR based detection of BBTV method. PCR confirmed presence of BBTV coat protein gene plants showed signals whereas plants which were negative to the BBTV in PCR did not produce any signal confirmed the PCR based BBTV detection is one of the best method to screen, diagonase and eliminate the BBTV affected plants. Based upon the results obtained from this study we can conclude the PCR based detection of the BBTV is one of the efficient sensitive molecular tool and it can be effectively utilised for BBTV screening in banana.

6. Acknowledgement

I thank the Chairman of Department of Plant Biotechnology, Centre on Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore for providing all the facilities for molecular work.

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