

Original Research Article

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Banana Bunchy Top Viral Coat Protein (CP) Gene Expression Studies at Molecular Level in Hill Banana cv. Sirumalai (AAB)

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ABSTRACT

Bunchy top disease caused by the banana bunchy top virus (BBTV) is a serious disease in hill banana. Detection of the BBTV infection in the planting material could help in the effective management of the disease. Primers were designed for the PCR amplification of BBTV coat protein (CP). In the present study, to confirm the virus infection and to quantify the viral load in the plants, samples from infected and healthy banana were subjected to PCR and southern blot hybridization. There was no distinguishable difference in viral load with respect to age of the plants was observed. The BBTV capsid protein (CP) gene located on component 3 was cloned in an expression vector pET28a (+). Expression of the CP in *E. coli* BL21 cells was induced by adding isopropyl-3-D-1-thiogalactoside (IPTG) to a final concentration of 1 mM. The expressed CP which migrated as a protein of approximately 19.5 kDa in Sodium Dodecyl Sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) was identified by its molecular weight. Hence it is concluded that the CP gene of BBTV was cloned and expressed in *E. coli* can be used for antibody rising or adding an affinity purification tag will enable us to produce pure monoclonal antibody in future.

Keywords

Banana, BBTV, CP, PCR, Cloning, Southern blot, Expression vector.

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Introduction

Banana (*Musa* spp.) is one of the most important fruit crop in the world (Dale *et al.*, 1987). It is the major staple food crop for approximately 400 million people. In India, banana is cultivated in Karnataka, Tamil Nadu, Gujarat, Maharashtra, 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 the states Gujarat has the highest productivity of 177.5 metric tonnes against India's average of 35.50 metric tonnes. Banana is affected by

various yield limiting factors such as pests, and diseases. Diseases like black sigatoka (*Mycosphaerella fijiensis*), wilt (*Fusarium oxysporum*), viruses like banana bunchy top virus (BBTV), banana streak virus (BSV), banana bract mosaic virus (BBMV) and nematodes cause significant yield losses (Tripathi *et al.*, 2004). Long generation time, various levels of ploidy, lack of genetic variability and sterility of most edible cultivars have hampered the development of disease-resistant *Musa* by conventional breeding.

Several approaches have been attempted to manage bunchy top disease in banana and none of the strategies was able to give 100% protection. The strategies include planting of disease free plantlets from disease free stock clumps, regular inspection for bunchy top disease, removal and destruction of diseased plants. Research progress in understanding the mechanism of resistance to this disease has been very slow in comparison with many other serious viral diseases because of the problems in purifying the virus.

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*) (Singh *et al.*, 1996; Valerie *et al.*, 2001). 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". The leaf edges often roll upwards and show a marginal yellowing. Dark green streaks are often found on midrib and petiole, extending down into the pseudostem. The most effective diagnostic symptom is short dark green dots and dashes (Fig. 1) along the minor leaf veins. In Tamil Nadu, it is a very serious disease in lower Pulney hills where the ecotypes of hill banana, Virupakshi and Sirumalai (AAB) are cultivated (Fig. 2).

Molecular investigations on BBTV genome revealed that BBTV has isometric virions (Harding *et al.*, 1991; Katul *et al.*, 1997; Su *et al.*, 2000), 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 (Burns *et al.*, 1994 and 1995; Karan *et al.*, 1997; Wanitchakorn *et al.*, 1997; Beetham *et al.*, 1999). BBTV virions have a single coat

protein of approximately 20 kDa (Thomas and Dietzen, 1991). Several approaches have been attempted to manage bunchy top disease in banana and none of the strategies was able to give 100% protection (Karan *et al.*, 1994; Horser *et al.*, 2001). The strategies include planting of disease free plantlets from disease free stock clumps, regular inspection for bunchy top disease, removal and destruction of diseased plants (Amain *et al.*, 2008). Research progress in understanding the mechanism of resistance to this disease has been very slow in comparison with many other serious viral diseases because of the problems in purifying the virus (Wu and Su, 1990). This is because of the presence of large amounts of latex and phenolic compounds in banana tissue which interfere with virus extraction and purification (Dale *et al.*, 1986).

Successful control of bunchy top disease depends on the availability of reliable BBTV detection methods which will help us 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. Enzyme Linked Immunosorbant Assay (ELISA) is convenient and easy method but it has a limitation of being less sensitive when the viral load is low (Landgraf *et al.*, 1991). In this context, Nucleic acid based polymerase chain reaction (PCR) (Edwards and Gibbs, 1994; Grieco and Gallitelli, 1999; Tettelin *et al.*, 1999; Olmos *et al.*, 2002; Stellrecht *et al.*, 2004) 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 (Mullis *et al.*, 1986; Henson and French, 1993; Xie and Hu, 1994; McManus and Jones, 1995; Hafner *et al.*, 1997a and b; Elnifro *et al.*, 2000; Nassuth *et al.*, 2000).

Materials and Methods

Collection of plant sample

Hill banana field trial was conducted at Horticulture Research Station (HRS), Tamil Nadu Agricultural University, Thadiyankudisai (Lower Pulney Hills). Thirty hill banana suckers infected with BBTv were collected from HRS and 30 tissue culture and certified hill banana suckers free from BBTv infection were collected from National Research Centre for Banana NRCB, Trichirapalli and used as planting material for field trial. The leaf samples were collected from the plants at two different stages viz., juvenile stage (3 months after planting) and vegetative stage (6 months after planting). Healthy tissue culture derived plants were grown negative control.

Total genomic DNA extraction from banana leaves

Fresh young emerging green leaves with midribs were collected from the field trial plants and DNA was isolated and stored at -20°C for further use. Genomic DNA was isolated using the modified CTAB (Cetyl Trimethyl Ammonium Bromide) protocol (Sambrook *et al.*, 1989). Prior to extraction, 100 to 300 mg of midrib of young hill banana leaves were cut into bits and transferred to a zip lock bag (7 x 9 cm). Extraction CTAB 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 kept at 65° C (heating blocks) for 10–12 min and then the tube was centrifuged for 10 minutes at 12,000 rpm and 450 µl of the supernatant was transferred immediately

to a new eppendorf tube, and 450 µl of ice cold IPA (Isopropyl alcohol) was added and after mixing, the tubes were kept in ice for 20 min. The tubes were centrifuged for 15 min at 12,000 rpm 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 kept at 65°C for 3 min (to suspend the pellet well) and stored at -20°C. The isolated DNA was checked for its purity by 0.8% agarose gel electrophoresis and quantified by UV Spectrophotometer.

Multiple sequence alignment and primer designing

ClustalW (Thompson *et al.*, 1997) program was used for multiple sequence alignment of retrieved sequences of different BBTv isolates. Conserved blocks in the BBTv genome sequence were identified after 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 worthiness of the designed primer was analyzed using Fast PCR software.

PCR screening of BBTv infection in hill banana

PCR amplification was performed to amplify the CP gene. DNA samples isolated from the hill banana leaf samples were used as template. PCR reaction 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, 1.5 mM MgCl₂), 0.5 µl of 200 mM dNTPs, 1 µl of 100 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 PTC-100TM Programmable Thermal Cycler (MJ Research, Inc., Watertown, USA). The temperature profile is 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, extension 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 0.8% agarose gels.

Agarose gel electrophoresis

The PCR products were resolved on 0.8% agarose gel was prepared and to this 2µl ethidium bromide was added from the stock (10 mg/ml). After cooling, the mixture was poured into a preset template using an appropriate comb. The comb was removed after solidification and gel with template was placed in a horizontal electrophoresis unit containing 1X TBE buffer.

The PCR product was mixed with 6X loading dye at 5:1 ratio and loaded. Electrophoresis was carried out at 60V (Sambrook *et al.*, 1989). After the gel electrophoresis, the amplified DNA bands were visualized under UV transilluminator.

PCR Amplification of coat protein (CP)

PCR analysis was performed to amplify the CP gene in the DNA samples isolated from the hill banana leaf samples collected from the field trial (Fig. 2). The reactions were carried out in a reaction volume of 20µl and by following the temperature profiles as described above. After amplification, 10 µl of the PCR product was electrophoresed in a 0.8% agarose gel and bands were visualized by ethidium bromide staining.

Cloning and sequencing of CP gene

Cloning of CP gene

PCR amplification of CP gene was done using the designed forward and reverse primer. PCR amplified product was resolved by agarose gel electrophoresis. DNA band of expected 513 bp size (Fig. 3) was excised from the gel and the DNA fragment was eluted using gel extraction kit by following manufacturer's instructions (Sigma, USA). 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 50 ng of vector DNA, 150 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. The recombinant white colonies were selected and screened for the recombinant plasmid by colony PCR analysis.

Colony PCR analysis

Colony PCR analysis was done to identify the recombinant clones by using the CP gene specific primers. Reactions were performed in a final volume of 20 µl (2 µl of diluted total genomic DNA, 2.0 µl of 10X PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl₂), 0.5 µl of 200 mM dNTPs, 1 µl of 100 ng of forward (ATGGCTAGGT ATCCGAAG) and reverse primer (TCAAACATGATATGTAATTC), 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 PTC-100TM Programmable Thermal Cycler (MJ Research, Inc., Watertown, USA).

The temperature profile used in the amplification was as follows; Initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 57°C for 1 min, 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.

Sequencing of the coat protein

The isolated recombinant plasmid was sequenced (NCBI accession number: FJ664271.1). 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.

NCBI accession number: FJ664271.1

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ATGGCTAGGTATCCGAAGAAATCCATC
AAGCAGAGGGCGGGTTGGGCGCCGGAA
GTATGGCAGCAAGGCGGTAACGAGCC
ACGACTACTCGTCGTTAGGGTCAATAT
TGGTTCCTGAAAACACCGTCAAGGTAT
TTCGGATTGAGCCTACTGATAAAACAT
TACCCAGATATTTTATCTGGAAAATGT
TTATGCTTCTTGTGTGCAAGGTGAAGC
CCGGAAGAATACTTCATTGGGCTATGA
TCAAGAGTTCTTGGGAAATCAACCAGC
CGACAACCTGTCTGGAAGCCCCAGGTT
TATTTATTAACCTGAACATAGCCATC
TGGTTAAACTGGTATGTAGTGGGGAAC
TTGAAGCAGGAGTCGCAACAGGGACA
TCAGATGTTGAATGTCTTTTGAGGAAG
ACAACCGTGTTGAGGAAGAATGTAAC
AGAGGTGGATTATTTATATTTGGCATT
CTATTGTAGTTCTGGAGTAAGTATAAA
CTACCAGAACAGAATTACATATCATGT
TTGA
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Southern blot analysis

Restriction digestion of genomic DNA

Restriction digestion was done according to Sambrook *et al.*, (1989). The banana genomic DNA was restricted with the restriction endonucleases, Hind III (2 units of enzyme per µg of DNA) in recommended buffer, at 37°C, overnight.

Reaction was analyzed by 0.8 % agarose gel electrophoresis along with undigested banana DNA as control.

Southern blotting

Equal amount of restricted genomic DNAs (10 µg/lane) were resolved on a 0.8 % agarose gel. After electrophoresis, the gel was immersed in 0.25 N Hydrochloric acid for 15 minutes for partial depurination of DNA. After depurination, incubating the gel in 0.4 M sodium hydroxide for 20 minutes denatured DNA. After soaking the gel in 10X SSC for 30 minutes, the gel was placed carefully on transfer platform covered with a Whatman 3 mm filter paper wick with its margins hanging in 10X SSC poured in a glass tray, a piece of nylon filter, cut marginally smaller than the gel, was placed carefully on the gel.

Nylon filter was covered immediately with 5 sheets of whatman 3 mm filter papers and then with a stack of crude filter papers, cut exactly to the dimensions of the gel. The stack of filter papers was covered with a glass plate, over which a weight of 500 g was placed.

The level of 10X SSC should be below the surface of the platform. The upward diffusion of the buffer was allowed for 18 hours. After transfer, the nylon filter was carefully removed, cross linked against UV light for 50 seconds, rinsed with 2X SSC and dried.

Preparation of radiolabeled probes

The DNA was amplified using CP gene PCR by, following standard procedure (Sambrook *et al.*, 1989). About 25 ng of DNA fragments was denatured with 0.5 µg of random hexanucleotide primers in a boiling water bath for 5 minutes and chilled immediately on ice. To the mixture, other reaction compounds are added to set a 25 µl reaction containing 25 µM each of 0.33 mM dATP, 0.33 mM dTTP, and 0.33 mM dGTP, 50 µCi of α [32P] dCTP (BRIT, CCMB campus, Hyderabad) and 10 units of klenow fragments of DNA polymerase I. The reaction was incubated at room temperature for three hours.

Southern Hybridization

Southern hybridization was performed as described by Sambrook *et al.*, (1989) with required modifications. Nylon filters carrying electrophoretically resolved DNA from banana tissue culture plant and infected were prehybridized in prehybridization solution (5X SSC, 5X Denhardt's solution, 1 mg/ml denatured salmon sperm DNA) at 62°C for 4 hours. The prehybridized filters were hybridized in hybridization solution (pre hybridization solution + radiolabelled probes at a concentration of 1X 10⁶ cpm/ml.) at 62°C for 18 hours. After hybridization, nylon filters were 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°C. Then filters were dried at room temperature and exposed for autoradiography.

Expression of recombinant coat protein gene in *E. coli*

Cloning in pET vector

To express the coat protein gene in *E. coli*, the CP gene of BBTV was subcloned into

expression vector pET28a (+) at EcoRI and HindIII site. The ligated product was transformed into competent *E. coli* strain BL21 cells. The recombinant colonies were selected by colony PCR and restriction digestion analysis.

Induction of coat protein gene in *E. coli*

The *E. coli* strain, BL21 harbouring the plasmid pET 28a (+) CP was streaked separately on LB agar plate with appropriate antibiotics and incubated at 37°C for 12-16 h so as to get isolated individual colonies. Single colony was inoculated in LB broth containing appropriate antibiotics and grown on a rotary shaker (200 rpm) at 37°C for 16 h. Fifty ml of LB broth was inoculated with 1% of overnight grown culture and it was incubated at 37°C and 200 rpm till the OD reaches 0.3 – 0.5 at 595 nm. One ml of the aliquot was taken in an eppendorf and centrifuged at 10,000 rpm for 5 min., and the pellet was dissolved in 50 µl of the laemmli buffer (0.06M Tris-HCl, pH6.8, 10% glycerol, 5% β-Mercaptoethanol and 2% SDS) and stored at –20°C for later use as uninduced culture. The remaining culture was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside), to a final concentration of 1mM. The culture was allowed to grow at 37°C and the aliquot was collected at 3 and 6 h., after incubation and the pellet was dissolved in Laemmli buffer (Laemmli, 1970) and stored at –20°C.

SDS-PAGE analysis

The glass plates were cleaned thoroughly with water followed by alcohol and placed in a casting unit. The separating gel mixture was poured in the glass plate assembly and overlaid with a film of isopropanol to avoid trapping of any air bubble and uniform gel surface. After polymerization, the alcohol layer was removed, rinsed with water and

blotted with filter paper. The stacking gel mixture was then poured and the comb was placed on top of the sandwich. After polymerization, the comb was carefully removed and wells were rinsed with electrode buffer before loading the samples. The protein samples were mixed with 1× loading dye, boiled for 2 min and then loaded. Electrophoresis was carried in a 1× buffer (Tris 0.025M; Glycine 0.192M and SDS 0.1%) at constant current of 15 mA till the dye front reached the separating gel. The current supply was then increased to a constant supply of 30 mA till the blue dye reaches the bottom. After the electrophoretic run, the gel unit was dismantled and gel was fixed using a fixative (Methanol; acetic acid; water, 4:1:5 ratio) and stained in a Coomassie Brilliant Blue solution for 12 hrs. The gel was destained until the background became colorless and the gel was documented using Alphaimager (AlphaInnotech, USA).

Results and Discussion

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. They are used for the complete amplification of the ‘CP’ gene in the BBTV infected hill banana plants. The PCR products were resolved on 0.8% agarose gel and an expected size of 513 bp was observed. 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 and named as pHBScp. The ligated product was then transformed into the *E. coli* strain (DH5 α) and was selected on ampicillin containing medium. The recombinant colonies were identified by colony PCR using the same ‘CP’ gene specific primer and one of positive clones was selected and further confirmed by releasing the cloned fragment using restriction

enzymes, KpnI and HindIII. The selected three recombinant clones were sequenced (Fig. 3). Sequencing has resulted in a length of 513 bp and the homology search of the sequence using NCBI blast (Altschul *et al.*, 1997) 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.

Southern hybridization analysis

To confirm the virus infection and virus load at two different (juvenile stage and vegetative stage) stages, the five plant samples each from infected and healthy banana samples were subjected to southern blot hybridization and virus infection was confirmed using CP gene specific probe of BBTV. DNA of the banana samples both infected and healthy was restricted with restriction endonucleases, HindIII, the products were electrophoretically resolved, transferred to nylon membrane and hybridized against radiolabeled probes prepared using the BBTV CP gene. Hybridization of the blot with BBTV CP gene probe showed that no signal was observed in case of healthy plant control indicating free from BBTV infection, whereas the infected sample showed strong hybridization signal indicates the presence of BBTV (Fig. 4). Due to the presence of excessive phenolic compounds, they formed smeared lanes with faint bands.

Expression of BBTV coat protein in *E. coli*

The BBTV coat protein expressed (Fig. 5) in *E. coli* was subjected to SDS-PAGE analysis. The expression of BBTV coat protein was found in very low at 1-6 hour of incubation in IPTG induction and low level of expression was found at 7 and 8 hours of incubation in IPTG induction (Fig. 6).

Figure.1 Dark green patches of BBTV infected leaf sheath

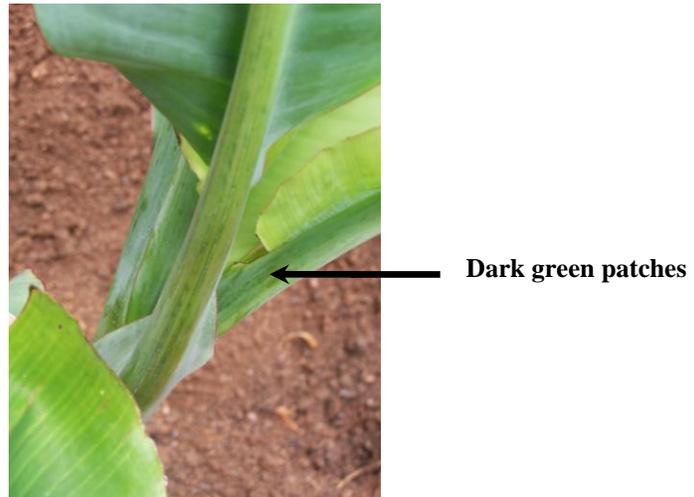


Figure.2 Field view of healthy and BBTV infected banana



Figure.3 Confirmation of Recombinant clones by Colony PCR analysis, M- 100 bp ladder, 1- Coat protein (CP) gene (513 bp), 2- Negative control (Tissue culture Plantain).

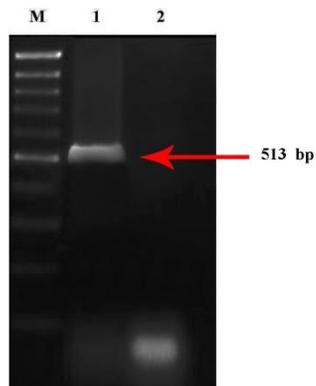


Figure.4 Southern blot analysis at Juvenile stage and Vegetative stage, PC – Positive control (Cloned Coat Protein Plasmid), NC – Negative control (Tissue culture Certified Plantain), I- BBTV infected plantain and H – Healthy plantain

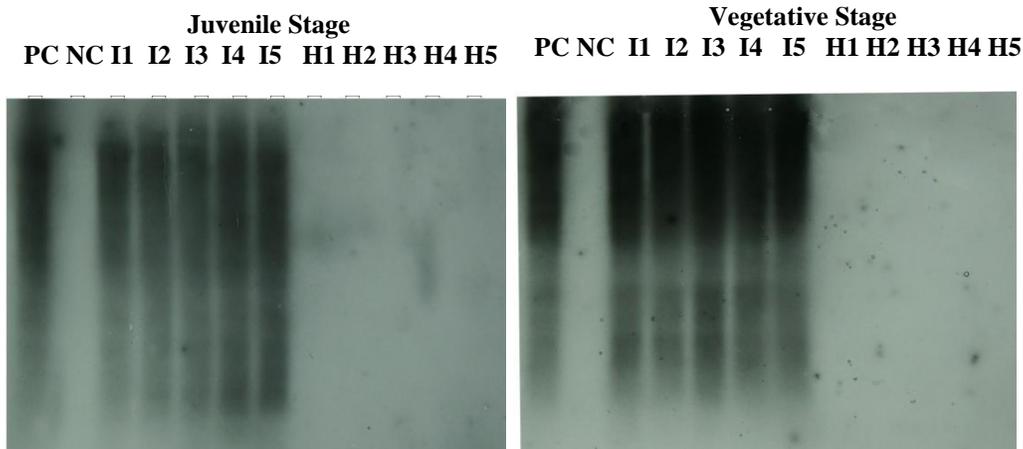


Figure.5 Cloning of pET -28a(+) vector with CP gene

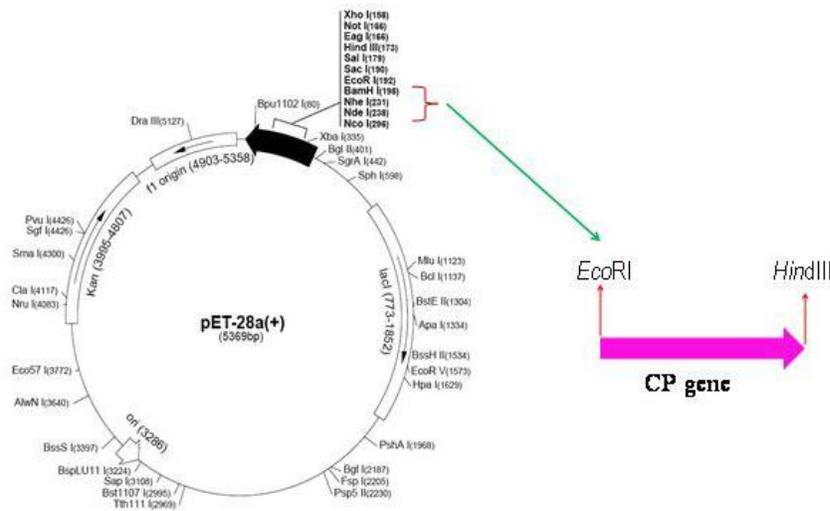


Figure.6 BBTV coat protein, M- Protein marker, C –pET 28a(+) vector (control), 3h, 6h – hours after IPTG induction

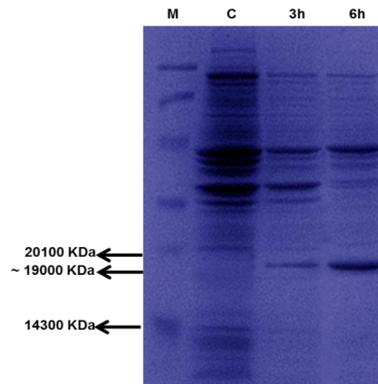


Table.1 Primer sequences for amplifying gene-specific sequences.

Primer name	Sequence	Base pairs
CP gene forward primer	5'-ATGGCTAGGTATCCGAAG-3'	18
CP gene reverse primer	5'-TCAAACATGATATGTAATTC-3'	20

Banana is one of the most important fruit crop grown in most of the southern states of India. Several banana cultivars are under cultivation in India and hill banana is one of the unique cultivar grown in the lower Paliney hills in Tamil Nadu. Hill banana cultivation is seriously affected by the disease caused by BBTv. BBTv is transmitted in a persistent manner by the banana aphid *Pentalonia nigronervosa* and causes a yellows disease in bananas, it was classified as a 'possible member' of the luteovirus group (Matthews, 1981). Further evidence supporting this classification is the reported occurrence of dsRNA in BBTv infected plants but not in corresponding healthy plants (Dale *et al.*, 1986). Selection of disease free planting material is one of the very important techniques in banana cultivation. Identification of BBTv infection in the planting material/suckers is very difficult as the symptom development is not visible at the early stage of infection. Several reports are available for the molecular detection of the plant virus in different crops using PCR and ELISA method. Immunological detection using technique such as ELISA is not effective as the virus is present in low titre in the banana plant. There is only one successful report of ELISA based detection of BBTv. Most of the recent literature showed that BBTv detection was effective using PCR method (Mansoor *et al.*, 2005). PCR based detection technique, had the advantage of amplifying the target nucleic acid present even at very low level and it had become an attractive technique for the diagnosis of many plant viral diseases (Henson and French, 1993; Xie and Hu, 1994; Hafner *et al.*, 1997a, Chandrasekar *et al.*, 2011).

The CP coded by the DNA-3 component of the BBTv has been used for virus detection because of its important role in viral particles packing and disease establishment and has been consistently associated with BBTD in Asian countries group. An attempt was made to develop BBTv detection protocol utilizing the CP gene specific primers.

In order to, PCR amplification using CP specific primer gave amplicon of expected size (513 bp) with DNA isolated from infected hill banana plant. A common problem in the use of PCR for diagnostic purposes is, that it needs a good quality DNA. Plant species which contains high phenolic shows false-negatives i.e., even though the target viral DNA is present but still fail to amplify because of the quality of the DNA taken for PCR analysis. So the amplification of a PCR by using the internal control primer will possibly demonstrates the absence of PCR inhibitors in banana DNA samples and can provide some level of confidence on a diagnostic PCR reaction that is negative for the presence of BBTv. BBTv detection method using internal control primer and BBTv Rep gene primer was reported by Mansoor *et al.*, (2005). Potential problems in PCR include false negatives due to reaction failure or false positives due to contamination (Chandrasekar *et al.*, 2011). False negatives were often revealed in Southern blot technique.

Harding *et al.*, (1991) used the Southern blot technique to detect the presence of BBTv in banana tissue and in aphids. In the present study, to confirm the presence of BBTv (DNA-3) CP gene, Southern hybridization

analysis was performed. Southern hybridization analyses against the radiolabeled probes prepared from BBTV CP gene. The experiment performed with samples collected at two banana growth stages viz., juvenile and vegetative stage. From each stage five samples of infected and healthy samples were collected. The result indicates that all found in both stages. Normally banana plants has more mucilage and phenolic compound, in Biotic stress condition the phenolic, abscisic acid, ethylene, jasmonic acid, and salicylic acid levels are increased, here the BBTV plays major role of stress factor, where the phenolic levels are more elevated. Due to the presence of excessive phenolic compounds, they formed smeared lanes with faint bands. Infected plants had similar level of hybridization signal in both stages, in the healthy plants no hybridization signal was seen. Harding *et al.*, (1991) have showed the virus like particles are associated with banana bunchy top disease contains small single-stranded DNA. They have used southern blot method to analyze the expression of virus like particles with extracts from sucrose gradient-purified BBTV, healthy banana plant and partially purified BBTV. Their studies have also shown that the expression was seen only at the infected plants not in the healthy plants. Coat protein expressed in *E. coli* showed very low level of coat protein accumulation as revealed by SDS-PAGE analysis. The low level of coat protein accumulation may be due to the small size of the protein not being stably present in the *E. coli* cells. In contrast, Harding *et al.*, (1991) and Wanitchakorn *et al.*, (2000) used the SDS-PAGE they have got expression at a molecular weight of 20100KDa.

Research involving human participants and/or animals

It does not involving in the any Human participants and Animals. It is confirmation of

banana bunchy top virus (BBTV) infection study. I confirm that all the research meets the ethical guidelines, including adherence to the legal requirements of the study country.

Abbreviations

BBTV- Banana Bunchy Top Virus

CP – coat protein

PCR- Polymerase chain reaction

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