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Cloning and Expression of Banana Bunchy Top Virus (BBTV) Coat Protein Gene in E. coli

B.S.Shilpa[1], Narayan Moger[2], Akhela Vani[2], Chidananda Rabinal[1], RV Hedge[3] and A S Byadagi[4]

[1]Institute of Agricultural biotechnology, College of Agriculture, UAS, Dharawad-580005,Karnataka, India.

[2] Indian Institute of Horticulture Research, Hessaragatta, Bangaluru, Karnataka, India-56

[3]Department of Horticulture, College of Agriculture, UAS, Dharawad-580005, Karnataka, India.

[4]Department of Plant Pathology, College of Agriculture, UAS, Dharawad-580005, Karnataka, India.

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ABSTRACT

Banana bunchy top virus (BBTV) is a destructive pathogen in banana cultivating areas worldwide. As the virus is located in the phloem tissue and its purification is difficult and gives low yield. Therefore, in the present study BBTV coat protein was bacterially cloned and expressed using recombinant DNA approach. The BBTV infected banana leaf samples were collected around Uttar Kannada district of Karnataka. Further, the total nucleic acid was extracted using dellaporta method. A 531bp PCR product containing coat protein coding region of BBTV was amplified using BBTVCPF and BBTVCPR primers and the amplified product was cloned into the pTZ57R/T and further sub-cloned in to the pQE30. After transformation in to JM 109 and M15 cells the clones were confirmed through PCR and sequencing. Amplification with expected size of 531bp and 100% homology with other isolates showed integrity of the clone. Further, the coat protein appeared to be expressed at 3hr after induction with 1mM IPTG. The expressed protein was analyzed through sodium-dodecyl sulphate-Poly acrylamide gel electrophoresis (SDS-PAGE). A band of 21kDa on the gel confirmed that coat protein was really fused to the His-tag. Further, 10mg/liter of the coat protein were purified using His-tag purification kit (Genei).

Introduction

Banana is one of the oldest tropical fruits cultivated by man from prehistoric times with great socio-economic significance. Globally banana (*Musa* spp.) ranks fourth after rice, wheat and maize in consumption. Banana is grown in about 121 countries (Anon., 2011) providing a major source of carbohydrates for over 400 million people in tropical countries and is one of the most important fruit crops in the world in terms of production and consumption (Bairu *et al*., 2006).

Due to intensification of banana cultivation in the recent years, an increased number of pests and diseases causing significant reduction in yield have been reported. Among these viral diseases have been causing considerable damage to banana production due to their systemic invasion and common spread through vegetative propagated planting materials and their subsequent transmission in nature by insect vectors (Geering, 2007). Among the viruses, Banana bunchy top virus (BBTV) is the most economically important virus infecting banana in the world over (Dale, 1987). BBTV is also the major viral disease that affects the banana cultivation in India (Anandhi *et al*., 2007).

BBTV is a member of the genus Babu vius of the family Nanoviridae (Vetten et al.,2005) and specifically transmitted by the black banana aphid *Pentalonia nigronervosa coquerelli* (Magee, 1953;Hu et al.,1996) in a persistent, circulative and non-propagative manner (Bressan and watanabe, 2011). The BBTV genome consists of atleast six components of monocistronic, single standed DNA of about 1.1kb in size encapsulated separately to form spherical virions of 20 nm in diameter with icosahedral symmetry (Burns et al., 1995;Vetten et al., 2005). As the virus is located in the phloem tissue and its purification is difficult and gives low yield.

The aim of this work is to express BBTV coat protein in *E.coli* and to get purified recombinant protein by His-tag purification using recombinant DNA technology.

Materials and methods

Sample collection

Naturally BBTV infected and healthy banana leaf samples were collected from Uttara Kannada district of Karnataka. The samples were made into small discs and grounded into powder using liquid nitrogen. Further, the samples were stored in -20^0 C for future use.

Total nucleic acid extraction using Dellaporta method

Total nucleic acid was extracted from healthy and infected banana tissues according to the method mentioned in Dellaporta technique (Dellaporta *et al*., 1983).

Primer designing

The Coding sequence of BBTV-CP gene was retrieved from NCBI database. Using vector NTI software gene specific primers were designed. Restriction sites *BamH*I and *Hind*III were introduced at 5' end of forward and reverse primers respectively and three spacer nucleotides were added before restriction sites. The designed primers were synthesized at Sigma-Aldrich chemicals Pvt. Ltd., Bangalore. The designed sequence of primers is as follows.

BBTV (forward) - 5'
TACGGATCCATGGCTAGGTATCCGAAG 3'

Tele:
E-mail address: bsshilpashilpa@gmail.com

BBTV (reverse) - 5'
TAGAAGCTTTCAAACATGATATGTAATTC 3'

PCR amplification of BBTV-CP gene from isolated total nucleic acid

The thermal profile used for amplification of BBTV-CP was Initial denaturation at 94.0 ^{0}C for 5mins for one cycle. Denaturationat 94.0 ^{0}C for1.0 min, annealing at 59.0 ^{0}C for 1.0 min. Extension at 72.0 ^{0}C for45secthese steps for 30 cycles and final extension at 72.0 ^{0}C for 10min

Cloning of BBTV- CP

The PCR-amplified BBTV/CP DNA was molecularly cloned in *E. coli* into pTZ57R/T. All ligation reactions were incubated overnight at 16°C and contained equi-molar amounts of PCR product (insert) and vector DNAs. Ligations were conducted at 300mM Tris-Cl, pH 7.8, 100 mM MgCl2, 100 mM DTT, 10 mM ATP and 3 units/ µl T4DNA Ligase. To each 50 µl competent *E.coli* DH5α cells, 2 µl of ligation mixture was added and mixed gently by tapping. Tubes were incubated on ice for 30 minutes, then heat shocked at 42°C for 45-50 seconds and placed again on ice for 2 minutes. To each transformation mix, 950 µl of Luria broth medium (tryptone 10g/lit, yeast extract 5g/lit, sodium chloride 5g/lit, agar 18g/lit) was added and the tubes were incubated at 37 °C for 1.5 hr with 150 rpm shaking. From each transformation tube, 100 µl were plated on LB plates containing ampicillin (50 µg/ml). Plates were inverted and placed in a 37°C incubator overnight. Positive clones were identified by blue and white colony assay and restriction.

Sequencing and sequence based analysis

The restriction confirmed clone (pBSNM13) was sent for sequencing to Chromous Biotech Pvt. Ltd, Bengaluru, India. Sequencing was done by using universal M13 forward and reverse primers. Sequences were processed to remove the vector contamination by using BioEdit software. Further, the homology search was done with BLAST algorithm available at http://www.ncbi.nih.gov

Expression of BBTV/CP fusion proteins in *E. coli*

The BBTV/CP gene was restriction digested from pTZ57r/t using restriction enzymes *Bam*H1 and *Hind*III. The gel purified 531 bp amplicon was ligated into pQE-30 (Qiagen). Protein expression in pQE-30 was controlled through a double lac operator system and is induced by addition of 1 mM IPTG. Recombinant plasmids containing the inserted PCR fragment were validated by restriction analysis and also validated by PCR to confirm the integrity of the cloned DNA. In this way the viral DNA sequence was inserted in frame downstream of the 6xHist-protein.

His-tag fusion protein purification

Fusion proteins were expressed and the recombinant protein was purified using 6X His-tag purification kit (Genei cat# KT65) purified protein was subjected to SDS polyacrylamide gel electrophoresis. The BBTV/CP gene was sequenced to confirm the integrity of the insert.

Protein quantification using Lowry's method

The Lowry's method was used to determine the concentration of soluble protein in solution. The principle is that the aromatic amino acids like L-phenylalanine, L-tyrosine and tryptophan present in proteins react with phosphomolybdo-phosphotungstate reagent (FCR) to produce a blue colored complex with max at 660nm.The depth of the color produced is proportional to the aromatic amino acids present in sample. Further, the concentration of protein was calculated using standard curve readings for protein (BSA) concentrations.

Results and Discussion

The most commonly used diagnostic tool for BBTV is enzyme-linked immunosorbent assay, which is dependent on the availability of highly specific antibody to differentiate the viruses (Abdelkadar *et al.*, 2004). Purification of BBTV in sufficient quantities is difficult as BBTV is phloem-limited virus found in very low concentration in virus-infected plants. This is the major obstacle in the production of specific antibodies for its detection (Wanichakorn *et al.*, 1997). By using the recombinant technology expressing the CP gene in *E. coli* provides the ability to obtain large amount of protein which can be used for range of studies (Ramesh, 2010). Production of high titre BBTV specific antibody has been tried in Australia (Wanichakorn *et al.*, 1997) and Egypt (Abdelkader *et al.*, 2004). Initially, total nucleic acid was extracted from collected leaf samples of infected and healthy banana plants .

PCR based cloning

A set of oligonucleotide primers BBTV (forward) - 5' TACGGATCCATGGCTAGGTATCCGAAG3' and BBTV (reverse) - 5' TAGAAGCTTTCAAACATGATATGTAATTC 3' were designed by using vector NTI software to the CDS region of BBTV-DNA3 for PCR amplification of full ORF of BBTV-DNA3 (coat protein gene) used in the present study. A 531 bp product from BBTV DNA which included the full ORF sequence of BBTV DNA 3 was amplified using the specific primers of BBTV CP with restriction sites Fig:1. Amplification of nearly similar length of fragment was reported by Hu *et al.* (1996) and El-Din *et al.* (2005). However, El-Din *et al.* (2005) and Abdelkader *et al.* (2004) reported the major ORF of BBTV-DNA3 component (BBTV-CP CDS) was 500 nucleotide in size. The reason for getting 20 bp extra amplification was because of addition of restriction site and spacer nucleotides in the forward and reverse primers of BBTV. Further, the BBTV-CP gene was cloned in to T/A cloning vector pTZ57R/T. The bulk PCR was done and then sharp amplicon (BBTV-CP gene) of the expected size (531bp) was eluted from the gel, which was then cloned into pTZ57R/T and named as pBSNM13 (Fig. 2). This construct was transferred into *E. coli* DH5α and transformants were confirmed by colony PCR (Fig.3) and restriction digestion analysis using *BamH*I and *Hind*III endonucleases giving rise to linear fragment of 513bp insert and 2.8 kb vector back bone (Fig.4)

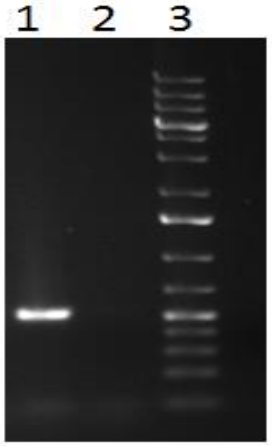


Fig1. PCR confirmation of infected sample
Lane 1, Infected sample
Lane 2, Healthy sample
Lane 3, 1Kbp ladder

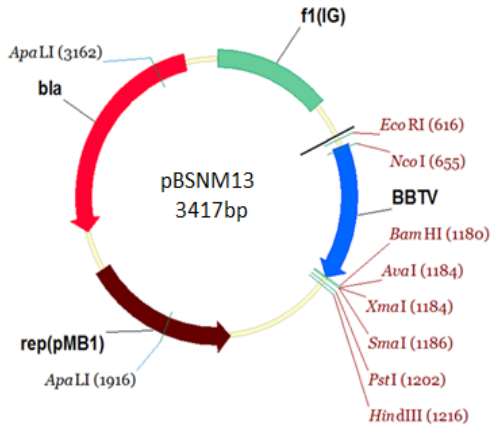


Fig 2. Construct map of pBSNM13 containing full length BBTV-CP in pTZ57R/T.

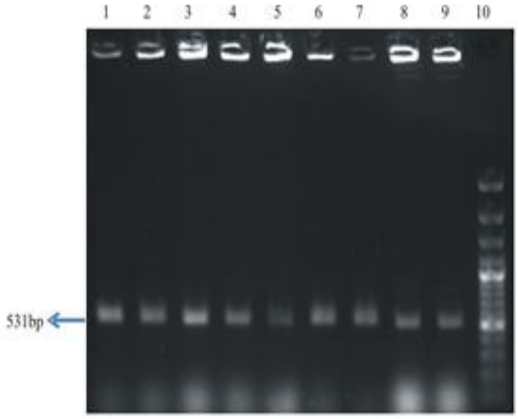


Fig3. Colony PCR confirmation of BBTV – CP gene in pTZ57R/T.

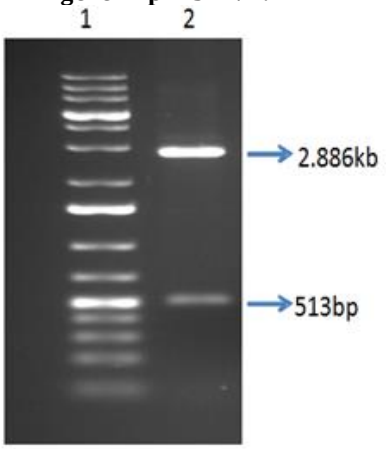


Fig4. Restriction confirmation of BBTV-CP clones in pTZ57R/T with *BamHI* and *HindIII*.

The construct pBSNM13 containing BBTV-CP gene was sequenced by employing M13 universal primers. The complete nucleotide sequence of BBTV CP gene is shown in Fig. 5. The available sequence information from cloned fragments was analyzed using BLAST algorithm available at http://www.ncbi.nih.gov.

1 atg gct agg gta tcc gaa gaa ata cca tca aga aga ggc ggg ttg 45
1 Met Ala Arg Val Ser Glu Glu Ile Pro Ser Arg Arg Gly Gly Leu 15

46 ggc gcc gga agt atg gca gca agg cgg caa cga gcc acg act act 90
16 Gly Ala Gly Ser Met Ala Ala Arg Arg Gln Arg Ala Thr Thr Thr 30

91 cgt cgt tag ggt caa tat tgg ttc ctg aaa aca ccg tca agg tat 135
31 Arg Arg End Gly Gln Tyr Trp Phe Leu Lys Thr Pro Ser Arg Tyr 45

136 ttc gga ttg agc cta ctg ata aaa cat tac cca gat att tta tct 180
46 Phe Gly Leu Ser Leu Leu Ile Lys His Tyr Pro Asp Ile Leu Ser 60

181 gga aaa tgt tta tgc ttc ttg tgt gta agg tga agc ccg gaa gaa 225
61 Gly Lys Cys Leu Cys Phe Leu Cys Val Arg End Ser Pro Glu Glu 75

226 tac ttc att ggg cta tga tca aga gtt ctt ggg aaa tca acc agc 270
76 Tyr Phe Ile Gly Leu End Ser Arg Val Leu Gly Lys Ser Thr Ser 90

271 cga caa cct gtc tgg aag ccc cag gtt tat tta tta aac ctg aac 315
91 Arg Gln Pro Val Trp Lys Pro Gln Val Tyr Leu Leu Asn Leu Asn 105

316 ata gcc atc tgg tta aac tgg tat gta gtg ggg aac ttg aag cag 360
106 Ile Ala Ile Trp Leu Asn Trp Tyr Val Val Gly Asn Leu Lys Gln 120

361 gag tcg caa cag gga cat cag atg ttg aat gtc ttt tga gga aga 405
121 Glu Ser Gln Gln Gly His Gln Met Leu Asn Val Phe End Gly Arg 135

406 caa ccg tgt tga gga aga atg taa cag agg tgg att att tat att 450
136 Gln Pro Cys End Gly Arg Met End Gln Arg Trp Ile Ile Tyr Ile 150

451 tgg cat tct att gta gtt ctg gag taa gta taa act acc aga aca 495
151 Trp His Ser Ile Val Val Leu Glu End Val End Thr Thr Arg Thr 165

496 gaa tta cat atc atg ttt 513
166 Glu Leu His Ile Met Phe

Fig 5. Complete amino acid and nucleotide sequence of BBTV-CP from pBSNM13.

The nucleotide sequence of CP of BBTV isolate had 99-100 per cent homology with CP gene of known isolates of other BBTV isolates from different parts of the India. Abdelkadar *et al.* (2004) found that sequence of ORF of Egypt BBTV isolate was 100 per cent homology with BBTV CP of Australian isolate (Burns *et al.*, 1995).

Sub cloning of BBTV- CP gene in prokaryotic expression vector

BBTV CP gene was amplified in the cloning vector pTZ57R/T and then cloned into *Bam*HI and *Hind*III sites of the expression vector pQE30 and transformed into *E. coli* JM109 cell which is a maintenance host for plasmid. The transformants with BBTV- CP were picked on Luria agar plates containing Amp_{100} and Nal_{10} selection pressure. The recombinants obtained were again confirmed by colony PCR (Fig:6) and by restriction digestion with *Bam*HI and *Hind*III. It was observed that linearization of vector and release of fragment (513bp) on agarose gel electrophoresis (Fig:7).

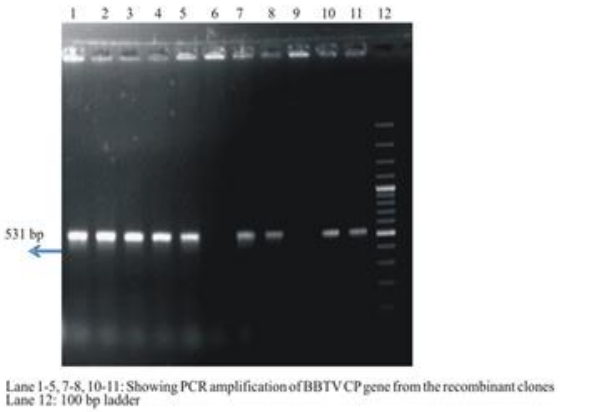


Fig 6. Colony PCR confirmation OF BBTV-CP gene in pQE30.

Later, the plasmid from the confirmed recombinant clones in *E. coli* JM109 cells was transformed in to expression host *E. coli* M15 (pREP4) for pQE30. The recombinant clone pBSNMCP30 (Fig:8)

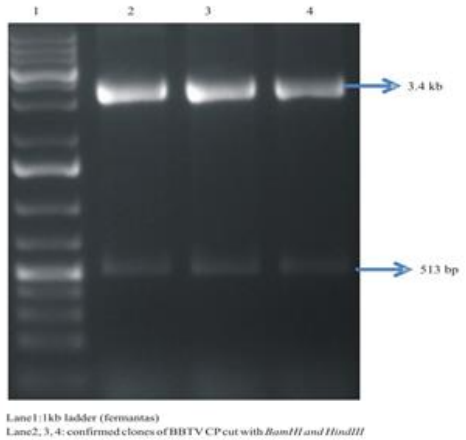


Fig 7. Restriction confirmation of BBTV-CP clones in *E.coli M15* (pREP4).

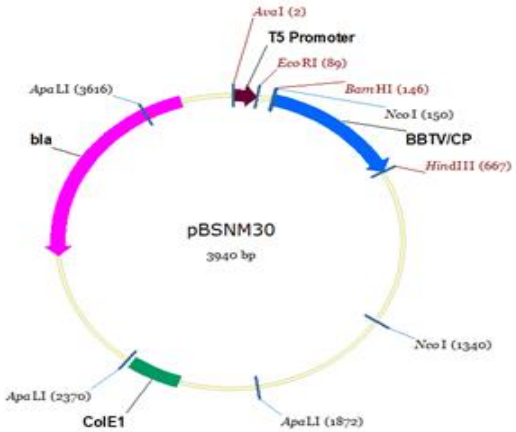
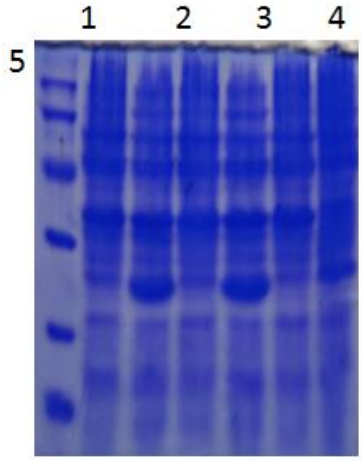


Fig8. Construct map of pBSNM30 containing full length BBTV-CP in pQE30.

Expression studies in *E. coli*

After checking the precision of the clones, the recombinant clone pBSNMCP30 was subjected to expression. The cells were induced by using 1mM IPTG for different time period (3hr, 5hr and 7hr) with 1hr time gap. The expressed protein was analyzed on SDS-PAGE. A protein band of approximately 21kDa was observed (Fig:9). It was found that at 1mM of IPTG concentration at 37 ^{0}C for 3 hr showed better expression of CP. However, Abdelkadar *et al.* (2004) also used 1mM IPTG for 3hr for induction of expression of BBTV coat protein in pQE-30 expression vector whereas Wanitchakorn *et al.* (1997) used 0.3M IPTG at 37^{0}C for induction of expression of BBTV CP in pMAL-c2 expression vector. Thus, it is suggested that individual standardization is required based on the vector and the promoter in the vector. Long time induction resulted in degradation of coat protein.



Lane1.GeNei Protein Molecular weight marker
Lane2,4,6. uninduced clone
Lane3,5,7. induced clone

Fig 9. SDS-PAGE analysis of BBTV-CP clones in *E.coli* M15 cells.

Subsequently the expressed protein was purified using genei His-tag purification kit and the purified protein was analysed on 12 per cent SDS-PAGE. After purification, single band of 21kDa was observed (Fig:10). Further the final yield of expressed protein was estimated using Lowry's method of protein estimation.Three hours of induction period yield corresponded to nearly 10mg/lit of culture.

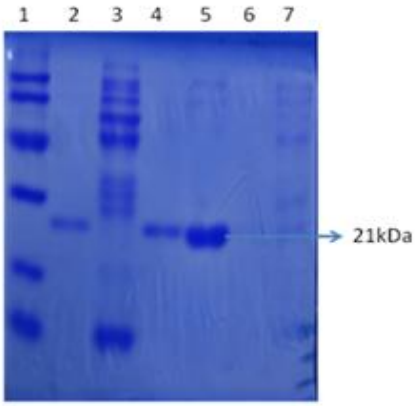


Fig 10. SDS-PAGE showing a purified fusion protein after.

Lane 1: GeNei Protein Molecular weight marker (PPMWM116632)
Lane 2: Elute1 Lane 3: Cell lysate
Lane 4: Elute2 Lane 5: Elute3
Lane 6: Elute4 Lane7:wash3

Conclusion

Intact virus purification is more challenging in case of banana bunchy top virus. As the phage display technology needs purified antigen to produce scFv monoclonal antibodies recombinant DNA approach was used in this study to get purified antigen.

Further, this purified BBTV coat protein can be used as an antigen to develop monoclonal antibodies using phage display technology.

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