Molecular cloning and expression of recombinant coat protein gene of banana bunchy top virus in *E. coli* and its use in the production of diagnostic antibodies

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ABSTRACT

Banana bunchy top virus is a multicomponent virus, comprising of at least six integral components, with a circular single-stranded DNA genome. In this study, specific rabbit polyclonal antibodies against bacterially expressed coat protein of Banana Bunchy Top virus (BBTV, genus Nanovirus) were produced using a recombinant DNA approach. The BBTV capsid protein (CP) gene located on component 3 was cloned in an expression vector pQE-30 (Qiagen). Expression of the CP with an N-terminal hexahistidine tag in Escherichia coli M15 cells was induced by adding isopropyl-3-D-1-thiogalactoside (IPTG) to a final concentration of 1 mM. About 13 mg of bacterially expressed CP was purified from 1 litre of bacterial liquid culture using a Ni-NTA resin column (Qiagen). The expressed CP which migrated as a protein of approximately 21 kDa in sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) was identified by its strong reaction with polyclonal antibodies produced against BBTV purified particles (BBTV-AS) and Ni-NTA-AP conjugate in Western blots. Expressed and purified CP (SDS-PAGE 21 kDa band) was injected into a white rabbit, using two subcutaneous and three intramuscular injections at weekly intervals. The antiserum produced was evaluated for BBTV and FBNYV detection in Western blot and dot blot immunoassays (DBIA). The antiserum raised against the expressed CP (BBTV-AS$_{6xHis}$) gave strong BBTV-specific DBIA reactions and very weak background reactions with non-infected tissues, similar to those produced by polyclonal antibodies raised against BBTV purified virion (BBTV-AS). Furthermore, (BBTV-AS$_{6xHis}$) polyclonal antibody reacted specifically with both the denatured recombinant protein and the disrupted BBTV virus particle as well as FBNYV purified particles in DBIA. These results showed that the (BBTV-AS$_{6xHis}$) polyclonal antibody is useful for the detection of BBTV in infected tissues by dot blot tests.

Key words: *BBTV-6x-His-tagged-fusion protein, prokaryotic expression vector, pQE-30, Polyclonal antisera, nickel affinity chromatography, western blotting, recombinant protein, PCR, DBIA.*

INTRODUCTION

Banana (*Musa* spp.) are grown in about 121 countries (F.A.O., 2001) providing a major source of carbohydrates for over 400 million people in tropical countries (Swennen *et al.*, 1995). Over 102 million metric tonnes are produced yearly (F.A.O., 2001). Banana also provides a major source of income for smallholders (Nweke *et al.*, 1988).

Banana bunchy top virus (BBTV) is the most economically important virus infecting bananas (Dale, 1987). BBTV is an important pathogen of bananas *(Musa* spp.) in Australia, the South Pacific, parts of Asia, Africa and Egypt (Fahmy, 1927; Sadik and Gad El-Karim, 1997). BBTV is a member of the Family *Nanoviridae*, which includes subterranean clover stunt virus (SCSV; Boevink *et al.*, 1995), faba bean necrotic yellows virus (FBNYV; Katul *et al.*, 1998), coconut foliar decay virus (CFDV; Rohde *et al.*, 1990) and milk vetch dwarf virus (MDV; Sano *et al.*, 1998). Banana bunchy top virus (BBTV) is a single-stranded DNA virus which infects members of the genus *Mus*a. BBTV has small isometric virions of 18-20 nm, and is persistently transmitted by the aphid *Pentalonia nigronervosa* (Harding *et al.*, 1991). Based on cytopathology and transmission characteristics, BBTV is considered phloem-limited (Sadik *et al.*, 1997). BBTV has a multi-component genome consisting of at least six ssDNA components (Burns *et al.*, 1995). Each DNA component has two conserved regions, the stem-loop common region (CR-SL) and the major common region (CR-M) Burns *et al.*, 1995). The CR-SL forms a stem-loop structure whose nucleotide sequence is similar to that of geminiviruses. The CR-M consists of 66 .92 nucleotides and is the binding site for DNA primers associated with complementary strand synthesis. Previous studies (Harding *et al.*, 1993; Wanitchakorn *et al.*, 1997, 2000) have shown that components 1, 3, 4 and 6 encode the replication-associated protein, coat protein, intercellular transport protein, retinoblastoma-binding protein and nuclear shuttle protein, respectively. The *Nanoviridae* members, including BBTV, have a number of characteristics or predicted characteristics in common with geminiviruses including replicative strategy and movement into and out of the nucleus as well as intercellular movement (Burns *et al.*, 1995; Hafner *et al.*, 1997b). It is likely, therefore, that the genes and the gene functions encoded by geminiviruses will have counterparts in BBTV. Enzyme-linked immunosorbent assay (ELISA) tests with monoclonal antibodies (Mabs) are commonly used for the accurate detection of BBTV (Wu and Su, 1990; Thomas and Dietzgen, 1991; Geering and Thomas, 1996). ELISA is convenient but limited in detecting sensitivity, especially with very low concentrations of BBTV. Therefore, a more sensitive assay based on polymerase chain reaction (PCR) has been developed (Xie and Hu, 1994; Hafner *et al.*, 1997b). In addition to the detection of BBTV, the PCR-based assay had used for the identification of various BBTV strains.

The aim of this work is to produce polyclonal antibodies by using recombinant coat protein expression technology to detect BBTV in infected banana plants and its comparison to conventional polyclonal antibodies raised against purified virus preparation and its use as diagnostic reagents for reliable laboratory based ELISA test.

MATERIALS AND METHODS

Purification of BBTV

BBTV was purified according to the technique described by Abdel-Salam *et al.* (2004).

Sources of virus isolates and antisera

Virus isolates used in this study were those reported by Abdel-Salam *et al.* (2004). BBTV isolates were from Giza (BBTV-G) and EL-Menya (BBTV- M) governorates. FBNYV isolate was obtained from Assiut governorate and designated as FBNYV-ASS. BBTV antisera raised for the whole virus *BBTV-AS,* or for disrupted virus protein *BBTV-*

AS/CP, used in this study were those reported by Abdel-Salam *et al.* (2004).

Extraction of total nucleic acids

Total nucleic acids were extracted from banana plants infected with BBTV as described by Karan *et al.* (1994). Approximately 1 g of leaf midrib tissue was ground to a powder in liquid nitrogen. 0.2 g of powdered tissue was mixed with 500 µl 1%SDS followed by extraction with phenol/chloroform (1:1) and then chloroform. The aqueous phase was collected and the nucleic acids were precipitated with absolute ethanol, washed with 70% ethanol and resuspended in 50 µl water.

Amplification of BBTV DNA-3 component

Oligonucleotide primers for PCR were derived from the published sequences of BBTV DNA-3 (Burns *et al,* 1995). The nucleotide sequences of the two primers used in the PCR amplification were as following: Forward primer (BTVCPF): 5'-GCTAGGTATCCGAAGAAATC-3' and reverse primer (BTVCPR):

5'-TCAAACATGATATGTAATTC-3'.

All PCR reactions (50 µl) were heated to 94°C for 3 min; then subjected to 35 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min; and finally 1 cycle of 72°C for 10 min.

Cloning and sequencing of BBTV DNA-3

The PCR-amplified BBTV/CP DNA was molecularly cloned in *E. coli* into pGEM-T-Easy. 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 $MgCl_2$, 100 mM DTT, 10 mM ATP and 3 Weiss units/ µl T4 DNA Ligase. To each 50 µl competent JM109 cells, 2 µl of pGEM-T-Easy ligation reactions were 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 room temperature SOC medium were 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 restriction endonucleases, and DNA sequence analysis. Cloned DNA was sequenced using cycle sequencing kit (Promega) as recommended by the manufacturer. Reaction products were analysed by electrophoresis in a 6 % (w/v) polyacrylamide gel containing 7 M urea. Gels were fixed, and stained using silver staining kit (Promega). The primer used for sequencing was universal sequencing primer (M13 forward).

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

The BBTV/CP gene was amplified by PCR from pGEM-T-Easy clone as a DNA template in the PCR reaction. Two restriction enzymes recognition sites *BamH1* and *HindIII* (underlined) were introduced into the forward (FQE30) and reverse (RQE30) primers respectively

(5′-TACGGATCCGCTAGGTATCCGAAGAAATC-3′).
(5′-TAGAAGCTTTCAAACATGATATGTAATTC-3′).

The amplified fragment 500 bp was purified from agarose gel by Geneclean (GFX-Gel Extraction Kit, Pharmacia). It was then cut by *BamHI/HindIII* restriction endonuclease enzymes and then ligated into *BamHI/HindIII* digested pQE-30 expression vector (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 selected 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. Fusion proteins were expressed and purified as described in the QIA expressionist manual and also described by (Wahle *et al.*, 1999). Fractions were analyzed by electrophoresis in 12% polyacrylamide gels as described by Laemmli, (1970). The *BBTV/CP* gene was sequenced to confirm the integrity of the insert.

SDS-PAGE of BBTV/CP

A total of 20 µl of each cell extract was fractionated by SDS–PAGE on a 12 % separating gel and electrotransferred onto a nitrocellulose membrane. After washing the membranes, immunoblotting with anti-CP serum was used to determine the exact molecular weight of the BBTV CP. Immunoblotting technique described by Harlow and Lane, (1988) was performed using anti-BBTV serum and/or Ni–NTA-AP conjugate (Qiagen) to identify the 6xHis-CP. The protein was purified from *E. coli* cells (centrifuged from a 250 ml culture) using Ni–NTA resin (Qiagen) under denaturing conditions (8 M urea) as described in the 'QIAexpressionist' (Qiagen). The suspensions were sonicated three times (30 s each with a 1 min interval) prior to stirring at room temperature. The relative efficiency of CP extraction by this method was examined by SDS–PAGE of supernatants (cleared lysates) after resuspension of an aliquot in Laemmli loading buffer and boiling for 10 min. For elution of 6xHis-CP under denaturing conditions, the elution buffer containing 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 4.5 was used.

Western blot analysis of BBTV fusion protein

Induction of the synthesis of the BBTV fusion protein was performed as recommended by QIA expressionist manual, (Qiagen). Briefly, exponential phase cultures of *E. coli* M15 cells containing the appropriate recombinant clones were induced by addition of 1 mM IPTG. Following 3 hr incubation period at 37 °C with vigorous shaking, cells were harvested by centrifugation (3 min at 13.000 rpm, 4 °C). The bacterial cells were directly resuspended in denaturing polyacrylamide gel electrophoresis (PAGE) loading buffer (125 mM Tris-Cl pH 6.8; 10 % SDS; 25 % B-mercaptoethanol) and lysed by boiling for 5 minutes. Protein extracts were finally clarified by a 10 min centrifugation at 13.000 rpm and either used directly or stored at -20 °C until used. Protein samples were separated on 12 % PAGE gels and transferred by semi-dry blotting on nitrocellulose membranes. The membrane was blocked in TBS (20 mM Tris-HCl pH 7.4, and 150 mM NaCl containing 3% BSA) for one hour at room temperature. The blot was incubated with the Ni-NTA alkaline phosphatase conjugate diluted (1:1000) and/or BBTV antibody (diluted 1:1000) (raised against the virion protein and/or expressed coat protein) in a blocking buffer for one hour at room temperature and washed three times for 15 minutes each in TBST containing 0.05 % tween 20. The colour reaction was started by incubating the membrane in 5-bromo-4-chloro-3-indolyl phosphate- nitrobluetetrazolium (BCIP/NBT) substrate for alkaline Phosphatase or stained with Napthol/Fast Red complex until the protein bands have reached the desired intensity. The reaction was stopped by washing the membrane in deionized water for several minutes. The membrane was air dried on a filter paper and photographed.

Purification of 6xHis-tagged BBTV fusion proteins

Purification of 6xHis-tagged BBTV fusion protein was performed under denaturing conditions by nickel- nitrilotriacetic acid (Ni-NTA) batch chromatography. The cell pellet from 200 ml of induced bacterial culture was resuspended in 4 ml of lysis buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 8.0). The cells were lysed and the cell lysate was centrifuged for 10 min at 15,000 xg to remove the cellular debris. To the supernatant, 1 ml of 50% Ni-NTA slurry was added and the lysate-resin mixture was loaded into an empty column. Endogenous proteins with histidine residues were washed out of the matrix twice by 4 ml of washing buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 6.3). The 6xHis-tagged fusion protein was eluted 4 times with 0.5 ml of elution buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 4.5). The 6xHis-tagged fusion protein was dissociated from the Ni-NTA resin at the acidic pH.

Preparation of antiserum for BBTV expressed coat protein

Denatured BBTV fusion coat protein (CP) was injected into rabbits for raising BBTV/CP antiserum (BBTV-AS_{6xHis}). Five weekly injections, each with 0.5 mg protein were used for rabbit immunization. The first and second injections were administrated subcutaneously. Complete and incomplete Freund's adjuvant were used in the first and second injections, respectively. The three remaining injections were emulsified with incomplete adjuvant, and administrated intramuscularly. After one week from the last injection, the rabbit was bled weekly for eight consecutive weeks. The *E.* coli-expressed CP was compared with native BBTV protein (in the form of purified BBTV virion protein) by SDS–PAGE, immunoblotting and western blotting. The *E. coli*-expressed CP antiserum (BBTV-AS_{6xHis}) was used to detect BBTV in infected tissues and in purified BBTV antigens using dot blotting immunoassay (DBIA) and western blotting techniques as described by Abdel-Salam *et al.* (2004).

Sensitivity determination

The sensitivities of the polyclonal antibodies for BBTV detection by antigen-coated dot-blot immunoassay were determined by sap dilution end-point of a BBTV-infected banana plants. BBTV- infected and healthy banana leaf tissue of 1 g, each, were ground in 4 ml of BBTV extraction buffer (PBS, 2 % (w/v) PVP and 1 % (w/v) Na_2SO_3). The 1:5 diluted leaf extracts were further diluted in a three-fold series from 1:15 to1: 3645, and tested for the presence of BBTV as described by Banttarri and Godwin (1985). The clarification step was as follows. To 0.5 ml of each serially diluted healthy and infected leaf sap extracts, 3 drops of Triton-X 100 were added and the mixture vortexed for 2 min. It was followed by the addition of equal volume (0.5 ml) of chloroform and the mixture emulsified by stirring in the cold room. The emulsified mixture was separated into two phases by centrifugation at 10000 x*g* for 10 min. The aqueous phase was decanted and referred to as chloroform-clarified sap. The chloroform-clarified and the crude sap extracts of the different serial dilutions were dotted on nitrocellulose membranes at 5 µl per sample and then allowed to air-dry. Polyclonal antibody raised against the expressed BBTV/CP (BBTV-AS_{6xHis}) at 1:1000 dilutions in PBS was used as detecting antibody.

RESULTS AND DISCUSSION

Banana bunchy top virus (BBTV), a complex circular single stranded DNA virus with multiple genomic components, is a destructive pathogen in banana-cultivating

areas worldwide. The symptoms induced by BBTV are similar to those caused by abiotic factors and other vascular diseases. This lack of unambiguous symptomatology necessitates production of reagents for the reliable diagnosis of BBTV infections.

The most commonly used diagnostic tool for BBTV is enzyme-linked immunosorbent assay, which is dependent on the availability of highly specific antisera to differentiate the viruses. While some BBTV can be purified easily, others remain difficult to extract in sufficient quantities for antiserum production. To test the ability of producing antiserum to BBTV by expressing the coat protein in *E.coli* and then immunizing the animal, we attempted to produce antibodies against the coat protein gene, one of the BBTV conservative regions by CP-based immunization, thereby eliminating the need for isolation and purification of the virus particles. The results of our study showed that antibodies produced against BBTV by CP-immunization were similar in sensitivity and specificity with the conventional polyclonal antibodies obtained by complete virion immunization. This information will be useful to scientists who are interested in producing diagnostic antibodies for plant viruses that are difficult to purify from infected plant tissues. PCR can also be used to rapidly and sensitively detect plant viruses. Indeed, the PCR method has proven to be more sensitive than ELISA and dot-blot hybridization (Hu *et al.*, 1996). Also Banana bunchy top virus (BBTV) can be effectively detected in its host and vector as well as from BBTV-infected *in vitro* cultures by (PCR) as described by (Shmloul *et al* 1999; Ismail *et al.*, 2003). The set of primers used in this study (Fig. 2a) have successfully amplified the CP/ORF flanking region of BBTV-Giza isolate from banana plants and thus can be used to improve the detection of BBTV in its host.

The correct 500 bp PCR product containing CP coding region of BBTV was amplified using BTVCPF & BTVCPR primers (Fig. 2a) and the amplified product was cloned into the plasmid pGEM-T-Easy. The transformation efficiency was calculated as the number of bacterial colonies appearing on selective plates after transformation divided by μg plasmids used in transformation. The efficiency of transformation was found to be $1.3x10^6$ colonies / μg. PCR performed on the presumptive recombinant DNA plasmids identified several colonies containing an insert with the expected size (500 bp) (Fig. 1). Fidelity of the clones was confirmed by sequencing. Comparing this very conservative region of the CP-Giza isolate with other known sequences we found 100% homology with the isolate of BBTV/CP (NC_003473-Burns *et al*, 1995), Fig. (3). At first, the PCR reaction was performed on the cloned BBTV/CP gene using specially designed primers FQE30 & RQE30 containing the extra part of sequences compatible to the distinct *BamHI/HindIII* recombination sites in the expression vector pQE-30 in which the BBTV/CP protein was expressed as a fusion protein with 6xHis tag in *E.coli* M15 cells. In the BBTV/CP construct, pQE30-CP; i.e. the coat protein gene was cloned and expressed under phage T5 promotor and the inducible *lac* operator (Fig. 2b). The expression of CP was induced by adding 1 mM IPTG. M15 cells *E.coli* strain carrying the *lac* mutation was used for expression of BBTV/CP. The presence the 6xHis-tag at the N-terminus of the produced protein made the screening and verification of CP expression easier by using Ni-NTA-AP conjugate antibody in western blotting assays (Fig. 5).

The CP protein was expressed in the bacteria after fractional analysis. The dynamics of recombinant CP expression in *E. coli* showed that the highest amount of

expressed CP appeared 3 hr after induction with 1 mM IPTG, suggesting that long time induction resulted in degradation of the fusion protein. A band of 21 kDa appeared, confirming that CP was really fused to the His-tag (Fig. 5A).

After optimization of the cultivation (time, temperature, and inductor concentration), the cells were harvested and the presence of the desired protein was proved by immunoblot using commercial available polyclonal antibodies against 6x-His-tag proteins (Fig. 5B). Ni-NTA batch chromatography was used to purify the 6xHis-tagged fusion coat protein from *E. coli* as shown in Fig. (4). The final yield of the expressed CP was estimated as 13 mg/l culture, which corresponds to approximately 6.2 % of the total proteins (~250 mg/l) of *E. coli*. SDS-PAGE and western blot analysis of the purified recombinant CP (Fig. 5) revealed the presence of a major protein band with an estimated molecular mass of 21 KDa. The different aliquots (total cell lysate, flow-through (unbound), wash, and eluted CP) collected during Ni-NTA batch chromatography were subjected to SDS-PAGE and western blot analysis as presented in Figure (5 A and B). In sap dilution end-point determination, the comparative BBTV-infected leaf sap dilution end-points of antigen in dot-blot immunoassay were 1:1215 when the nitrocellulose membrane was probed with AS_{6xHis} diluted 1:1000 in blocking buffer, when crude sap was used. However, when chloroform clarified sap was used; the sap dilution end- point was 1:135 (Fig. 6). The eluted 6xHis-tagged fusion CPs (O), (N), (E1), and (E2) bound to the nitrocellulose membrane showed a high positive reaction with the polyclonal antibody raised against purified virion of BBTV in dot-blot-immunoassays (Fig. 7). Surprisingly, this antibody also showed cross reactivity with FBNYV in the same assay. The Qiagen expression and purification method is much simpler and faster, and is surely the method of choice for high-throughput for prokaryotic expression, but unfortunately is more expensive. In all tested clones, the recombinant protein remained in insoluble fraction, which is favorable for its purification. Expressed fusion proteins were easily purified from bacterial lysates by affinity chromatography using Ni-NTA resin. The 6xHis-tag does not interfere in the structure or function of the purified protein as demonstrated for a wide variety of proteins, including enzymes, transcription factors, and vaccines. The 6xHis-tag system has been used successfully in many applications such as molecular immunology (Stüber *et al.*, 1990), the production of vaccines (Kaslow and Shiloach, 1994) and studies involving protein-protein (Holzinger *et al.*, 1996) and DNA-protein interactions (Hochuli *et al.*, 1988).

Cultures of *E. coli* M15 cells transformed with pQE30-BBTV/CP were induced with 1 mM IPTG, and fusion proteins were all expressed to high levels in soluble form in *E. coli*. Expression level was evaluated by immunological methods and analysed by PAGE followed by western blotting. The results showed that all BBTV antisera raised against BBTV complete virion protein and/or expressed BBTV coat protein recognized bands of the expected size 20 to 21 KDa (6xHis=0.84 KDa; BBTV/CP=20 KDa; Burns,*et al*, 1995). Our results indicated that both the antisera BBTV-AS and BBTV-AS_{6xHis} can detect the two isolates of BBTV (G and M) successfully in DBIA as shown in Fig. (8) as well as FBNYV and BBTV purified virions in western blotting as shown in Fig. (9). Moreover, the BBTV-AS produced against disrupted BBTV particles can recognize the denatured CP in western blot experiments and *vise versa*. So that, part of the epitopes is probably non-conformational

one and corresponds to a short sequence of amino acids. Although this may not be a general characteristic, peptide scanning experiments performed with other nanovirus have shown that a significant proportion of the epitopes recognized on nanovirus CPs are probably linear (continuous) epitopes (Abdel-Salam *et al.*, 2004). BBTV/CP produced by this method was used for preparation of polyclonal antibodies, can be used not only for research of antigenic properties of this virus, but also for purposes of routine diagnosis. The results showed that the BBTV-CP polyclonal antibody (BBTV-AS_{6xHis}) is useful for the detection of BBTV and FBNYV in infected tissues by dot blot -immunoassays.

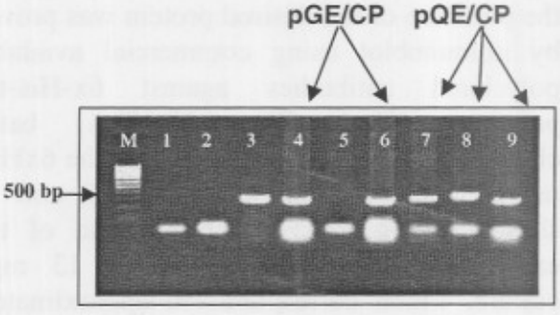

Fig. (1): 1% agarose gel electrophoresis showing the PCR amplification products of the DNA component 3 (CP) of BBTV. Lane 1: PCR negative control (no DNA template). Lane 2: Healthy banana. Lane 3: PCR product of amplified coat protein gene from infected banana tissues (500bp) using the primer pair BTVCPF & BTVCPR. Lanes 4 & 6: PCR products amplified from the clone (pGE/CP) pGEM-T vector containing BBTV/CP using the same primers. Lanes 7, 8, and 9: PCR products amplified from the clone (pQE/CP) pQE-30 vector expressing BBTV/CP using FQE30 & RQE30 primers. Lane M: Molecular weight DNA Marker, Roche diagnostics.

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228
ATG                BTVCPF
TATC CGAAGAAATC CATCAAGAAG
GTTG GGCGCCGGAA GTATGGCAGC
AAGGCGGCAA CGAGCCACGA CTACTCGTCG
TCAGGGTCAA TATTGGTTCC TGAAAACACC
GTCAAGGTAT TTCGGATTGA GCCTACTGAT
AAAACATTAC CCAGATATTT TATCTGGAAA
ATGTTTATGC TTCTTGTGTG CAAGGTGAAG
CCCGGAAGAA TACTTCATTG GGCTATGATC
AAGAGTTCTT GGGAAATCAA CCAGCCGACA
ACCTGTCTGG AAGCCCCAGG TTTATTTATT
AAACCTGAAC ACAGCCATCT GGTTAAACTG
GTATGTAGTG GGGAACTTGA AGCAGGAGTC
GCAACAGGAA CATCAGATGT TGAATGTCTT
TTGAGGAAGA CAACCGTGTT GAGGAAGAAT
GTAACAGAGG TGGATTATTT ATATTTGGCA
TTCTATTGTA GTTCTGGAGT AAGTATAAAC
TACCAGAACA GAATTACATA TCATGTTTGA 740
TATGTTTATG          BTVCPR
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Fig. (2a): Nucleotide sequences of the amplified coat protein gene of BBTV. The boxes show the forward and reverse primers designed for PCR amplification. The initiation (ATG) and stop (TAT) codons are underlined. Numbering refers to the nucleotide sequences of BBTV coat protein gene.

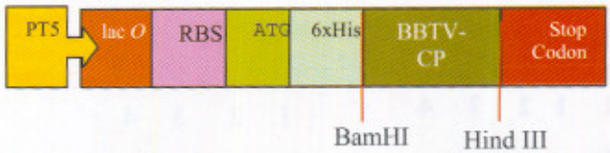

Fig. (2b): Schematic diagram showing the cloning strategy of BBTV/CP into an expression vector (pQE-30). The ends of the amplified PCR product of the BBTV coat protein gene and the pQE-30 vector were prepared by restriction digestions with BamHI and Hind III restriction enzymes to facilitate cloning. The BBTV/CP gene was cloned in the correct reading frame as a fusion protein fragment with 6xHist-tag without the need of the initiation and termination codons of the inserted fragment. PT5: T5 promoter, lac O: lac operator, RBS: ribosome binding site, ATG: start codon, 6xHis: His tag sequence.

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BBTV/CP(Eg)           MARYPKKSIKKRRVGRRKYGSKAATSHDYSSSGSILVPENTVKVFRIEPTDKTLPRY
BBTV/CP (NC_003473)   MARYPKKSIKKRRVGRRKYGSKAATSHDYSSSGSILVPENTVKVFRIEPTDKTLPRY

BBTV/CP(Eg)           FIWKMFMLLVCKVKPGRILHWAMIKSSWEINQPTTCLEAPGLFIKPEHSHLVKLVCS
BBTV/CP (NC_003473)   FIWKMFMLLVCKVKPGRILHWAMIKSSWEINQPTTCLEAPGLFIKPEHSHLVKLVCS

BBTV/CP(Eg)           GELEAGVATGTSDVECLLRKTTVLRKNVTEVDYLYLAFYCSSGVSINYQNRITYHV*
BBTV/CP (NC_003473)   GELEAGVATGTSDVECLLRKTTVLRKNVTEVDYLYLAFYCSSGVSINYQNRITYHV*
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Fig. (3): Amino acid sequence alignment of the coat protein gene of BBTV- Accession NO. NC_003473 (Burns,et al, 1995) and amino acids that have been translated from the amplified coat protein gene of the Egyptian isolate (Eg) using DNAMAN2 program. Codon length = 170 residues, 100 % aligned.

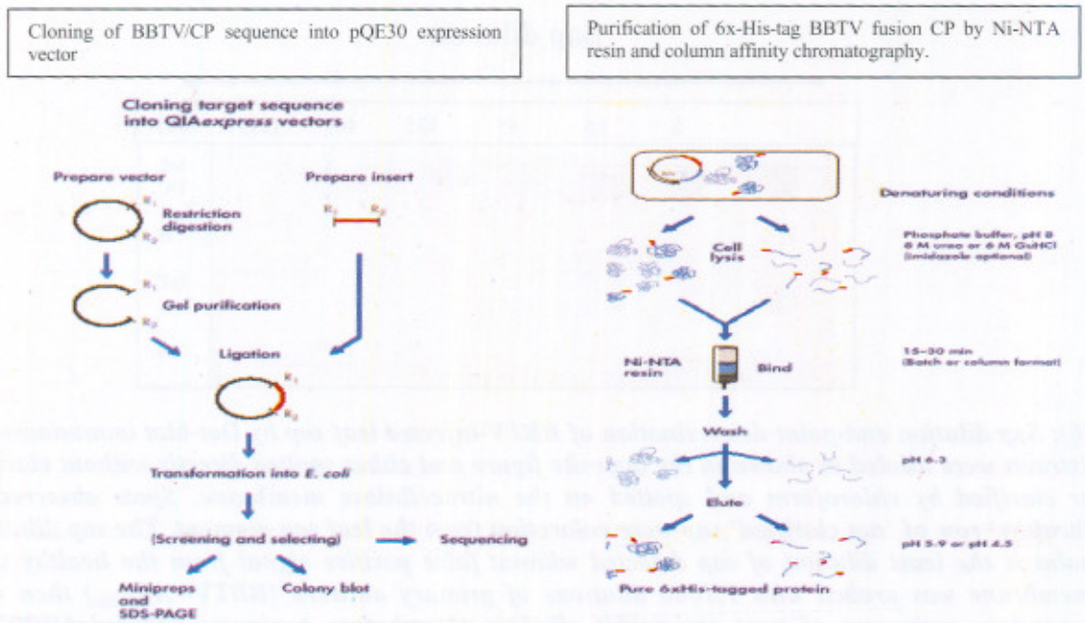

Fig. (4): Schematic presentation showing the expression and purification strategies used for BBTV/CP-6x-His-tag fusion protein from pQE30. The protocols were described by the QIAexpressionist hand book for high level expression and purification of 6xHis-tagged proteins (Qiagen).

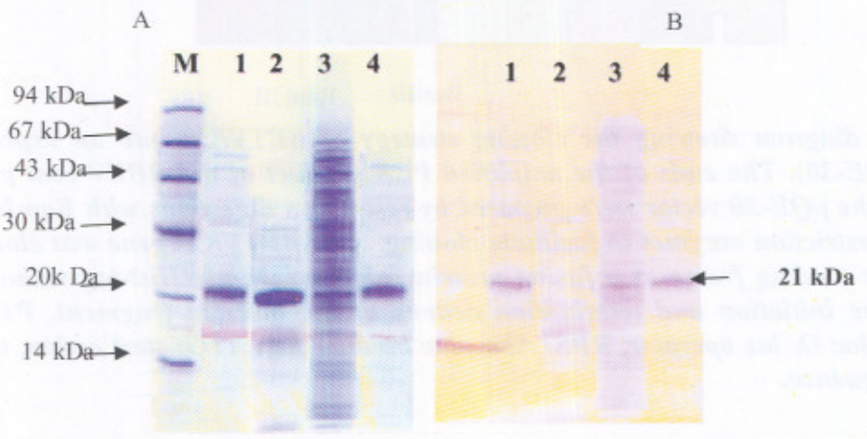

Fig. (5A): 12% SDS-PAGE showing a purified fusion coat protein of BBTV of M_r 21 KDa (lane 1) (first elution), Lane (2): BBTV purified virion proteins which appeared as one major protein band of M_r 20 KDa, Lane (3) Total protein (cleared lysate) of the expressed BBTV coat protein from E.coli, Lane (4): second elution of BBTV fusion coat protein. (B) Western blotting showing the biding of 6x-His tag fusion coat protein of BBTV with 1-1000 dilution of Ni-NTA alakaline phosphatase conjugate (Qiagen). Only proteins which in fusion with 6x-Histidine strongly reacted with Ni-NTA-AP conjugate as shown in Lanes 1, 3 and 4. The colour was developed after 5-15 minutes using BCIP-NBT substrate.

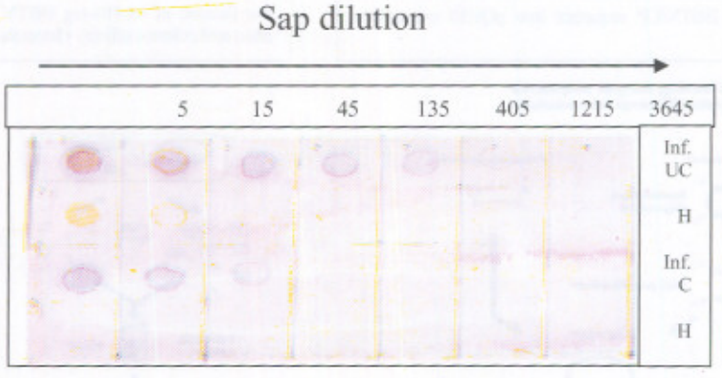

Fig. (6): Sap dilution end-point determination of BBTV-infected leaf sap by Dot-blot immunoassay. Sap extracts were diluted as shown in the opposite figure and either spotted directly without clarification or clarified by chloroform and spotted on the nitrocellulose membrane. Spots observed in the 'healthy' row of 'not clarified' sap were coloration from the leaf sap pigment. The sap dilution end-point is the least dilution of sap detected without false positive signal from the healthy sap. The membrane was probed with 1:1000 dilutions of primary antisera (BBTV- AS_{6xHis}) then with the secondary antiserum of goat anti-rabbit alkaline phosphatase conjugate (diluted 1/5000). The membrane was developed using NBT/BCIP substrate. Inf UC: Infected, un-clarified. Inf. C: Infected, Clarified. H: Healthy banana.

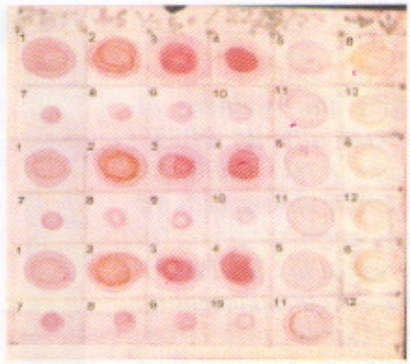

Fig. (7): DBIA showing the reactions of BBTV-AS with :1- BBTV (Menya), 2- BBTV (Giza), 3- Purified BBTV (Giza), 4- Purified FBNYV (Menya), 5- Healthy banana (leaf), 6- Healthy banana (fruit), 7- BBTV/CP (O), 8- BBTV/CP(N), 9- BBTV/CP(E1), 10- BBTV/CP(E2), 11- Healthy banana (leaf), 12- Healthy banana (fruit). Each blot was repeated three times.

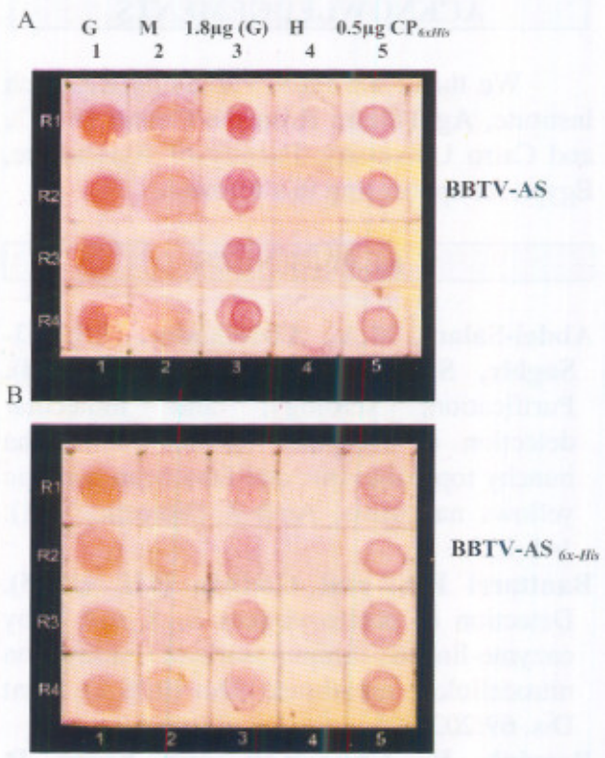

Fig. (8): Dot blot immuno-binding assay (DBIA) showing the reactions of two antisera for BBTV raised against (A) BBTV-AS obtained from purified BBTV-Giza isolate and (B) BBTV-AS$_{6xHis}$. Antigens in (A) and (B) are as follow: 1, 2; BBTV-Giza and BBTV-Menya isolates, respectively from leaf tissues of infected banana; 3, 1.8 µg of purified BBTV-Giza isolate: 4, healthy sap (control) from banana; 5, 0.5 µg of BBTV-CP expressed in E.coli. A dilution of 1/1000 was used for the two tested primary antisera (BBTV-AS and BBTV-AS$_{6xHis}$); while a dilution of 1/5000 was used for the secondary antiserum-conjugated alkaline phosphatase. Blots were stained with Napthol /Fast Red complex. Each treatment was repeated four times (R1 – R4).

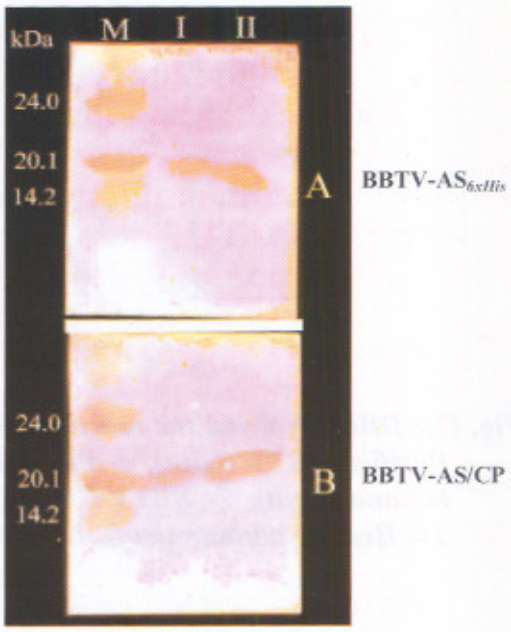

Fig. (9): Western blotting showing the reactions of BBTV (I) and FBNYV (II) with A, BBTV-AS$_{6xHis}$ (diluted 1/1000) and B, the BBTV-AS prepared from disrupted BBTV virions (diluted 1/1000). Disrupted virions performed by SDS and mercaptoethanol were run in SDS-PAGE, and then transferred onto PVDF membranes. The membranes were blocked with PVA, treated first with the primary antisera then with the secondary antiserum of goat antirabbit alkaline phosphatase conjugate (diluted 1/5000). Blots were finally stained with Fast Red/Naphthol complex. M: Molecular weight markers in kDa.

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الملخص العربي

الكلونة الجزيئية و التعبير الجيني للغلاف البروتيني لفيروس تورد القمة في الموز داخل بكتيريا القولون و استخدامه في إنتاج أجسام مناعية تشخيصية

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يعتبر فيروس تورد القمة (BBTV) واحدا من أهم الفيروسات المدمرة لزراعات الموز فى مصر والعالم . ويتكون فيروس تورد القمة من عديد من الجزيئات و يحتوى على الأقل على ستة جزيئات متكاملة و خيط مفرد مستدير من الحمض النووي الدنا .

في هذه الدراسة- تم إنتاج أجسام مناعية عديدة التخصص عن طريق استخدام تقنية الدنا المطعم والتعبير الجيني للغلاف البروتيني لفيروس تورد القمة داخل بكتيريا القولون حيث تم كلونة جين الغلاف البروتيني لفيروس تورد القمة في الموز والذي يقع على الجزىء رقم 3 من الدنا والتحامه مع ناقل التعبير الجينى و المعروف باسم pQE-30. وتمت عملية التعبير الجينى للغلاف البروتيني المدمج مع الطرف الأميني لنتابع الهستدين في الخلايا البكتيرية من النوع M15 بعد إضافة تركيز 1 mM من المستحث IPTG. و قد تم الحصول على تركيز عالى من البروتين المنقى يصل إلى حوالي 13 mg من اللتر الواحد من البكتيريا المستحثة وذلك باستخدام عمود النينتا أثناء التنقية.

وباستخدام تكنيك التفريد الكهربيى الرأسي وجيل البولىاكريلاميد المحتوى على *SDS* تم تحديد الوزن الجزيئى للغلاف البروتينى المنقى من البكتيريا (21 kDa) عن طريق استخدام أجسام مناعية متخصصة منتجة ضد جزيئات فيروس تورد القمة الكامل والمنقى و أيضا باستخدام جزيئات النيكل المرتبطة و المعلمة بأنزيم الفوسفاتيز القلوي (Ni-NTA-AP) و ذلك في تكنيك *Western blot*. و قد تم حقن هذا البروتين المنقى والمنتج في البكتيريا بتقنية التعبير الجيني في الأرنب بالحقن تحت الجلد مرتين وفى العضل ثلاث مرات أسبوعيا .

وقد تم اختبار الأجسام المناعية المنتجة بهذه الطريقة في الكشف عن فيروس تورد القمة في الموز وفيروس موت واصفرار نبات الفول باستخدام تكنيك *Western blot* و تكنيك *DBIA*. وقد أعطت الأجسام المناعية المنتجة بطريقة التعبير البروتيني تفاعلا ايجابيا قويا مع الفيروس في الأنسجة المصابة مع وجود بعض التلوين في الخلفية مع النباتات غير المصابة مثل تفاعلات تلك الأجسام المناعية المنتجة ضد الفيروس الكامل المنقى . وعلاوة على ذلك فان الأجسام المناعية المنتجة بطريقة التعبير البروتيني تفاعلت نوعيا مع كل من البروتين المهندس وراثيا و بروتين الفيروس المتفكك و أيضا مع جزيئات فيروس موت واصفرار نبات الفول في اختبار *DBIA* . و بالتالي فان النتائج أثبتت أن الأجسام المناعية المنتجة بطريقة التعبير البروتيني مفيدة اقتصاديا في الكشف عن فيروس تورد القمة في نباتات الموز المصابة باستخدام تكنيك dot blotting .