MOLECULAR CHARACTERIZATION AND PHYLOGENETIC STUDIES OF COMPONENT SIX OF BANANA BUNCHY TOP BABUVIRUS (BBTV DNA-6)

HANAN A. NOUR EL-DIN¹, S. KHALIL² AND SHIMAA M ABDELSALAM³

Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC). Giza, 12619, Egypt.

1. Department of microbial molecular biology, Agric. Genet. Eng. Res. Ins. (AGERI), (ARC), Egypt
2. Department of plant genetic transformation, Agric. Genet. Eng. Res. Ins. (AGERI), (ARC), Egypt
3. Department of Botany, Women’s Collage for Arts, Science and Education, Ain Shams University, Cairo, Egypt

Banana and plantations (Musa sp.) are the most fourth most important global food crop after rice, wheat and maize (Pietersen and Thomas, 2001). Banana plantations are subjected to various natural climates but diseases; in particular, viral diseases constitute a major setback to this crop worldwide. Among viral infections, Banana Bunchy Top Virus (BBTV) is the most serious virus affecting banana in Egypt as well as the world (Dale, 1987; Othman et al., 1996; Harish et al., 2008) and is responsible for the massive reduction in banana crop yield. The symptoms of BBTD are yellowing, dark green streaks on midrib, petioles, extending down into pseudostem, dark green dots and dashes along the minor leaf veins, stunted growth with bunchy top appearance (Magee, 1940; Vetten et al., 2005). BBTV belong to genus Babuvirus and family Nanoviridae with isometric particles of 18-20 nm in diameter. It is naturally transmitted, in persistence manner, by banana aphid Pentalonia nigronervosa (Hu et al., 1996). BBTV is identified as a phloem-limited virus used to occur in low concentration in banana-infected plants (Harding et al., 1991 and 2000). The virus has a single coat protein with molecular weight (Mr.) of about 20-21kDa (Dietzgen and Thomas, 1991; Harding et al., 1991; Sadik et al., 1999).

The viron consists of at least six components of circular ssDNA each component of the BBTV genome contains one large (monocistronic) transcriptional active open reading frame (ORF) except BBTV DNA 1 contains two ORFs one master replication protein (Rep) ORF (Hafner et al., 1997) and a small ORF internal to major ORF (Beetham et al., 1997). Additionally, two conserved region: stem-loop common region (CR-SL) and major common region (CR-M), potential TATA box at 3’ of the stem-loop and polyadenylation signal (Burns et al., 1995; Beetham et al., 1997 and 1999).

The nucleotide sequence of the component six of Australian isolate BBTV

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has 1089 nt. (Burns et al., 1995). The encoded protein of BBTV DNA-6 is a nuclear shuttle protein and is preferentially targeted to the nucleus when expressed alone however in the presence of the movement protein it targeted to the cell periphery (Wanitchakorn et al., 2000a).

The aim of this study is to amplify, clone and sequence the full length of BBTV DNA-6 and compare it with other published sequences of the corresponding sequence.

MATERIALS AND METHODS

Virus source

Infected banana plants were collected from El-Behera Governorate (BBTV-B) in Egypt. The presence of the virus was confirmed serologically by using indirect enzyme-linked immunosorbant assay (I-ELISA) as described by Wu and Su (1990).

Genomic DNA extraction from infected banana plants

The total DNA was extracted from the infected banana midrib by using DNeasy plant mini kit (cat. No. 69104 from QIAGEN).

PCR amplification of the full length of BBTV DNA-6

Two oligonucleotide primers were designed to amplify the full length of BBTV DNA-6, according to the published data of Karan et al. (1997) as the following:

Forward primer (FDNA-6)

5’GACAAGAAGGATCCTATTAGTAA CAGCAACAA3’

Reverse primer (RDNA-6):

5’ATAAGCTTTCTAACCCTTGCT CT3’

The nucleotides in bold represent BamH1 site in the forward primer and Hind III in the reverse primer.

One hundred nanograms of the extracted DNA, from infected banana plants, were used as a template in the PCR reaction. PCR was conducted in a volume of 50 µl containing 1X reaction buffer with MgSO₄, 200 µM each dNTPs, 0.4 pmoles of each primers and 0.025 units of pfu DNA polymerase (Promega). PCR amplification was performed in a Perkin-Elmer thermocycler (Gene Amp PCR system 2400) for 35 cycles after initial denaturation for 2 min at 95°C. Each cycle consisted of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min. The primer extension was extended to 7 min at 72°C in the final cycle. The PCR amplified products were detected by agarose gel electrophoresis (1.2%) at 80 volts for 1 hour (hr) (Sambrook et al., 1989).

Cloning the full length of the Egyptian BBTV DNA-6

The PCR fragment was tailed with adenine (A) to be ligated in pJET1.2 blunt vector (2974 bp) (Fermentas) by incubat-
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Sequencing and computer analysis of the full length of BBTV DNA-6

A recombinant clone (pJETH) was sequenced using Genetic Analyzer at AGERI, Giza, Egypt, ARC (ABI prism 310, version 3.4, Semi Adaptive, version 3.2). DNA sequence was translated to protein using EditSeq of DNA star software program. DNA and protein sequences of the BBTV-B (Egy6m) full length were aligned and compared using MegaAlign of DNA star program with eleven isolates.

RESULTS AND DISCUSSION

PCR amplification of the full length of BBTV DNA-6

By using two specific primers for the full length of BBTV DNA-6, 1.1 Kbp fragment was detected in the BBTV-infected banana plants. This fragment was absent in the healthy plant as a negative control (Fig. 1). Burns et al. (1995) and Islam et al. (2010) reported that the full length of BBTV DNA-6 was 1089 bp.

Recently, the molecular detection of plant viruses is now available and the PCR was successfully used for detecting several plant viruses (Wetzel et al., 1992; Hadidi et al., 1993) and BBTV (Xie et al., 1994; Harding et al., 2000). Therefore, the early detection of BBTV particular in the symptomless banana plant materials produced via tissue culture and the BBTV in banana samples showed the characteristic symptoms of BBTV and also in banana aphid could be detected.

Harding et al. (2000) and Abdel-Hamid et al. (2003) reported the importance of BBTV detection as early step for virus control, in particular, in the plants produce via tissue culture as well as symptomless plants.

Cloning the Egyptian BBTV DNA-6 full length

The purified A-tailed PCR product, which represent the full length of Egyptian BBTV DNA-6 was cloned into pJET1.2/blunt vector to creat the plasmid pJETH. After mini-preparation, the purified plasmid DNA was subjected to double digestion with BamHI and HindIII enzymes as a confirmatory test. A segment of about 1.1 Kbp was released from all recombinant plasmids (Fig. 2). The success of cloning process was confirmed by using molecular techniques as reported by Wanitchakorn et al. (1997).
Sequencing and computer analysis of Egyptian BBTV DNA-6 full length

The sequence analysis of the amplified product revealed that the full length of BBTV DNA-6 (Fig. 3). It contains a sequence of 61 nt sequence which represented the stem loop common region (CR-SL) in which a stem loop structure consists of 31 nt (from position 1-31) as show in Fig. (4), Burns et al. (1995) deduced that this sequence was a highly conserved in all BBTV components and contained the pentanucleotide sequence TACCC which has been shown to be the site for initiation of viral strand DNA synthesis.

The second common region, which is located at 5’ of the (CR-SL), is called the major common region (CR-M) at the position (801-891) and it consists of 91 nt. In this region, from nts 4 to 19 and 21 to 36, an almost complete 16 nt of direct repeat (ATACAAc/gACg/aCTATa/gA) were observed. Further a 15 nts GC-rich sequence (average of 86.6% G+C) was located from nts 77 to 91, this result came in harmony with Burns et al. (1995). The presence of the GC-rich region in the CR-M make it believed to resemble the SP1 binding sites found in promoters of genes in animal cells and viruses (Fenoll et al., 1990). Hence, it was assumed to be the binding site for DNA primers associated with complementary strand synthesis (Hafner et al., 1995; Su et al., 2003). A potential TATA box of nine nucleotide sequence (CTATTAATA). Alberts et al. (1994) stated that this box is found in the gene promotors from 20 to 60 bp before the start of transcription, and any removing or altering in this sequence markedly reduces the promotor activity. After this box by 46 bases, one large ORF was identified which started with the translation initiation codon ATG at position 277 to 741 in the virion sense of 465 nts in size. In the Egyptian BBTV DNA-6, two potential polyadenylation signals were observed associated with the 3’ end of the major ORF. A GT-rich region contained the trinucleotide TTG was located after this polyadenylation signal. Similarly found by Burns et al. (1995) who stated that the combination of a polyadenylation signal and GT-rich region contained the trinucleotide TTG may be required for efficient termination. No other ORFs were identified in the component in either the virion or complementary sense which potentially encoded proteins greater than 10 KDa and had appropriately located potential TATA boxes and polyadenylation signals suggesting that this component encodes a single gene. Burns et al. (1995) reported that the full length of BBTV DNA-6 of the Australian isolate had one ORF that was associated with the potential TATA box and a polyadenylation signal and this assumption was confirmed by Beetham et al. (1999).

The sequenced full length of Egyptian BBTV DNA-6 was compared with the nucleotide sequences of eleven different geographical isolates of BBTV DNA-
6. From the phylogenetic analysis based on the nucleotide sequences of BBTV DNA-6 full length and the eleven isolates, the tree was divided into two clusters. The first cluster represents Asian group and included the isolate from China and Taiwan. The second cluster represents the South Pacific group including Indian, Australian, Pakistan and Egy 6m (in this study) isolates (Fig. 5).

The comparative analysis revealed the presence of high degree of homology between the studied isolate (Egy 6m) and Pakistan (isolate TJ1) and India isolates (isolates 1, 2 and 3), the percent of identities were 98.4, 97.7, 97.3% and 97.3%, respectively. However, the lowest identity was obtained in comparison with China 3 isolate percent of identity 78.9% as shown in Table (1).

Karan et al. (1994 and 1997) and Wanitchakorn et al. (2000b) reported that there were two groups of BBTV isolates, Asian and south Pacific groups based on sequence variation in DNA-1, DNA-6 and DNA-3, respectively.

As mentioned before the DNA-6 of the Egyptian isolate of BBTV was found to have one large ORF. The predicted protein of this ORF was consisted of 154 amino acids and had a M.wt of 17.39 KDa.. Burns et al. (1995) reported the same results.

The Phylogenetic analysis of BBTV DNA-6 full length, based on the amino acid sequences, of the Egyptian isolate (in this isolate) with the eleven different isolates resulted in the presence of two clusters. The first group was the Asian group which enclosed the isolates from China and Taiwan and the other group which contained the south pacific group (isolates from Australia, India and Pakistan). The Egyptian isolate was found to be grouped in the south pacific group as shown in Fig. (6).

The comparative analysis (based on the amino acid sequences) showed the highest degree of identity between the Egyptian isolate (Egy 6m) with Australia, India (isolates 4 and 5) and Pakistan TJ1 with a percent of identities 99.4%.

The results confirm the existence of two groups of BBTV isolates (Asian and South pacific groups) based on the nucleotide and amino acid sequences of full length of DNA-6 and the Egyptian isolate belong to the south pacific group.

The two oligonucleotides belonging to the BBTV component-6 which synthesized in this study can use as specific primers in BBTV detection. The PCR technique is play an important role in BBTV detection and could be introduced as an early step for virus control especially in tissue culture stage which the existence of virus used to occur in low concentration.

SUMMARY

Banana bunchy top babuvirus, the causal agent of banana bunchy top disease (BBTD), is the most serious viruses infecting banana alloover the world as well as
in Egypt. In this study, component 6 (DNA-6) of Banana bunchy top virus (BBTV) encoding a protein of 17.39 KDa was investigated. The full length of BBTV DNA-6 was amplified via polymerase chain reaction (PCR), cloned into the pJET1.2/blunt vector and sequenced. Results showed that the size of the full length of BBTV DNA-6 was 1087 nucleotides (nts) with one open reading frame (ORF) and it's molecular length was 465 nt. The full length nucleotide sequences for DNA-6 of the Egyptian isolate (Egy6m) were aligned with eleven overseas isolates (three isolates from China, two from Pakistan, one from Taiwan, four from India and one from Australia). Results showed that the isolates were clustered into two groups, the first cluster called South Pacific group that includes the Egyptian, Indian, Australian and isolates from Pakistan. The second cluster called Asian group that contained isolates from Taiwan and China. The Egyptian DNA-6 (Egy6m) was found to be grouped in the south pacific group with high similarity with isolate TJ1 from Pakistan based on the level of the nucleotide sequence and the amino acid sequence of the deduced protein.

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Table (1): Sequence distance between the studied isolate of BBTV DNA-1 (A) and the other published eleven sequences based on the nucleotide (nt) and amino acids sequences (aa).

| BBTV groups | Overseas BBTV isolates | % Identities |
|-------------|------------------------|--------------|
|             | Isolates               | Accession #  | Length | Nt  | aa  |
| Asian       | China 1                | EF470243     | 1080   | 79.9| 93.5|
|             | China 2 (Hainan 571101)| AY494787     | 1081   | 81.9| 91.6|
|             | China 3 (Haikou)       | FJ463047     | 1082   | 78.9| 91.6|
|             | Taiwan                 | DQ826396     | 1086   | 79.5| 91.7|
| South Pacific | Pakistan (TJ1)   | EF529519     | 1088   | 98.4| 99.4|
|             | Pakistan 2 (clone CP IM 14) | AM418568 | 1096   | 97.1| 97.4|
|             | Australia              | L41577       | 1089   | 95.1| 99.4|
|             | India 1                | EU190970     | 1087   | 97.7| 98.7|
|             | India 2(BBTV-LKO)      | EU391633     | 1096   | 97.3| 96.8|
|             | India 3 (Bihar)        | FJ609644     | 1090   | 97.3| 96.1|
|             | India 4 (BT-1)         | AY948438     | 1080   | 91.1| 99.4|
Fig. (1): PCR amplification of the full length of Egyptian BBTV DNA-6. M: 1Kb DNA marker. Inf: infected banana tissue and H: healthy banana tissue.

Fig. (2): Digestion of mini-prepared plasmid DNA from recombinant plasmid with BamH1 and Hind III. M: 1 kb DNA marker, 1: non-recombinant plasmid. 2, 3 and 4: recombinant plasmids.
| Sequence                | Description                                      |
|------------------------|--------------------------------------------------|
| AGCACGGGGG ACTATTATTA CCCCCCGTGC TCGGGACGGG | 40 |
| ACATGACGTC AGCAAGGATT ATAATGGGCT TTTTATTAGC | 80 |
| CATTATATTG AGTTGGGCGCG GTTTTGTCA TTTTACAAAA | 120 |
| GCCCGGTCCA GGATAAGTAT AATGTCACGT GCCGAATAAA | 160 |
| AGTTTGCTTC GCCCTGAAGA AACCTATAT GAGGTAGCGT | 400 |
| ATTTAATTAC CGTATATGTA AGACCTGTA TATAATGGATG | 800 |
| GCCCGGTCCA GGATAAGTAT AATGTCACGT GCCGAATAAA | 160 |
| AGGTGGCTTC GCCTCGAAGA AACCTAATAT GAGGTAGCGT | 200 |
| ATTGAATTTC TTTATTTCG AAGGAAAGGG GAAAGTTGAA | 600 |
| TGTTATATAT CATCAGAGTA ATCAAGGTT AGATATTAGT | 680 |
| GGACGAGAGA CATGGAAGTT AGTATTAGT ACAGCAACAA | 800 |
| CGACCGGAGT TCTAGTGCAC TCTCGGTGATG TGTATGTTTT | 1000 |
| TTCCTCTACG TATCGATCAG AGACGATGAC GGTAGCGT | 1200 |
| TTCCAGTACT CATGAGTCAA TTCTGGAGCT GGAAGAAGT | 1400 |
| TAATGATTTC TATTATTTCG AAGGAAAGGG GAAAGTTGAA | 1600 |
| TGTTATATAT CATCAGAGTA ATCAAGGTT AGATATTAGT | 1800 |
| AAACATCAAC ACCGCTATGAA ATACAAGACG CTATGACAA | 2000 |
| TGTAGCGGTA TCTATGGAGAT TTTTTGTAC GCCTGAAGGG | 2200 |
| CGCACGGGGG TGAAAGAATA ATACATGGA ATAAACTGTA | 2400 |
| AGATAATAT TACAGAGATG TGATCATGAA ACCTAAACAT | 2600 |
| AGAAAGGCCC ACCAACTAAA AGGTGGGGAG ATGTCCCGA | 2800 |
| TGACGTA                                      | 3000 |

Fig. (3): The sequence of the Egyptian BBTV DNA-6 full length

- The **CR-SL** is in *italics and underlined* with the stem sequence arrowed (1-31).
- The potential **TATA boxes** are in **bold and double underlined** (222-230).
- The **CR-M** is in **bold and italics** (801-891).
- The **polyadenylation** sequences were represented in **shaded letters** (737,764).
- The **ORF** was represented in **bold** (277-741).
- The start codon was **underlined** at the beginning of the ORF.
- The possible termination signal was **underlined** at the end of the ORF.
Fig. (4): The possible stem-loop structure of BBTV DNA-6 full length of the Egyptian isolate examined in this study. Underlined numbers indicate the nucleotide position on it.

Fig. (5): Phylogenetic analysis of BBTV DNA-6 full length of the Egy 6m with the eleven different isolates based on the nucleotide sequences.
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Fig. (6): Amino acid sequences of the translated ORF of Egyptian BBTV DNA-6 of the Egyptian isolate.

Fig. (7): Phylogenetic analysis of BBTV DNA-6 full length of Egyptian isolate (Egy 6m) with eleven different isolates based on the amino acids sequences.
