Molecular Cloning, Sequence Analysis, and Detection of Banana Bunchy Top Virus in Hawaii

W. S. Xie and J. S. Hu

Department of Plant Pathology, University of Hawaii, Honolulu 96822.

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ABSTRACT

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The Hawaiian isolate of banana bunchy top virus (BBTV) was purified from infected banana cultivar Williams. Three single-stranded DNA (ssDNA) components were cloned and sequenced; they were named component 1, 3, and 4, respectively. Component 1 is 1,110 nucleotides in length and shares 98% nucleotide sequence identity with the BBTV DNA component 1 of the Australian isolate. This component contains two open reading frames (ORF) capable of encoding a protein of 33.5 kDa, which may function as a replicase, and a protein about 15.2 kDa, with unknown functions. Component 3 is 1,057 nucleotides in length and does not contain any ORFs larger than 10 kDa. Component 4 is 1,017 nucleotides in length and potentially encodes a protein of 18.9 kDa. All three ssDNA components share the same stem-loop sequence and have a conserved noncoding region. The sequence of each of these three components is different from that of BBTV DNA components of two Taiwanese isolates. BBTV-specific clones were used in dot-blot hybridization assays for detection of BBTV in plants using radioactive and nonradioactive probes. A polymerase chain reaction (PCR) assay was developed for detection of BBTV in banana samples and single aphids. Dot-blot hybridization assays were as sensitive as enzyme-linked immunosorbent assay (ELISA), while PCR was 1,000 times more sensitive than dot-blot and ELISA assays for detection of BBTV in banana.

Additional keywords: NTP-binding motif, subterranean clover stunt virus, coconut foliar decay virus, geminiviruses.

Banana bunchy top disease is the most devastating viral disease of bananas in many banana-producing areas, including Asia, Africa, and the South Pacific (4). Banana plants infected by banana bunchy top virus (BBTV) produce distorted leaves; new growth from infected plants is stunted with a "bunchy top" appearance and does not produce fruit (4). The virus is transmitted by the banana aphid (*Pentalonia nigronervosa*) in a persistent manner (11,16). In the 1920s, this virus disease nearly destroyed the banana industry in southeast Queensland and northern New South Wales in Australia (4). It has spread and caused devastating problems in many countries (4, 8), including Fiji (1927), India and Egypt (1953), the Philippines and Taiwan (1961), Tonga and Samoa (1967), Vietnam (1969), China (1979), and Guam (1982) (4). It was first found in 1989 in Hawaii (8), where it remains as a serious threat to the banana industry. Since 1989, the Hawaii Department of Agriculture has been attempting eradication of the virus, an approach that worked well in Australia. However, the eradication approach is insufficient for management of BBTV in Hawaii because BBTV has been found in farms and residential areas throughout the island of Oahu and because elimination of infected plants is not mandatory.

The genome of BBTV is single-stranded circular DNA (11). BBTV has been studied since the 1920s but was not purified until 1988 (24), due to its low titer in infected banana plants. The virus has isometric particles about 18 nm in diameter, a single coat protein with an *Mr* value of 20,500, and at least six single-stranded, circular DNA components, each about 1 kb (3,11,23). The virus has a buoyant density of 1.28–1.29 g/ml in Cs_2SO_4 and sediments at 46S in sucrose density gradients (23). It has been isolated from diseased banana plants in Taiwan, Australia, Indonesia, Tonga, Hawaii, China, and India (5,23,25). Portions of the BBTV genome have been cloned in Australia and Taiwan and four DNA components have been cloned and completely sequenced (11,27). Component 1 (from the Australian isolate) contains 1,111 nucleotides and encodes a putative replicase protein of 33.6 kDa (11). Component 2 (from a Taiwanese isolate) is 1,095 in length (28), and components I and II (from another Taiwanese isolate) contain 1,106 and 1,096 nucleotides, respectively (26).

Currently, the recommended strategy for control of BBTV is to identify virus-infected plants as early as possible, remove the diseased plants, and replant with virus-free banana plants (4). In order for this approach to be successful, it is necessary to have sensitive, rapid, and reliable indexing assays for detection of BBTV from banana plants. These detection assays are also essential for the study of BBTV epidemiology. BBTV-specific polyclonal and monoclonal antibodies have been produced (23,25), and BBTV has also been successfully detected by dot-blot hybridization using radioactive probes (11).

Serological studies indicate that BBTV isolates from different countries are closely related (23). Serology and molecular hybridization results have shown that the Hawaiian isolate of BBTV is closely related to the Australian and Taiwanese isolates (13). Based on sequence analysis of one replicase-like component, Karan et al (15) concluded that BBTV may have two geographic strains: one in Australia, Hawaii, and other Pacific Islands; the other in China, Taiwan, and Southeast Asia. In this study, we report the cloning and sequencing of three components of the Hawaiian BBTV isolate. The sequences of these components were compared with those of the Australian and Taiwanese BBTV isolates. Dot-blot hybridization using nonradioactive probes and polymerase chain reaction (PCR) assays was developed and compared with enzyme-linked immunosorbent assay (ELISA) and dot-blot hybridization using radioactive probes for detection of BBTV in banana plant samples. A preliminary report of this work has been published (26).

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MATERIALS AND METHODS

BBTV and aphid sources. BBTV-infected banana samples and viruliferous banana aphids (*P. nigronervosa*) were obtained from an infected Cavendish banana (cv. Williams, AAA genotype) on the island of Oahu. The infected banana plants displayed characteristic symptoms of BBTV infection. Healthy Williams banana plants and BBTV-free banana aphids were collected from the island of Hawaii, where BBTV is not present.

Purification of BBTV. BBTV was partially purified from diseased banana midrib or pseudostem tissue as described previously (23,24). Briefly, banana corm tissue was pulverized using liquid nitrogen and a tissue grinder, extracted with 2 volumes 0.2 M phosphate buffer (pH 7.4) containing 0.5% Na_2SO_3 and clarified by chloroform/butanol (1:1; 0.1 vol) extraction. After two cycles of differential centrifugation, the BBTV preparation was further purified by centrifugation in 10-40% sucrose density gradients at 70,000 *g* for 4 h at 4 C. Fractions (0.4 ml each) were collected using an ISCO density gradient fractionator (ISCO, Lincoln, NE) and BBTV concentration in each fraction was monitored by ELISA as described below. BBTV DNA was isolated from those fractions that contained the highest concentration of virus.

Cloning and sequence analysis. Partially purified virus fractions were treated with 1% sodium dodecyl sulfate and extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Nucleic acids were recovered by precipitation with ethanol. Remaining RNA was removed by RNaseA (100 μg/ml) digestion at 37 C for 2 h. The recovered single-stranded DNA(ssDNA) was used for double-stranded DNA (dsDNA) synthesis and cloning essentially as described by Gubler and Hoffman (10). Approximately 1 μg of BBTV ssDNA was boiled for 10 min with 5 μg random primers (Promega, Madison, WI) and then quenched on ice. The dsDNA was synthesized at 37 C for 1 h in a reaction mixture containing 100 mM HEPES (pH 6.9), 10 mM $MgCl_2$, 2.5 mM dithiothreitol, 70 mM KCl, 0.5 mM each dNTP and 5 units of Klenow fragment from DNA polymerase 1 (Promega). The dsDNA was then treated with T4 DNA polymerase (Promega) and ligated into *Eco*RV-cut pBluescript SK+ (Stratagene, La Jolla, CA), and transformed into *E. coli* XL1-Blue competent cells (Stratagene). Transformants were identified by screening on media containing 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) as described by Sambrook et al (19). Restriction digests of plasmids were analyzed by electrophoresis in 1% agarose gels in Tris-acetate-EDTA buffer (TAE). Virus-specific clones were identified by Southern blot hybridization to nucleic acid extracts from BBTV-infected plants and sequenced by the dideoxynucleotide chain-termination method (20) and the primer-directed dideoxynucleotide sequencing method (21). A sequencing kit (Sequenase version 2.0, US Biochemical, Cleveland, OH) was used in the sequencing reactions. Sequence data were compiled and analyzed using the University of Wisconsin Genetics Computer Group (GCG) computer program, available from GCG, Inc., Madison, WI.

Southern and dot-blot hybridizations. Banana samples (0.1 g) were ground in 0.3 ml of extraction buffer containing 176 mM NaCl, 176 mM glycine, 20 mM EDTA, and 2% sodium dodecyl sulfate (pH 9.0), and treated with phenol/chloroform/isoamyl alcohol (25:24:1). Total nucleic acids were recovered by ethanol precipitation and dissolved in 100 μl TE (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). For Southern blot analysis, nucleic acids were electrophoresed in 1% agarose gels in TAE buffer and then blotted onto GeneScreen membrane (DuPont, Boston, MA) in 1 N NaOH using a vacuum blotter following the instructions of the manufacturer (Pharmacia, Piscataway, NJ). For dot blot analysis, total nucleic acid extracts were diluted 1:10 in 10 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate; pH 7.0), denatured by boiling for 5 min, and 100 μl were applied to GeneScreen membrane using a Bio-Dot apparatus as recommended by the manufacturer (Bio-Rad, Hercules, CA). The membrane was then fixed for 5 min under a UV transilluminator (302 nm).

An insert isolated from BBTV-specific clone (pBT2) was labeled by random priming for both radioactive and nonradioactive probes. For radioactive labeling and detection, insert DNA was labeled using [^{32}P]-dCTP and Klenow fragment of DNA polymerase 1 (Boehringer Mannheim, Indianapolis, IN). For nonradioactive labeling and detection, a DIG DNA Labeling and Detection Kit (Boehringer Mannheim) was used with chemiluminescent Lumi-Phos 530. Prehybridizations and hybridizations were done following the procedures provided by the vendor (Boehringer Mannheim).

PCR amplification and cloning. Leaf lamina and midribs of banana, and aphids were ground in an extraction buffer containing 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 2% hexadecyltri-methylammonium bromide (CTAB) and 0.2% mercaptoethanol (7). For plant samples, about 80 mg of leaf lamina or leaf midrib was ground in 0.3 ml CTAB buffer. For aphid samples, single aphids in a 1.5-ml microfuge tube were ground in 50 μl CTAB buffer using a glass pestle. Extractions were incubated at 60 C for 60 min and then emulsified with phenol/chloroform/isoamyl alcohol (25:24:1). Total DNA was recovered by precipitation with ethanol and dissolved in 100 μl TE (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). One microliter of the sample was used pre PCR reaction. PCR was conducted in a reaction containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 0.2 mM each dNTP, 2 mM $MgCl_2$, 50 pmol each primer (primers 5 and 7, see below) and 2.5 units of Taq DNA polymerase (Promega). Cycling conditions were denaturation at 94 C for 4 min followed by 30 cycles consisting of denaturation at 94 C for 1 min, annealing at appropriate temperature (depending on different PCR reactions, see Results) for 2 min and extension at 72 C for 3 min, and final extension at 72 C for 10 min. Amplified products were analyzed by electrophoresis in 1% agarose gels in 1X TAE. For cloning, PCR products were isolated from gel slices(22) and treated with Klenow fragment, digested by restriction enzymes, and ligated into the plasmid vector pBluescript SK+. Transformants were screened and characterized as described above.

ELISA. Double antibody sandwich ELISA was performed essentially as described by Wu and Su (25). Microtiter plates were coated with specific monoclonal antibodies (MAb 2H6, kindly provided by H. J. Su at National Taiwan University, Taiwan) in a sodium bicarbonate coating buffer at a concentration of 1 μg/ml, 100 μl per well. The plates were then incubated overnight at 4 C and washed with a mixture of phosphate buffered saline (PBS) plus Tween 20 three times, with the plates sitting for at least 3 min each time. Banana samples (0.1 g) were prepared with 1 ml of extraction buffer (1 × PBS and 0.2% sodium diethyldithiocarbamate) and added to the plate at 100 μl per well. The plates were again incubated overnight at 4 C and then washed with PBS–Tween 20 three times for 3 min each. Alkaline phosphatase–labeled specific monoclonal antibodies (MAb 2H6) in enzyme conjugate buffer at a dilution of 1:2,000 were added and incubated for 4 h at 30 C. The plates were washed again with PBS–Tween 20 three times for 3 min each. Substrate (p-nitrophenyl phosphate at 1 mg/ml) in 100 μl substrate buffer was added to each well and incubated 2 h at room temperature. Absorbance at 405 nm was measured with a Model 450 Microplate Reader (Bio-Rad Laboratories, Richmond, CA). Controls with virus extraction buffers, healthy samples, and virus-infected samples were included in all tests. A reaction was considered positive only if the absorbance was > 0.1, which was at least three times the mean of the healthy control.

RESULTS

Cloning of BBTV. BBTV ssDNA was isolated from partially purified virus particles after sucrose gradient centrifugation and RNA was removed by RNaseA digestion. The BBTV ssDNA preparations were analyzed by agarose gel electrophoresis and there was no detectable plant genomic DNA or RNA contamination (data not shown). The dsDNA was synthesized from

purified BBTV ssDNA and cloned into pBluescript SK+. Eight clones were analyzed and two, pBT1 and pBT2, were found to contain inserts of about 450 and 200 bp, respectively. Both clones hybridized to BBTV-infected banana nucleic acid extracts but not to BBTV-free banana extracts in a Southern blot analysis (data not shown). They were, therefore, specific to BBTV ssDNA components. To select other clones with larger inserts, these two clones were used to probe the library. Using pBT1 and pBT2, three BBTV ssDNA components were identified and characterized.

Sequencing of BBTV component 3. When pBT1 was used as a probe to screen the library, one clone (pBT3) was selected and found to contain an insert of about 1 kbp. Clone pBT3 was sequenced and found to contain a 1,022-bp insert. Since BBTV ssDNAs are circular, full-length DNA should be amplified by PCR (12). Based upon the nucleotide sequence of pBT1, two abutting oligonucleotides (primer 1: 5′-GTATAGGGGTGTCCCGAGG-3′; primer 2: 5′-GTCATCATTATCAGGGGTA-3′) were synthesized and used as primers in PCR using the purified viral BBTV DNA as template. Cycling conditions were as reported above with an annealing temperature of 37 C. A single DNA fragment was obtained of about 1 kbp (data not shown). When the amplified DNA product was cloned, subcloned, and sequenced, it was found that 35 extra nucleotides were contained in the PCR clone that were absent from clone pBT3. This was confirmed by sequencing another cDNA clone (pBT4) identified from the library. Clone pBT4 contained a sequence that overlapped with clone pBT3 from nucleotide 203 to 315 and it contained the 35 bp that were missing from clone pBT3. Three other overlapping clones (pBT1, pBT6, and pBT7) were also sequenced. The complete sequence of BBTV component 3 is 1,057 nucleotides (Fig. 1).

Sequencing of BBTV component 4. When clone pBT2 was used as a probe to screen the cDNA library, a cDNA clone was selected that contained an insert of about 950 bp (pBT5). The nucleotide sequence of clone pBT5 was determined and found to be 948 bp in length. The same PCR approach described above for component 3 was used to amplify the full-length DNA component 4 (12). Two abutting oligonucleotides (primer 3: 5′-GGTCCATGTCCCGAGTTA-3′; primer 4: 5′-CTACGTTGCCAGCTCATA-3′) derived from the sequence of clone pBT5 were synthesized and used as primers in PCR as described above with annealing temperature of 48 C. The template DNAs were isolated from BBTV-infected banana leaf. A single DNA of about 1 kb was produced (data not shown). This amplified DNA fragment was cloned into pBluescript SK+. Sequence analysis of two PCR clones showed the presence of an additional 65 bp that were not present in clone pBT5. The entire nucleotide sequence of this BBTV DNA component is shown in Fig. 2.

Sequencing of BBTV component 1. Sequence analysis showed that clone pBT2 contained an insert of 208 bp with sequences

GAGGTTGAATGAACTTCTGCTGACGTAG**GCGCTGGGGCTTATTATTACCCCCAGCGC**CGG 60

GACGGACATGGGCTTTTTAAATGGGCTTTGAGAGTTTGAACAGTTCAGTATCTTCGTTAT 120

TGGGCCAACCCGGCCCAATAATTAAGAGAACGTGTTCAAATTCGTGGTATGACCGAAGGT 180

CAAGGTAACCGGTCAACATTATTCTGGCTTGCGCAGCAAGATACACGAATTAATTAATTA 240

ATTCGTAGGACACGTGGACGGACCGAAATACTCCTGCATCTCTATAAATACCCTAATCCT 300

GTCAAGGATAATTGCTCTCTCTCTTCTGTCAAGGTGGTTGTGCCGAGGCGAAGATCGCCA 360

GCGGCGATCGTCGGAACGACCTGCATCTAGAGAGGCGGCGAGGAAACTACGAAGCGTATA 420

TCGGGTATTTATAGACTTATAGCGTAGCTAGAAGTTTACACTGTACAGATATTGTATTTT 480

GTAAATTACGAAGAAAGTCGTATTTGATATTAATAAAACAACTGGGTTTGTTAATGTTTA 540

CATTAACTAGTATCTTTTATGTACAAATTAAAATATAGTATACGGAACGTATACTAATCA 600

AAAAATAAATGACAGGCGACAAGTGATTAACAGGTGTTTAGGTATAATTAAAATAATTAT 660

GTCAAGTAATTATAATACGGAAAATGAATAAGTATGAGGTGAAAGAGGAGATATTAGAAT 720

ATTTAAAAACCCAATTATATTATTTTGGAACGAAATACAACACACTATGAAATACAAGAC 780

GCTATGACAAATGTACGGGTATCTGATTAGGTATCTGAACGTATAAGGGCCGCAGGCCCG 840

TCAAGTTGAATGGACGGTCCAGATTAATTCCTTAGCGACGAAGAAAGGAATCTTAAAGGG 900

GACCACACAATATACAGCTGTCACTGATTTATTAAATAATATAATAACCAAAAGACCTTT 960

GTACCCCTGATAATGATGACGTATAGGGGTGTCCCGAGGTAATTTAACATAGCTCTAAAA 1020

AGAGATATGGGCCGTTGGATGCCTCCATCGGACGATG 1057

Fig. 1. Nucleotide sequence of banana bunchy top virus DNA component 3 of the Hawaiian isolate. The potential stem-loop sequence (bold), the conserved noncoding region, the possible TATA box (TATAAA), and potential a poly(A) signal (AATAAA) are underlined. Two primers used in polymerase chain reaction are primer 1 from nucleotides 981–999 and primer 2 from complementary nucleotide sequence 980–962. GenBank accession no. U18078.

distinct from pBT5. Clone pBT2 must, therefore, represent another component of BBTV. This expectation is supported because pBT2 shares 97% nucleotide sequence identity with the putative replicase gene from nucleotides 1,053 to 151 of DNA component 1 of the Australian isolate, suggesting that clone pBT2 may represent part of the putative replicase gene of the Hawaiian isolate. To compare the sequence identity of the putative replicase gene of the Australian isolate with that of the Hawaiian isolate, full-length BBTV DNA component 1 of the Hawaiian isolate was obtained using PCR. Based on the nucleotide sequence of the clone pBT2, two abutting oligonucleotide primers (primer 5: 5′-GGCGAATTCTATAAATAGACCTCCC-3′; primer 6: 5′-GGCAAGCTTGCAAATGTCCCGTCCCG-3′) were designed and used in PCR as described above with annealing temperature of 37 C and purified viral BBTV DNA as a template. A PCR product of about 1.1 kbp was produced and cloned into pBluescript KS+. One clone of the PCR product was sequenced. The entire sequence (1,110 nucleotides) of this BBTV ssDNA component is shown in Fig. 3.

Sequence analysis of the three BBTV components. Based on the sizes of the three DNA components of the Hawaiian isolate and sequence homology with those of the Australian and Taiwanese isolates, we have tentatively referred to the Hawaiian isolate BBTV DNA components reported here as BBTV DNA component 1 (BBTV-H1, 1,110 nt), DNA component 3 (BBTV-H3, 1,057 nt) and DNA component 4 (BBTV-H4, 1,017 nt). DNA component 1 of the Australian BBTV isolate (BBTV-A1) has been characterized. It is 1,111 nucleotides in length and encodes a putative replicase (12). During the preparation of this manuscript, the nucleotide sequence of three other BBTV DNA components were determined for two Taiwanese BBTV isolates. The components were named BBTV component 2 and DNA I and DNA II, respectively (26,28).

Sequence comparison reveals that DNA component 1 of the

CCATGTCCCGAGTTAGTGCGCCACGTAAG**CGCTGGGGCTTATTATTACCCCCAGCG**CTCA 60

GGACGGGACATCACGTGCAACTAACAGACGCACGTGAGAATGCAGTAGCTTGCAGCGAAA 120

GATAGACGTCAACATCAATAAAGAAGAAGGAATATTCTTTGCTTCGGCACGAAGCAAAGG 180

GTATAGATATTTGTTCGAGATGCGAAAATGGAGGCTATTTAAACCTGATGGTTTTGTGAT 240

TTCCGAAATCACTCGTCGGAAGAGAAATGGAGTTCTGGGAATCGTCTGCCATGCCTGACG 300
M E F W E S S A M P D D

ATGTCAAGAGAGAGATTAAGGAAATATATTGGGAAGATCGGAAGAAACTTCTGTTCTGTC 360
V K R E I K E I Y W E D R K K L L F C Q

AGAAGTTGAAGAGCTATGTCAGAAGGATTCTTGTTTATGGAGATCAAGAGGATGCCCTTG 420
K L K S Y V R R I L V Y G D Q E D A L A

CCGGAGTGAAGGATATGAAGACTTCTATTATTCGCTATAGCGAATACTTGAAGAAACCAT 480
G V K D M K T S I I R Y S E Y L K K P C

GTGTGGTAATTTGTTGTGTTAGCAATAAATCAATTGTGTATAGGTTAAACAGCATGGTGT 540
V V I C C V S N K S I V Y R L N S M V F

TCTTTTATAATGAATACCTTGAAGAACTAGGTGGTGATTACTCAGTATATCAAGATCTCT 600
F Y N E Y L E E L G G D Y S V Y Q D L Y

ATTGTGATGAGGTACTCTCTTCTTCATCGACAGAGGAAGAAGATGTAGGAGTAATATATA 660
C D E V L S S S S T E E E D V G V I Y R

GGAATGTTATCATGGCATCGACACAAGAGAAGTTCTCTTGGAGCGATTGTCAGAAGATAG 720
N V I M A S T Q E K F S W S D C Q K I V

TTATATCAGACTACGATGTAACATTACTCTAATGTAATATCCATTATCATCAATAAAATA 780
I S D Y D V T L L *

ATGGAATGTTGATTATGTATTTATCATAAATACATAATGGTATACGTATAGCATAAAATA 840

CATTAACCAACATACAACACACTATAAAATACAACACACTATAACAAATGTAGGGTATCT 900

GATTGGGCTATATTAACCCCTTAAGGGCCGAAGGCCCGTTTAAATATGTGTTGGACGAAG 960

TCCAAGCACAAAAAAGTAAGCAGAACAATGGAATAATATGAGCTGGCAACGTAGGGT 1017

Fig. 2. Nucleotide sequence of banana bunchy top virus DNA component 4 of the Hawaiian isolate. The deduced amino acid sequence is indicated by the single letter amino acid code below the nucleotides. The potential stem-loop sequence is bold and underlined; the conserved noncoding region, the potential TATA box (TATTTAAA), and the potential poly(A) signal (AATAAA) are also underlined. The stop codon is marked by an asterisk. Two primers used in polymerase chain reaction are primer 3 from nucleotides 1,015–15 and primer 4 from complementary nucleotide sequence 1,014–997. GenBank accession no. U18079.

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G
ATATGTCCCGAGTTAGTGCGCCACGTAAGCGCTGGGGCTTATTATTACCCCCAGCGCTCG   60
                                                   A
GGACGGGACATTTGCATCTATAAATAGACCTCCCCCCTCTCCATTTCAAGATCATCATCG  120

ACGACAGAATGGCGCGATATGTGGTATGCTGGATGTTCACCATCAACAATCCCACAACAC  180
      M  A  R  Y  V  V  C  W  M  F  T  I  N  N  P  T  T  L
                  A     T
TACCAGTGATGAGGGATGAGATCAAATACATGGTATATCAAGTGGAGAGGGGACAGGAGG  240
 P  V  M  R  D  E  I  K  Y  M  V  Y  Q  V  E  R  G  Q  E  G
      T           T  T  C                 C
GTACTCGGCATGTGCAAGGATACGTGGAGATGAAGAGACGAAGTTCTCTGAAGCAGATGA  300
 T  R  H  V  Q  G  Y  V  E  M  K  R  R  S  S  L  K  Q  M  R

GAGGCTTCTTCCCAGGCGCACACCTTGAGAAACGAAAGGGAAGCCAAGAAGAAGCGCGGT  360
 G  F  F  P  G  A  H  L  E  K  R  K  G  S  Q  E  E  A  R  S
                                                   T
CATACTGTATGAAGGAAGATACAAGAATCGAAGGTCCCTTCGAGTTTGGTGCATTTAAAT  420
 Y  C  M  K  E  D  T  R  I  E  G  P  F  E  F  G  A  F  K  L
                                                   S

TGTCATGTAATGATAATTTATTTGATGTCATACAGGATATGCGTGAAACGCACAAAAGGC  480
 S  C  N  D  N  L  F  D  V  I  Q  D  M  R  E  T  H  K  R  P

CTTTGGAGTATTTATATGATTGTCCTAACACCTTCGATAGAAGTAAGGATACATTATACA  540
 L  E  Y  L  Y  D  C  P  N  T  F  D  R  S  K  D  T  L  Y  R

GAGTACAAGCAGAGATGAATAAAACGAAGGCGATGAATAGCTGGAGAACTTCTTTCAGTG  600
 V  Q  A  E  M  N  K  T  K  A  M  N  S  W  R  T  S  F  S  A

CTTGGACATCAGAGGTGGAGAATATCATGGCGCAGCCATGTCATCGGAGAATAATTTGGG  660
 W  T  S  E  V  E  N  I  M  A  Q  P  C  H  R  R  I  I  W  V
                                          A                 A
TCTATGGCCCAAATGGAGGAGAAGGAAAGACAACGTATGCTAAACATCTAATGAAGACGG  720
 Y  G  P  N  G  G  E  G  K  T  T  Y  A  K  H  L  M  K  T  G
                                                            R
                                                         C
GAAATGCGTTTTATTCTCCAGGAGGAAAATCATTGGATATATGTAGACTGTATAATTATG  780
 N  A  F  Y  S  P  G  G  K  S  L  D  I  C  R  L  Y  N  Y  E
                  T A                                 G
AGGATATTGTTATATTTGATATCCCTAGATGCAAAGAGGATTATTTAAATTATGGTTTAT  840
 D  I  V  I  F  D  I  P  R  C  K  E  D  Y  L  N  Y  G  L  L

TAGAGGAATTTAAGAATGGAATAATTCAAAGCGGGAAATATGAACCCGTTTTGAAGATAG  900
 E  E  F  K  N  G  I  I  Q  S  G  K  Y  E  P  V  L  K  I  V

TAGAATATGTCGAAGTCATTGTAATGGCTAACTTCCTTCCGAAGGAAGGAATCTTTTCTG  960
 E  Y  V  E  V  I  V  M  A  N  F  L  P  K  E  G  I  F  S  E

AAGATCGAATAAAGTTGGTTTCTTGCTGAACAAGTAATGACTTTACAGCGCACGCTCCGA 1020
 D  R  I  K  L  V  S  C  *
                                         G T    T
CAAAAGCACACTATGACAAAAGTACGGGTATCTGATTAGATATCCTAACGATCTAGGGCC 1080

GTAGGCCCGTGAGCAATGAACGGCGAGATC                               1110
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Fig. 3. Nucleotide sequence of banana bunchy top virus DNA component 1 of the Hawaiian isolate. Only the predicted amino acid sequence of the largest ORF1 is shown. The potential stem-loop sequence (bold), the potential TATA box (TATAAA), the potential poly(A) signal (AATAAA), the putative NTP-binding motif (GGEGKT), and the conserved noncoding region are underlined. The different amino acids encoded by DNA component 1 of the Australian isolate are underlined and shown below the predicted amino acid sequence of that of the Hawaiian isolate. The different nucleotide sequence of the Australian isolate is shown on top of nucleotide sequence of the Hawaiian isolate. The stop codon is marked by an asterisk. Two primers used in polymerase chain reaction are primer 5 from nucleotides 77–94 and primer 6 from complementary nucleotide sequence 76–59. GenBank accession no. U18077.

Hawaiian isolate shared 98% nucleotide sequence identity with the DNA component 1 of the Australian isolate (19 nucleotides difference) (Fig. 3; Table 1). The Hawaiian isolate BBTV DNA component 1 contains two ORFs that have the potential to encode proteins larger than 10 kDa. The largest ORF has the potential to encode a 33.5-kDa protein, which contains the conserved NTP-binding motif of GGEGKT, indicating that it may function as a putative replicase (9). Like the BBTV DNA component 1 of the Australian isolate, a possible TATA box (TATAAA) at nucleotides 79–84 and a polyadenylation signal (AATAAA) at nucleotides 968–973 were present (12). This putative replicase shares 99% sequence homology with ORF1 of DNA component 1 of the Australian isolate, suggesting that they are closely related (12). The second ORF of component 1, is located in the complementary orientation between nucleotide positions 628–221, and potentially encode a protein of 15.2 kDa (136 amino acids). The corresponding ORF in component 1 of the Australian isolate also occurs in the complementary orientation but encodes a protein of 13.8 kDa (122 amino acids). The function of these proteins is unknown.

Sequence analysis of the DNA component 3 of the Hawaiian isolate showed that it does not have any ORFs capable of encoding proteins greater than 10 kDa. The sequence was confirmed by sequencing pBT3 several times and sequencing other overlapping DNA clones. Component 2 of the Taiwanese isolate does not encode any proteins greater than 15 kDa. However, the Hawaiian BBTV component 3 and the Taiwanese BBTV component 2 are different. They share only 39% sequence identity except in the stem-loop and conserved noncoding regions (Table 1). The DNA component 3 of the Hawaiian isolate also has a possible TATA box (TATAAA) at nucleotides 283–288 and a polyadenylation signal (AATAAA) at nucleotides 604–609. Component 4 of the Hawaiian isolate contains a single long ORF of 483 nucleotides spanning nucleotides 267–749 that potentially encodes a protein of 161 amino acids with a calculated molecular weight of 18.9 kDa. There may be a possible TATA box (TATTTAAA) at nucleotides 216–223, and a polyadenylation signal (AATAAA) at nucleotides 772–777 (14). Hawaiian BBTV components 3 and 4 share less than 45% nucleotide sequence identity with components I and II of another Taiwanese BBTV isolate (Table 1).

All three BBTV DNA components of the Hawaiian isolate contained a putative stem-loop structure at nucleotides 29–57, which is composed of an 18 nucleotide stem and an 11 nucleotide loop, which is identical to that of BBTV-A1 of the Australian isolate (12) but different from that of DNA components of the Taiwanese isolates, especially in the stem region (27) (Fig. 4). In addition to the stem-loop region, sequence comparisons show that all three BBTV DNA components of the Hawaiian isolate and DNA component 1 of the Australian isolate share a high degree of sequence identity (>82%) downstream from the coding region. This region, spanning about 70 nucleotides, shares considerably less sequence identity (33–46%) with the Taiwanese isolates (Fig. 5A). DNA component 4 of the Hawaiian isolate shares 99% nucleotide sequence identity with DNA component 1 of both the Hawaiian isolate and the Australian isolate from nucleotides 3 to 71, which

TABLE 1. Percentage of nucleotide sequence identity of the DNA components of the Hawaiian, Australian, and Taiwanese isolates of banana bunchy top virus (BBTV)

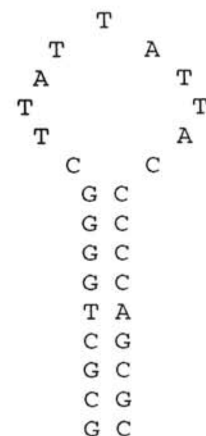
	A1	H1	H3	H4	T2	TI	TII
A1[a]		98.3	42.4	44.3	49.8	49.9	47.9
H1			41.5	44.7	50.5	50.2	48.1
H3				42.9	38.8	39.3	39.4
H4					41.2	40.9	41.0
T2						97.1	66.8
TI							63.6
TII							

[a] A1 = DNA component 1 of the Australian BBTV isolate; H1, H3, and H4 = DNA components 1, 3, and 4 of the Hawaiian BBTV isolate; T2 = DNA component 2 of one Taiwanese BBTV isolate (28); TI and TII = DNA components I and II of another Taiwanese isolate (26).

spanned the stem-loop region (Fig. 5B).

Comparison of detection assays. Samples extracted from diseased banana plants were used to compare the sensitivity of dot-blot, ELISA, and PCR assays for detection of BBTV (Figs. 6 and 7). In ELISA, BBTV was detectable when samples were diluted 1: 250, which is equivalent to 0.4 mg banana leaf tissue (Fig. 6A). BBTV was detected at concentrations equivalent to 0.08 mg or 0.4 mg banana leaf tissue in dot-blot assays, using radioactive or nonradioactive probes, respectively (Fig. 6B,C). In both cases, the BBTV DNA probe only hybridized to samples from BBTV-infected banana leaf midrib tissue and not from healthy tissues (Fig. 6B,C).

Initially, primers 1 and 2 (for component 3) or primers 5 and 6 (for component 1) were used for PCR amplification of BBTV. BBTV DNA could not be amplified using total nucleic acid extracts of banana leaf samples, although amplification was successful when purified viral ssDNA was used as a template. A new oligonucleotide (primer 7: 5′ CGGAGCGTGCGCTGTAAA 3′) was designed, which is located downstream of the coding region of the replicase gene (DNA component 1) in complementary orientation from nucleotides 1,019 to 1,002. With this primer and oligonucleotide primer 5 BBTV infection was detected in both banana plant tissue and aphid samples using PCR with an annealing temperature of 58 C. A PCR product of about 960 bp was amplified from DNA extracts isolated from BBTV-



A1 5' GCGCTGGGGCTTATTATTACCCCCAGCGC 3'
H1 5' GCGCTGGGGCTTATTATTACCCCCAGCGC 3'
H3 5' GCGCTGGGGCTTATTATTACCCCCAGCGC 3'
H4 5' GCGCTGGGGCTTATTATTACCCCCAGCGC 3'
T2 5' CCGAGGTGGCTTAGTATTACCCACCTCGG 3'
TI 5' CCGAGGTGGCTTAGTATTACCCACCTCGG 3'
TII 5' GGAGGAGCGGCTAGTATTACCCGCTCCTCC 3'
CFDV 5' GCCGCGGGGGCTAGTATTACCCCCCGCGGC 3'
TGMV 5' GGCCATCCGTTTAATATTACCGGATGGCC 3'

Geminivirus TAATATTAC

Consensus TANTATTAC

Fig. 4. The possible stem-loop structure of banana bunchy top virus (BBTV) DNA components 1, 3, and 4 (H1, H3 and H4) of the Hawaiian isolate. Stem and loop sequences were compared with that of BBTV DNA component 1 of the Australian isolate (A1), BBTV DNA component 2, I, and II of the two Taiwanese isolates (T2, TI, and TII), coconut foliar decay virus and tomato golden mosaic geminivirus. Loop regions are underlined. Note all these single-strand DNA viruses share a conserved loop sequence of TANTATTAC.

A

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BBTV-A1   1023  AAAGCACACTATGACAAAAGTACGGGTATCTGATTGGGTTATCTTAA.CGATCTAGGGCCGTAGGCCCGT 1091
BBTV-H1   1023  AAAGCACACTATGACAAAAGTACGGGTATCTGATT.AGATATCCTAA.CGATCTAGGGCCGTAGGCCCGT 1090
BBTV-H3    774  ACAAGACGCTATGACAAATGTACGGGTATCTGATTAGG.TATCTGAA.CGTATAAGGGCCGCAGGCCCGT  841
BBTV-H4    871  ACAACACACTATAACAAATGTA.GGGTATCTGATTGGGCTATATTAACCCCTTAAGGGCCGAAGGCCCGT  939
BBTV-T2    941  ATAACACGCTATGACAATCGTACGCTATCAGGATATGCATCTAAAAATCATTATAATTAATATTTGAATT 1010
BBTV-TI    923  AGAACACTCTATCACGGGGACACGCTATGACAATCGTACGCTAAAAATCATTATAATTAATATTTGAATT  992
BBTV-TII   972  CCACACGGTAGCTTGCAGAACACGCTATCATTAAATGCATCAGAAAATCATTATAATTAATAAATCTCTT 1041
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B

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BBTV-H1      3  ATGTCCCGAGTTAGTGCGCCACGTAAGCGCTGGGGCTTATTATTACCCCCAGCGCTCGGGACGGGACAT  71
                                                                        *
BBTV-H4      3  ATGTCCCGAGTTAGTGCGCCACGTAAGCGCTGGGGCTTATTATTACCCCCAGCGCTCAGGACGGGACAT  71
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Fig. 5. A, Sequence alignment of the conserved noncoding region in individual banana bunchy top virus (BBTV) DNA components from different isolates. BBTV-H1 = Hawaiian isolate component 1; BBTV-H3 = Hawaiian isolate component 3; BBTV-H4 = Hawaiian isolate component 4; BBTV-T2 = Taiwanese isolate component 2; BBTV-TI = Taiwanese isolate DNA I; BBTV-TII = Taiwanese isolate DNA II; BBTV-A1 = Australian isolate component 1. **B,** Sequence alignment of a common region between components 1 and 4. The potential stem-loop sequence is underlined. The single nucleotide difference between the two components is marked by an asterisk.

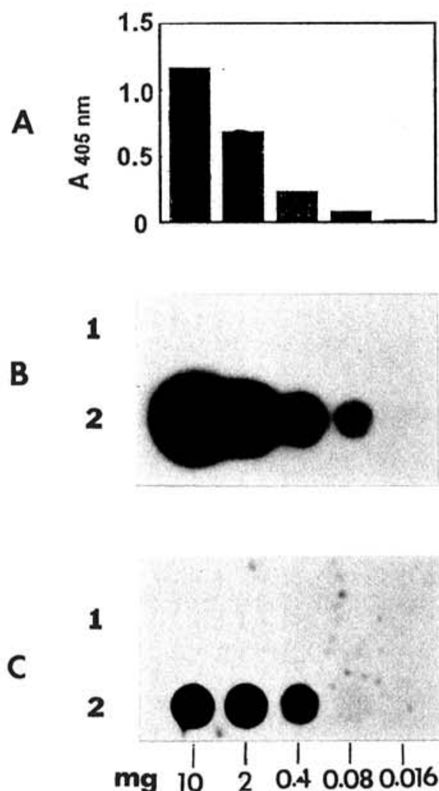

Fig. 6. Sensitivity comparison for detection of banana bunchy top virus (BBTV) by enzyme-linked immunosorbent assay (ELISA) and dot blot assays using radioactive- and nonradioactive- labeled probes. **A,** Histogram of ELISA analysis for detection of BBTV. Values are A_{405} after 2 h. **B and C,** Detection of BBTV using dot-blot assays with radioactive- **(B)** and nonradioactive- **(C)** labeled probes. Total nucleic acids were extracted from banana leaf midrib and a five-fold dilutions were made that corresponded to 10 mg, 2 mg, 0.4 mg, 80 μg, and 16 μg of leaf midrib tissue, and blotted onto GeneScreen membrane. 1: BBTV-free banana leaf midrib extracts. 2: BBTV-infected banana leaf midrib extracts.

infected but not from healthy banana leaf midribs (Fig. 7A). BBTV was detectable when samples were diluted $1{:}10^4$, equivalent to about 80 ng banana leaf tissue (Fig. 7A). PCR is therefore about 1,000 times more sensitive than ELISA and dot-blot assays for detection of BBTV from banana samples. The PCR assay was also used for the detection of BBTV from single banana aphids. A DNA product was obtained with PCR when DNA extracts of viruliferous aphids were used, but not when virus-free aphids were used (Fig. 7B).

DISCUSSION

It has been reported that the BBTV genome consists of at least 6 circular ssDNA components with sizes from 1,000 to 1,200 nucleotides (3). The nucleotide sequences of four of these components have been sequenced completely (12,26,28). In this study, nucleotide sequences of three DNA components of the Hawaiian BBTV isolate were determined and compared with the sequences of the four components characterized in Australia and Taiwan. DNA component 1 of the Hawaiian isolate shares 98% and 99% sequence identity at nucleotide and amino acid levels, respectively, with DNA component 1 of the Australian isolate, indicating that the Hawaiian BBTV isolate is closely related to the Australian isolate. This component contains an ORF that may encode a replicase with the conserved NTP-binding motif GGEGKT (9,12). Based on the sequence analysis of this ORF in many BBTV isolates, two distinct populations of BBTV, South Pacific–African and Asian regions, have been distinguished (15). Within each population the degree of sequence identity is 99%, whereas between the populations the degree of sequence identity is 90%. However, a recent report based on the sequence from an incomplete BBTV fragment from Taiwan indicated that the degree of sequence identity between the Australian component 1 and the Taiwanese component 1 is nearly 100% (28).

DNA component 3 of the Hawaiian BBTV isolate does not encode any protein greater than 10 kDa. The functions of the small polypeptides are unknown. DNA components 3 and 4 of the Hawaiian BBTV isolate share less than 45% nucleotide sequence idenity with other BBTV components. Therefore, they are considered different DNA components in BBTV genome. DNA component 2 of a Taiwanese BBTV isolate shares more than 97% nucleotide sequence identity with DNA component II of another Taiwanese BBTV isolate. However, component II contains a large ORF but component 2 does not. It is possible that the component 2 is a defective mutant of the component II. Similarly, DNA component 3 of the Hawaiian BBTV isolate does not encode any protein greater than 10 kDa. Therefore, it is possible that DNA component 3 of the Hawaiian BBTV isolate is a defective mutant of one component in the BBTV genome.

An analysis of the nucleotide sequences of BBTV components reveals that DNA components 1, 3, and 4 of the Hawaiian isolate contained a stem-loop region identical to that of DNA component 1 of the Australian isolate but different from DNA components of the Taiwanese isolates (12,26,28). Karan et al (15), however, found that isolates collected from Australia, the Philippines, Taiwan, and Burundi have a fully conserved 31-bp stem-loop

sequence. We found that three DNA components of the Hawaiian isolate contain a 29 base stem-loop sequence identical to that of the Australian isolate, but share less than 70% sequence identity with that of the Taiwanese isolates. Based on the stem-loop region, our results suggest that the Hawaiian isolate is more closely related to the Australian isolate than to the Taiwanese isolates. All BBTV components share loop sequences similar to that of coconut foliar decay virus (CFDV) and the geminiviruses. The conserved sequence of TANTATTAC is also common to all (17,18) (Fig. 5). The stem-loop structure is considered to be essential for the replication of the geminiviruses (17).

Nucleotide sequence comparison showed that DNA components 1, 3, and 4 of the Hawaiian isolate share another region of sequence identity, in addition to the stem-loop region, with DNA component 1 of the Australian isolate and possibly DNA components of the Taiwanese isolates. This region, which extends for 67–70 nucleotides, is a conserved noncoding region in individual DNA components of the BBTV genome (Fig. 6). This region may play an important role during the replication of BBTV genome DNA, as does the common region of geminiviruses (17).

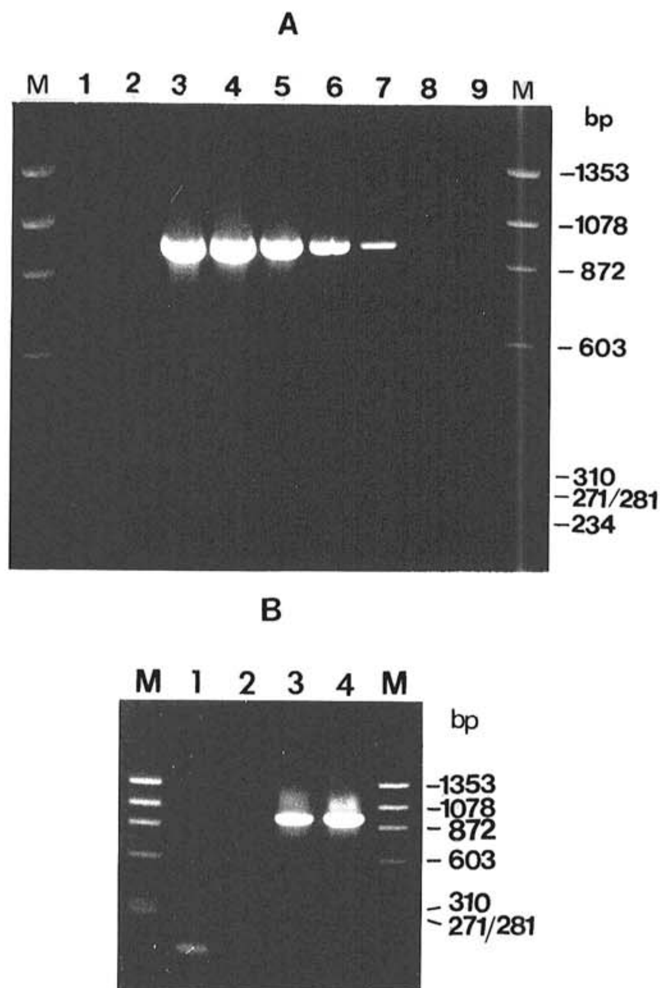

Fig. 7. A, Agarose gel electrophoresis of polymerase chain reaction (PCR) products derived from banana leaf DNA using primers 5 and 7. Lane M: DNA size markers (150 ng, Promega); lane 1: water control without DNA template; lane 2: DNA extracted from banana leaf tissue free of banana bunchy top virus (BBTV); lanes 3–9: DNA extracted from BBTV-infected banana leaf tissue corresponding to 800 μg, 80 μg, 8 μg, 800 ng, 80 ng, 8 ng, and 0.8 ng of tissue. **B,** Agarose gel electrophoresis of PCR products derived from aphid DNA. Lane M: DNA size marker (150 ng, Promega). Lanes 1 and 2: DNA extracted from banana aphids collected from BBTV-free banana plant; lanes 3 and 4: DNA extracted from individual viruliferous banana aphids collected from a BBTV-infected banana plant. Values of DNA size markers in bp are indicated to the right.

Based on the sequence identity of this conserved region, once again, the Hawaiian isolate is more closely related to the Australian isolate (>80%) than to the Taiwanese isolates (50%). It was noted that BBTV DNA components 1 and 4 have a common region that shares 99% sequence identity, is 69 nucleotides in length, and spans the stem-loop region. These two components contain large ORFs and have the common region, while BBTV DNA components 2 and 3 do not contain large ORFs and lack the common region, implying that this common region might function in gene expression. However, DNA components I and II of the Taiwanese BBTV isolate do not have the common region, although they contain large ORFs.

Our results further confirm that the genome of BBTV has multiple ssDNA components. Two other plant ssDNA viruses, subterranean clover stunt virus (SCSV) and CFDV, share characteristics with BBTV (1,2,19). Both SCSV and CFDV virions are 18–20 nm isometric particles and contain circular ssDNA genomes. SCSV consists of 7 circular ssDNA components each about 1 kb in length (2). Considering the similar properties of BBTV, SCSV, and CFDV, they may belong to a new ssDNA plant virus group, distinct from geminiviruses (2,12,17,18).

All the assays compared in this study are sensitive and specific for detection of BBTV in diseased banana plant samples. PCR is by far the most sensitive assay. It is capable of detecting BBTV from the equivalent of 80 ng banana leaf tissue and also from individual aphids. For routine tests of large numbers of samples, ELISA and dot-blot assays are similar in sensitivity and are convenient and practical. In dot-blot assays, although radioactive probes are slightly more sensitive than nonradioactive probes, nonradioactive probes are more economical and safer. Studies on other plant viruses have also shown that nonradioactive chemiluminescent detection methods provide safe and sensitive assays and will allow the wider use of nucleic acid probes in laboratories that do not have facilities for handling radioisotopes (6). The clone (pBT2) used in this study contains the common region of DNA components 1 and 4. It might also react with other BBTV components containing the same conserved region. Because purification of BBTV and production of high titer antiserum are difficult, BBTV-specific clones could be used as nonradioactive probes for detection of BBTV for routine indexing tests and epidemiology studies of BBTV.

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