Molecular characterization of banana bunchy top virus isolate from Sri Lanka and its genetic relationship with other isolates

W. A. R. T. Wickramaarachchi¹ · K. S. Shankarappa² · K. T. Rangaswamy³ ·
M. N. Maruthi⁴ · R. G. A. S. Rajapakse¹ · Saptarshi Ghosh⁴

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Abstract Bunchy top disease of banana caused by *Banana bunchy top virus* (BBTV, genus *Babuvirus* family *Nanoviridae*) is one of the most important constraints in production of banana in the different parts of the world. Six genomic DNA components of BBTV isolate from Kandy, Sri Lanka (BBTV-K) were amplified by polymerase chain reaction (PCR) with specific primers using total DNA extracted from banana tissues showing typical symptoms of bunchy top disease. The amplicons were of expected size of 1.0–1.1 kb, which were cloned and sequenced. Analysis of sequence data revealed the presence of six DNA components; DNA-R, DNA-U3, DNA-S, DNA-N, DNA-M and DNA-C for Sri Lanka isolate. Comparisons of sequence data of DNA components followed by the phylogenetic analysis, grouped Sri Lanka-(Kandy) isolate in the Pacific Indian Oceans (PIO) group. Sri Lanka-(Kandy) isolate of BBTV is classified a new member of PIO group based on analysis of six components of the virus.

Keywords Banana · Sri Lanka · BBTV · Polymerase chain reaction · Sequence · Phylogenetic tree

Introduction

Banana is one of the important commercial horticultural crops. The crop suffers from many diseases caused by fungus, bacteria as well as viruses. Of the different viral diseases, Banana bunchy top disease caused by BBTV is widely present across the banana growing regions of the world except Latin America [14]. BBTV consists of 18–20 nm diameter icosahedral particles and a multi-component genome consisting of at least six circular single stranded DNA components encoding for six major proteins [23]. The disease was first recognized in Fiji in 1889 and subsequently in Taiwan, Egypt, and Australia in 1890, 1901, and 1913 respectively. It was first reported from Sri Lanka in 1913 [18, 38] and later from Southern India in the 1940s [46] but the disease is now spread to all the banana-growing continents [15] except in Latin America and Caribbean [24]. The disease is known to cause devastating yield losses in many countries including Fiji, India, Sri Lanka, Egypt, the Philippines, Taiwan, Hawaii, China and Pakistan, reducing banana production by up to 80 % in affected areas [12, 15]. Due to high destructive potential, BBTV is regarded as ‘quarantine pest’ of very high importance and Invasive Species Specialist Group (ISSG) of the World Conservation Union-International Union for conservation of nature (IUCN) listed BBTV in the 100 of the World’s Worst Invasive Alien Species [30].

BBTV is transmitted by banana aphid *Pentalonia nigronervosa* Coquerel [22, 31] in a persistent, circulative and non-propagative manner [8]. The disease is spread long distances by the movement of infected planting material

✉ K. S. Shankarappa
ksshankarappa@gmail.com

✉ K. T. Rangaswamy
ktr_uasb@rediffmail.com

¹ Division of Plant Pathology, Department of Agriculture, Horticulture Crops Research and Development Institute, Gannoruwa, Peradeniya 20400, Sri Lanka

² Department of Plant Pathology, K. R. C. College of Horticulture, Arabhavi, University of Horticultural Sciences, Bagalkot, Karnataka, India

³ Department of Plant Pathology, University of Agricultural Sciences, GKVK, Bengaluru 560 065, India

⁴ Natural Resources Institute, University of Greenwich, Central Avenue, Chatham Maritime, Kent ME4 4TB, UK

such as suckers, corms and tissue cultured plantlets by humans. The genome of BBTV comprises six circular, single stranded DNA components of approximately 1.1 kb in size referred to as DNA-R (encoding the replication initiation protein), DNA-U3 (the function of a potentially encoded protein remains unknown), DNA-S (encoding the capsid protein), DNA-M (encoding a movement protein), DNA-C (encoding a cell cycle link protein) and DNA-N (encoding a nuclear shuttle protein) [11, 33, 42, 43]. These DNAs encode a single open reading frame (ORF) in virion sense except DNA-R, which has an additional small internal ORF the function of which is not known. Each DNA component has a conserved major common region (CR-M), a stem-loop common region (CR-SL), a potential TATA box and a polyadenylation signal associated with each ORF [5, 20].

BBTV isolates from all continents have been characterized based on the genomic DNA components of the virus [39]. Based on phylogenetic analysis of DNA-R, Karan et al. [26] divided BBTV isolates into two groups such as a PIO group and South-East Asian (SEA) group. BBTV isolates from India were found to be belonging to the PIO group [6, 37, 41]. Similarly, Amin et al. [3] characterized the Pakistan (Sindh) isolate of BBTV by analyzing of sequences of DNA components and indicated that BBTV present in Pakistan belongs to the PIO group. The virus showed the highest levels of sequence identity to BBTV isolates originating from Egypt, India and Australia. Bunchy top disease of banana was first reported from Sri Lanka in 1913 [18, 38] and caused by BBTV [46], but none of the isolates of the virus has been characterized till recently. In the present study we report complete sequence information of the six DNA components of BBTV isolate from Kandy, Sri Lanka (BBTV-K) and its molecular relationship with isolates from around the world.

Materials and methods

Virus source, total DNA extraction and amplification of BBTV components by PCR

The leaf samples from naturally infected banana plants showing typical symptoms of BBTV infection were collected from banana variety 'Embul' (Mysore AAB) during February 2010 from Seed Farm, Department of Agriculture, Kandy, Sri Lanka. The total DNA was extracted from leaf samples using CTAB method [29, 32]. The details of primers used for amplification of different DNA genomic components of BBTV were given in the Table 1. PCR amplifications were conducted in thermo-cycler (Eppendorf Mastercycler gradient, 22,331, Hamburg, Germany) in separate 25 µl reaction mixture that contained 2.0 µl total DNA extracted from infected banana leaf tissues (200–300 ng), 0.2 µl Taq DNA polymerase (Bangalore Genei Pvt. Ltd, Bengaluru, India) (5 U/µl), 2.5 µl of 10X PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂), 0.5 µl of 25 mM MgCl₂, 2.0 µl each primer (10 mM), 2.0 µl dNTPs mix (2.5 mM each) and sterile water to make up the volume. The mixture was subjected to one cycle of initial denaturation at 94 °C for 4 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 0.5 min, extension at 72 °C for 1.5 min and a final extension at 72 °C for 10 min. DNA from healthy banana plants and nuclease free water instead of template DNA were used as experimental controls.

Cloning and sequencing of DNA components

The PCR amplicons obtained using specific primers for respective DNA components were ligated into pTZ57R/T plasmid vector using InsTAcloneTM PCR Cloning Kit (Cat.

Table 1 Primers used for amplification of different components of BBTV by polymerase chain reaction

Component	Primer name	Primer sequence (5'-3')	Expected amplicon size (≈bp)	References
DNA-R	BBTV-DNA1-F	5'-GGA AGA AGC CTC TCA TCT GCT TCA GAG AGC-3'	1110	Harding et al. [21]
	BBTV-DNA1-R	5'-CAG GCG CAC ACC TTG AGA AAC GAA AGG GAA-3'		
DNA-U3	BBTV-DNA2-F	5'-GGA CGG ACC GAA ATA CT-3'	1050	Jun and Liu [25]
	BBTV-DNA2-R	5'-ACG TGT CCT ACG AAT TAA-3'		
DNA-S	BBTV-DNA3-F	5'-GGT ATT TCG GAT TGA GCC TAC-3'	1058	Furuya et al. [17]
	BBTV-DNA3-R	5'-TTG ACG GTG TTT TCA GGA ACC-3'		
DNA-M	BBTV-DNA4-F	5'-CGA TGG CAT TAA CAA CAG AGC GGG TG-3'	1050	Vishnoi et al. [41]
	BBTV-DNA4-R	5'-CGT TAG GAA CAT AGG TCC AGC GTT TCC-3'		
DNA-C	BBTV-DNA5-F	5'-AAC GGA ATA ATA TGA GCT GGC AAC-3'	1000	Burns et al. [11]
	BBTV-DNA5-R	5'-TAC TGC ATT CTC ACG TGC TGC TGT-3'		
DNA-N	BBTV-DNA6-F	5'-GTA TTA GTA ACA GCA ACA AC-3'	1110	Jun and Liu [25]
	BBTV-DNA6-R	5'-TAA CTT CCA TGT CTC TGC TCC-3'		

No. K1214, Fermentas, Germany) at 16 °C for overnight. The ligation reaction mixture of 15 µl (1 µl of cloning vector pTZ57R/T (55 ng/µl), 3 µl of 5X ligation buffer, 5 µl of gel eluted PCR product, 5 µl of nuclease free water and 1 µl of T4 DNA ligase (5 U/µl)) was set up in 200 µl centrifuge tubes. One reaction mixture which was set up without insert DNA served as control. The ligation mixtures (recombinant plasmids) were used for transformation of *Escherichia coli* DH5α competent cells by heat and cold shock method. The transformed colonies were selected on Luria Broth agar plates containing ampicilline (100 µg/ml). Positive clones of each amplicon were sequenced using the automated sequencing facility at Chromous Biotech Pvt Ltd, Bengaluru, India. The sequence data obtained from two clones of each component analyzed and consensus data were deposited in gene bank (Table 2).

Analysis of sequence data

BBTV-K sequences were compared with the respective DNA sequences from various BBTV isolates from all over the world which were available in gene bank using BLAST search program for sequence identification. Open reading frames (ORFs) and translation of ORFs was determined using expasy proteomic server tool. The nucleotide (nt) sequences of all the six fragments of the BBTV isolates were concatenated sequentially and multiple alignments of nucleotide (nt) sequences were performed using CLUSTALW in MEGA 6.0 program. Maximum likelihood phylogenetic trees were generated using GTR + G substitution model with 1000 bootstrap replicates using MEGA 6.0 [40].

Results

PCR amplification, cloning and sequencing

All six DNA components of BBTV-K were amplified using specific primers and as expected all amplicons were approximately 1.0–1.1 kb in size. After sequencing of two cloned inserts of each component were analyzed and their features of six components of the BBTV-K isolate along with gene bank accessions (Table 2).

Sequence analysis of BBTV genomic components

Stem-loop common region

The analysis of sequence data showed that all DNA components of BBTV-K isolate shared conserved regions defined as stem-loop common region (CR-SL) which having 69 nt in DNA-R, DNA-S, DNA-M and DNA-C, where as 48 and 60 nt in DNA-S and DNA-N respectively.

The CR-SL of all components of BBTV-K isolate contained a predicted stem-loop structure with the conserved nonanucleotide (TATTATTAC) loop sequence that likely marks the origin of virion-strand DNA replication.

Major common region

The second common region was located at various distances 5' of the CR-SL and was called the major common region (CR-M). The CR-M of components of BBTV-K ranged from 64 nt (DNA-R) to 92 nt (DNA-C). A 15 nt GC-rich sequence was conserved in CR-M of all components and located extreme rightward of the CR-M.

Table 2 Features of the major genomic components of Sri Lankan (Kandy) isolate of BBTV

Component	Accession Number	Size (nt)	Predicted CDS size (location)	Predicted protein (s) (aa/kDa)	TATA box location	Poly (A) location	CR-M size (location)	CR-SL size (location)
DNA-R	JN 250593	1111	861 (102–962) 129 (403–531)	Rep (286/31.46) *(42/4.62)	51–59	941–946	64 (1001–1064)	69 (1087–44)
DNA-U3	JN 250594	1061	234 (144–376)	*(77/8.47)	256–264	543–548; 644–650	87 (731–817)	48 (1057–43)
DNA-S	JN 250595	1075	528 (213–740)	CP (175/19.25)	189–197	755–760	87 (789–875)	69 (1051–44)
DNA-M	JN 250596	1047	354 (284–637)	MP (117/12.87)	263–271	662–667	90 (759–848)	69 (1023–44)
DNA-C	JN 250597	1016	486 (240–725)	Clink (161/17.71)	188–196	745–750	92 (820–911)	69 (992–44)
DNA-N	JN 250598	1090	468 (278–745)	NSP (155/17.05)	222–230	741–746; 768–773	91 (805–895)	60 (1075–44)

Rep replication initiation protein, CP coat protein, MP movement, Clink cell cycle link protein, NSP nuclear shuttle protein

* denotes for protein of unknown function

Table 3 Percent nucleotide sequence identity of BBTV- Sri Lanka-(Kandy) isolate with other isolates belonging to South-East Asian (SEA) group, Pacific-Indian Oceans (PIO) group and out group (ABTV)

BBTV components	PIO group (%)	SEA group (%)	Out group (ABTV) (%)
DNA-R	95.3–98.5	90.3–90.7	78.8
DNA-U3	81.8–98.1	79.3–83.8	55.5
DNA-S	94.5–98.9	81.3–85.9	62.2
DNA-M	93.8–98.8	83.6–85.1	56.4
DNA-C	94.7–98.9	86.8–88.2	60.1
DNA-N	89.7–98.8	85.2–86.1	64.7

Fig. 1 Phylogenetic tree showing the genetic relationship of BBTV- Sri Lanka-(Kandy) isolate to other isolates of BBTV and out group (ABTV-Abaca bunchy top virus) based on concatenated sequences of

the six DNA fragments of BBTV. Maximum likelihood phylogenetic trees were generated using GTR + G substitution model with 1000 bootstrap replicates

Potential TATA boxes

A potential TATA box was identified in six components of BBTV-K isolate. In each of these components, the potential TATA box was a nine nucleotide sequence, CTATa/ta/tAt/aA and was located downstream from the stem-loop sequence.

Poly (A)

The Poly (A) location was observed in all six components of BBTV-K isolate. Whereas Poly (A) was observed at two locations in DNA-U and DNA-N components of the BBTV-K isolate.

Component DNA-R

Based on sequence analysis, BBTV-K grouped into the PIO. The sequences were 95.3–98.5 % nt identity to isolates from Australia, Egypt, Fiji, India, Pakistan, Myanmar and Tonga which belonged to PIO group, and 90.3–90.7 % nt identity with isolates from China, Indonesia, Japan, Philippines and Taiwan which were part of the SEA group (Table 3). When DNA-R sequence of BBTV-K was compared with Tamil Nadu, Lucknow and North

Eastern Region (NER) isolates of India, it showed 97.7, 97.7 and 95.3 % respectively.

Component DNA-U3

BBTV-K DNA-U3 shared 81.8–98.1 % nt identities with isolates from Australia, Egypt, India, Malawi, Pakistan, Rwanda, and Tonga and grouped with the PIO group. It shared 79.3–83.8 % nt identities with the BBTV isolates from China and Taiwan which were grouped under SEA group (Table 3).

Component DNA-S

BBTV-K DNA-S shared 94.5–98.9 % nt identities with the isolates from India, Pakistan, Myanmar, Australia, Egypt, Burundi, Tonga, Fiji, Cameroon, Rwanda, Malawi, Gabon and Congo which were together grouped in the PIO group. It was 81.3–85.9 % nt identity to isolates from China, Myanmar, Japan, Indonesia, Vietnam and Taiwan in the SEA group (Table 3).

Component DNA-M

BBTV-K DNA-M shared 93.8–98.8 % nt identities with the isolates from Australia, India, Egypt, Malawi, Rwanda,

Pakistan and Tonga and clustered into the PIO group. It shared 83.6–85.1 % nt identities with the isolates (China, Thailand and Taiwan) belonging to SEA group (Table 3).

Component DNA-C

BBTV-K DNA-C shared 94.7–98.9 % nt identities with the isolates from Australia, India, Egypt, Malawi, Rwanda, Pakistan and Tonga and clustered with the PIO group. It shared 86.8–88.2 % nt identities with China and Taiwan isolates in the SEA group (Table 3).

Component DNA-N

BBTV-K DNA-N showed highest level of identity (89.7–98.8 %) with the isolates from Australia, India, Egypt, Malawi, Rwanda, Pakistan and Tonga in the PIO group. It shared 85.2–86.1 % identities with the isolates (China and Taiwan) in SEA group (Table 3).

Phylogenetic analysis

Phylogenetic analysis based on nt sequence of all six components of BBTV-K revealed that it clustered with isolates belongs to PIO group (Fig. 1).

Discussion

Bunchy top disease is caused by BBTV is the major constraint in banana cultivation. There was reduction of banana yield up to 80 % in affected areas [12, 14].

All six DNA components of BBTV-K were amplified using specific primers and as expected all amplicons were approximately 1.0–1.1 kb in size through PCR. In the present study, all six components of BBTV-K isolate were evaluated. Sequence analysis of BBTV-K isolate revealed that it is as similar as other reported genomes of BBTV [41]. Burns et al. [10] demonstrated that the genome of BBTV comprises at least five ssDNA components based on the restriction digests of putative full-length clones synthesized from purified virions. Thus, BBTV is a multi-component virus and appears to be most similar to subterranean clover stunt virus (SCSV) which has small isometric virions and a genome comprising of at least seven circular ssDNA components each of about 1 kb [13]. Six components of BBTV have been consistently associated with BBTV worldwide suggesting that they are integral components of the BBTV genome [27].

All six components of BBTV-K isolate shared two common regions defined as CR-SL and CR-M in the putative intergenic or untranslated region. The CR-SL is involved in the regulation of replication of components

[19]. The second region is CR-M which is the binding site for ssDNA primers reportedly prime the synthesis of transcriptionally active dsDNA [20]. The CR-SL consisted with the conserved stem-loop structure and the loop sequence of 11 nt were conserved in all BBTV components. The nine nucleotide sequence within the 11 nt conserved sequence were identical to the invariant loop sequence in geminiviruses [28]. A model for implicating the loop sequence in rolling circle replication has been described for Gemini viruses [36]. Thus, it is possible that the loop sequence of the BBTV has a similar function. The major common region (CR-M) was indentified in all five components and was located 5' of the CR-SL and 3' of the major ORF except for DNA-U3 in which no major ORF was indentified. The CR-M contained a 15 nucleotide GC-rich sequence and two direct GC-repeats which resembled the Sp1 binding sites found in promoters of genes in animal cells and viruses [16].

Karan et al. [26] reported a distinct separation of BBTV isolates based on DNA-R, which was highly conserved within the PIO group of BBTV isolates (96.5 % identity) and within the Asian group isolates (98 % identity), but highly variable between the isolates in two groups with only 68 % identity.

ORFs of DNA-R and DNA-N have potential conserved TATA boxes and polyadenylation signals. The potential TATA boxes are highly conserved with the nonanucleotide sequence of CTATA/tA/tAa/tA which was essentially similar to that described by Bucher [9]. The phylogenetic analysis of six components of the BBTV-K isolate with other BBTV isolates and out group *Abaca bunchy top virus* (ABTV) of the family *nanoviridae* clearly separated BBTV isolates into two groups possibly due to their distribution within two different regions of South Pacific and Asian regions. These two groups of BBTV, the PIO group (isolates from Australia, Burundi, Egypt, Fiji, India, Tonga and Western Samoa) and the Asian group (Vietnam, Philippines and Taiwan) were described based on sequence analysis of BBTV DNA-R, DNA-S and DNA-N [25, 27, 44]. Wanitchakorn et al. [45] confirmed the presence of two distinct groups of BBTV isolates by analyzing DNA-S sequences from six geographical isolates. In the present study, BBTV-K isolate clustered with all other isolates from India, Pakistan, Fiji, Australia, Egypt, Malawi and Rwanda based on six components of BBTV.

Use of rep and coat protein gene sequences to reconstruct phylogenies has been the prime approach for elucidating the evolutionary history of BBTV and other *Nanoviruses* [23]. Such an approach could reflect the genealogy of individual genes of BBTV. However, the phylogenies for one component might not be applicable to other components since different components can have different phylogenies [23]. The chimeric BBTV isolate

(TW4) having a mixture of the Asian and the Pacific components in a single isolate. It likely contains DNA-U3, S, and M of the Pacific group and DNA-N of the Asian group by genome *reassortment*, as supported by the CR-M phylogeny and the ORF phylogenies. Interestingly, this isolate has both the Pacific and the Asian type of DNA-R. The reason for maintaining two copies of DNA-R could be due to a necessity for the two types of components [23].

Wardlaw [46] described that BBTv has been introduced into South India from Fiji through Australia by infected planting materials. The BBTv-K isolate showed greater nt sequence identity with the BBTv isolates of India. In India there are reports of the occurrence of BBTv isolates belongs to PIO. On the basis of these findings the possible scenario for the introduction of BBTv from Sri Lanka to India could be introduction through virus infected suckers. Strong cultural and trading links with Sri Lanka and poor control over illegally imported plant material may have helped in introduction of virus. It has been estimated that more than 50 % of emerging plant diseases were driven by introduced pathogens [5]. The determination of newly introduced pathogen is often based on genetic analysis of the pathogen at its new location compared to other areas where it is found. Such an approach allows for the identification of the probable origin of introductions [2, 24]. Information on a single isolate would be insufficient to determine the genetic relationship and to identity of a virus in a particular region. Therefore characterization of some more representative isolates from the country is needed to draw a definite conclusion.

The measure of variability not only has implications for the virus and disease but may also impact upon virus diagnosis and the development of transgenic resistance [44]. CP-mediated resistance was one of the most common pathogen derived resistance strategies employed against plant viruses and has proven success against many viruses [15]. However CP mediated resistance may only be effective against closely related viruses which share a high level of sequence homology [1, 4, 19, 34, 35]. High level of conservation of 96–100 % observed in the CP gene of BBTv isolates of South Pacific group analyzed in the present study suggests that any BBTv CP transgene on the South Pacific isolates likely to provide effective CP mediated resistance against South Pacific BBTv isolates including Sri Lankan isolate.

The nucleotide sequence of BBTv DNA-S (encoding the capsid protein) varies by up to 6 % among Asian isolates [45]. Bell et al. [7] showed the level of variation of BBTv within Vietnam based on sequences of DNA-R was approximately doubled that previously reported for Asian BBTv isolates.

We report BBTv-K isolate is new member of PIO group based on molecular analysis of six components of BBTv.

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