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Cloning, Sequencing and Identification of a *Banana Bunchy Top Virus* Isolate from Bangalore

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ABSTRACT

Banana bunchy top disease is one of the most devastating viral diseases of bananas (*Musa* spp.) in Asia. It is wide-spread in all banana-producing areas and causes serious problems in most countries. *Banana bunchy top viruses* (BBTV) are isomeric particles with a single coat protein and contain six single-stranded, circular DNA components. PCR amplification was performed on isolates from Bangalore with primers specific to coat protein, which amplified a DNA fragment of 1058 bp. The PCR amplified products of coat protein gene of BBTV were cloned into *ptz57R/T* vector and sequenced. Sequence analysis resulted in a sequence of 1058 nucleotides which correspond to the DNA II component of the BBTV genome. The sequence comparison resulted in a maximum similarity (97%) with the isolates obtained from India and a minimum similarity (68.1%) with the isolates obtained form Taiwan. Based on the sequence analysis it can be inferred that the isolate from Bangalore was characterized under 'S' type (Severe strain) of BBTV that causes distinct bunched atrophy of leaves and dwarfness.

Keywords: BBTV, coat protein gene, DNA-2 component, pTZ57R/T vector
Abbreviations: Diversity among BBTV isolates

INTRODUCTION

Bananas and plantains (*Musa* spp.) are two of the most important tropical crops, both as table fruits and staples, with approximately 100 million tons produced annually worldwide (Furuya *et al*. 2005). The productive potential is affected by the banana bunchy top disease, which is one of the most destructive diseases in tropical Asia, Australia and South Pacific countries (Dale 1987; Dietzgen and Thomas 1991). The infection is caused by the pathogen *Banana bunchy top virus* (BBTV), a single-stranded DNA virus with isometric virions 18-20 nm in diameter. It infects most banana cultivars, retards the growth of infected plants, and causes substantial economic losses to banana production (Dale 1987). BBTV occurs in the phloem tissues of banana and incites symptoms such as leaf chlorosis, vein clearing, dwarfing and leaf atrophy (Wu and Su 1990). Because it is transmitted by banana aphids (*Pentalonia nigronervosa* Coq.) and through vegetative production, BBTV, along with other major virus diseases such as *Cucumber mosaic virus*, *Banana bract mosaic viruses*, and *Banana streak virus* (Jones 2000), are serious threats to banana production.

The BBTV genome consists of at least six components of circular single-stranded DNA, each of 1-1.1 kb (Burns *et al*. 1994). 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. A single gene is transcribed from all six BBTV components except for DNA-1 where a small mRNA is also transcribed from within the major gene (Beetham *et al*. 1997). Previous studies (Harding *et al*. 1993; Wanitchakorn *et al*. 1997, 2000) have shown that components encode the replication-associated protein, coat protein, intercellular transport protein, retinoblastoma binding protein and nuclear shuttle protein.

Based on their sequence analysis BBTV isolates are classified into two groups by Karan *et al*. (1994) represented as Asian and South Pacific groups. The maximum nucleotide sequence difference was approximately 3.0% between isolates from the same group and up to 14.5% between isolates from the two groups with certain regions of the sequences more variable than others. Based on the symptoms in the field, the isolates are classified into two types as S-type and R-type. The S-type or severe strain results in distinct bunched atrophy of leaves, dwarf ness, no yield and causes 90% of total destruction. The R-type or rough strain remains symptomless and does not affect yield.

Enzyme linked immunosorbent assay (ELISA) tests with monoclonal antibodies are commonly used for the accurate detection of BBTV (Thomas and Dietzgen 1991; Geering and Thomas 1996). ELISA is convenient but limited in detection 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*. 1997). In this paper, we describe the genetic analysis of DNA-2 component of BBTV obtained from Bangalore, India and compared with several isolates collected from India and South Asian regions.

MATERIALS AND METHODS

Nucleic acid extraction from banana tissues

Total DNA was extracted from the symptomatic leaf sample (**Fig. 1**) and healthy leaf sample (**Fig. 2**) for control of banana collected from Bangalore, India according to a modified Cetyl trimethyl ammonium bromide (CTAB) method (Simon *et al*. 2007). Two g of infected leaf sample was powdered in liquid nitrogen to extract

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Fig. 1 Banana plants infected by *Banana bunchy top virus*.

Fig. 2 Healthy banana plants.

DNA. The powder was mixed with 10 ml extraction buffer, preheated to 65°C, and containing 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 3% (w/v) CTAB, 2% Polyvinyl pyrrolidone and 1% β-mercaptoethanol, then incubated at 65°C for 1 h. The mixture was cooled to room temperature, 6 ml cold 24:1 (v/v) chloroform:isoamylalcohol was added, and the contents were mixed well. After centrifugation at 7,500 × *g* for 12 min at 4°C, the supernatant was transferred to a fresh tube and the chloroform: isoamylalcohol step was repeated until a clear supernatant was obtained. 5 M NaCl was added to the supernatant (0.5 v/v) and mixed gently followed by addition of 1 volume of cold isopropanol to precipitate the DNA. The mixture was incubated overnight at 4°C, and then centrifuged at 6,500 × *g* for 5 min. The resulting pellet was washed with 70% (v/v) ethanol, air dried, and dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The DNA was quantified using a spectrophotometer (Nano Drop Technologies, Wilmington, USA).

Primers and PCR conditions

The four primer pairs used, and their sequences, are listed in **Table 1**. All these primer pairs were designed to amplify PCR fragments of 1-1.1 kb. PCR was performed using 25 µl of reaction mixture containing 200 ng of template DNA, 150 µM each dNTP, 1.5 mM $MgCl_2$, 1 unit *Taq* DNA polymerase (Sigma Aldrich Chemicals, Bangalore, India), 50 ng forward and 50 ng reverse primers, in PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.05% (v/v) NP40 and 0.05% (v/v) Triton X-100). The thermal cycle conditions for FPCR4 and FPC primer pairs were: one cycle at 94ºC for 3 min; 30 cycles at 94ºC for 1 min, 56ºC for 1 min, and 72ºC for 2 min; then followed by a 72ºC extension for 10 min. The thermal cycle conditions for the S-CR and CBT3 primer pairs were the same except that the annealing temperature was 60ºC. Amplifications were performed in a MJ Research PTC-100 Thermocycler (Bio-Rad Laboratories, Bangalore, India). The PCR products were analysed by electrophoresis using 1.5% agarose in TBE buffer [40 mm Tris-borate (pH 8.0), 1 mm EDTA] with ethidium bromide (0.5 µg/ml) and visualized by Alpha Digidoc system (Alpha Innotech, San Leandro, CA, US). The 1000-bp DNA Ladder set (Sigma Aldrich Chemicals, Bangalore, India) was included as size markers.

Table 1 The nucleotide sequences of three primer pairs used for differentiation of strains of *Banana bunchy top virus*.

Primer pairs	Nucleotide sequence
S-CR	
Forward	5′-GGGGCTTATTATTACCCCCAGC-3′
Reverse	5′-AGCGCTTACGTGGCGCAGCACTAACT-3′
FPCR4	
Forward	5′-GGAAGAAGCCTCTCATCTGCTTCAGAGARC-3′
Reverse	5′-TTCCCAGGCGCACACCTTGAGAAACGAAAG-3′
CBT3	
Forward	5′-GGTATTTCGGATTGAGCCTAC-3′
Reverse	5′-TTGACGGTGTTTTCAGGAACC-3′
FPC	
Forward	5′-GGAAGAAGCCTCTCATCTGCTTCAGAGARC-3′
Reverse	5′-TTCCCAGGCGCACACCTTGAGAAACGAAAG-3′

Table 2 BBTV isolates used in the DNA-2 analyses.

BBTV isolate	Country of origin	Accession number
BI	India	EU046323
IND-1	India	AY884173
IND-2	India	AY884172
IND-3	India	AY960129
CHINA	China	AY606084
TAI-1	Taiwan	DQ826392
TAI-2	Taiwan	DQ826391

Cloning, sequencing and analysis of BBTV DNA-2

The PCR fragment amplified by the S-CR primer pair of approximately 1.1 kb of BBTV DNA-2 was eluted from the gel using a QIAGEN Gel Extraction kit (Qiagen, Hilder, Germany). The eluted PCR amplified DNA was cloned into the plasmid vector pTZ57R/T using PCR cloning kit (Cat# K1214, MBI, Fermentas) following the manufacturer's instruction. Two clones of PCR fragment were sequenced using the automated sequencing facility at MWG Biotech, Bangalore, India. Sequencing was done in both directions using M 13 forward and reverse primes in an ABI Prism 377 DNA sequencer with an ABI Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), the sequences were determined in an automated sequencer using dideoxynuleotide chain termination procedure (Sanger *et al*. 1977). Sequence data were compiled were aligned using software package GENEDOC version 2.6, for a consensus sequence. Multiple alignments for sequence comparisons were conducted with six isolates from two other countries (**Table 2**) using CLUSTAL W 1.7 software (Higgins *et al*. 1996). A phylogenetic tree was constructed among the isolates using neighbour-joining and parsimony methods using PAUP version 4.0 (Swofford 1993). Genetic distance, the neighbor-joining method was performed using Kimura's two-parameter distance method (Kimura 1980).

RESULTS

In the survey of banana diseases in and around Bangalore, India the most commonly observed symptom of the plants were stunting and had narrow leaves, sometimes with chlorosis on the leaf edge and vein clearing (**Fig. 1**). The samples were collected in this study were based on their morphological symptoms and confirmed with PCR primers (**Table 1**). The primer pair S-CR produced approximately 1.1 kb amplified BBTV DNA-2 fragments in all the BBTV-positive samples (**Fig. 3**). Full length BBTV DNA-2 of two isolates from Bangalore were cloned and sequenced. Both the DNA-2 fragments resulted in 1058 nt. The sequences obtained (Bangalore isolate) was compared with isolates from India, China and Taiwan (**Table 2**). The similarity

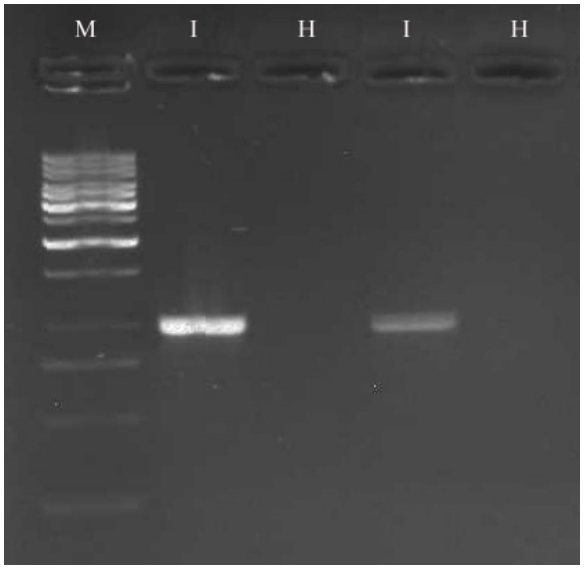

Fig. 3 PCR amplification of infected and healthy banana samples by primer pair S-CR. I: BBTV infected samples; H healthy samples; M: standard 1000 bp DNA marker.

Table 3 Similarity matrix among the BBTV isolates based on nucleotide sequence variability of DNA-2 component.

Isolates	BI	IND-1	IND-2	IND-3	CHINA	TAI-1	TAI-2
BI	1	84.1	67.5	97.0	80.0	71.3	68.1
IND-1	--	1	78.2	85.7	70.7	72.2	69.7
IND-2	--	--	1	68.9	57.5	60.3	58.1
IND-3	--	--	--	1	80.5	72.5	69.0
CHINA	--	--	--	--	1	79.1	76.3
TAI-1	--	--	--	--	--	1	89.0
TAI-2	--	--	--	--	--	--	1

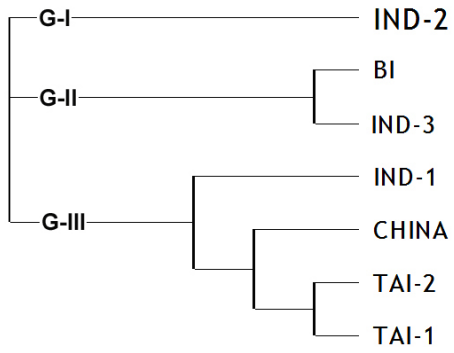

Fig. 4 Phylogram based on nucleotide sequence variability of DNA-2 component. The three major groups (G-1, G-II and G-III) are marked.

matrix showed a maximum of 97% and a minimum of 57.5% between the isolates (**Table 3**). The cluster analysis grouped the isolates into three groups principally based on the region from which they were obtained (**Fig. 4**). The Bangalore isolate had a closer relationship to the IND-2 from India.

DISCUSSION

BBTV constrains banana production worldwide (Wardlaw 1972; Jones 2000). In this study, virus of banana with mainly bunchy top symptoms was identified in Bangalore, India and the DNA-2 component was evaluated. Bananas with typical bunchy top symptoms were frequently observed, and the causal virus, BBTV, was easily detected by PCR (**Fig. 3**). Use of coat protein gene sequences to reconstruct phylogenies has been the prime approach for elucidating the evolutionary history of BBTV and other nanoviruses (Jer-Ming *et al*. 2007). Our results indicate that although such an approach could reflect the genealogy of individual genes of BBTV, the phylogenies might not be applicable to other components since different components can have different phylogenies.

Analysis of the BBTV DNA-2 sequences from six geographical isolates of BBTV has confirmed the presence of two groups of BBTV isolates previously identified by Karan *et al*. (1994, 1997). The Asian group comprised isolates from the Philippines, Taiwan and Vietnam while the isolates from Australia, Burundi and Fiji formed the South Pacific/African group. Since bananas are believed to have originated in south and south-east Asia and have since been widely distributed throughout the world, it may also be possible that BBTV has originated from the Asian continent and has been introduced to other regions through infected planting material. However, since the emergence of this disease has been a relatively recent event, given the long history of banana propagation as well as the absence of BBTV from many banana-producing regions, notably Central and South America, it is not inconceivable that the virus has moved into bananas from an alternate host (Karan *et al*. 1994, 1997).

The entire single stranded DNA-2 component was amplified by the primer pair S-CR from Bangalore isolates of BBTV. Two independent, full-length clones of DNA-2 from the geographical region of Bangalore, India were sequenced and compared to the published sequence of the India, China and Taiwan. Two clones of the isolate were sequenced in both directions to obtain a consensus sequence comprising 1058 nt (EU046323). When compared at the nucleotide level, the DNA-2 sequence of the Bangalore isolate (BI) showed a minimum of 3% difference with the isolate obtained form India (Ind-3), 20% with China isolate and a maximum of 31.9% difference with the isolates obtained from Taiwan (Tai-2) (**Table 3**).

Phylogenetic network analysis is designed for analyzing non-tree-like phylogenies in the case of reticulate evolution such as hybridization, horizontal gene transfer, and recombination (Huson and Bryant 2006). It has also been proven very useful in studying virus evolution (Holmes *et al*. 1999; Van *et al*. 2005). The comparisons of the full sequences of BBTV DNA-2 from seven isolates confirmed the division of BBTV isolates into the three groups (**Fig. 4**). Group I consists of one isolate (Ind-2) showing a similarity of 67.5% with the Bangalore isolate. Group II consists of two isolates (BI and Ind-3) closely related to each other with a similarity of 97%. Group III consists of four isolates (Ind-1, China, Tai-1 and Tai-2) showing greater variability (15.9-31.9%) with the Bangalore isolate. Similarly, a variability of 21 to 38% was observed among the coat protein gene by Jer-Ming *et al*. (2007). The two isolates from Taiwan were linked together with a 89% similarity.

These results suggest that the Indian isolates are clustered in all the groups and more diversely related to each other than they are to the other Asian isolates. This phenomenon could reflect either the period of time in which these isolates have been evolving in bananas or that there has been more than one introduction of BBTV into bananas in the Asian region (Karan *et al*. 1997). The Taiwan isolates with similar sequence probably have a much more recent common progenitor strengthening the probability that the movement of BBTV commencing from India through China has been recent and derived from a single source.

Sequential studies of worldwide viruses such as BBTV provide information on virus dispersion through host migration. In this study, we determined the genetic status and origin of BBTV by comparing Bangalore isolates with other Asian isolates. The recent use of tissue-cultured banana seedlings should raise awareness about the need to protect against BBTV invasion. Therefore, further studies of BBTV occurrence throughout the Indian sub-continent are necessary. Furthermore, molecular analysis among the other components of Bangalore isolates is essential for accurate diagnosis and comparison with other BBTV isolates.

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