Simultaneous detection of episomal *Banana streak Mysore* virus and *Banana bunchy top* virus using multiplex RT–PCR

Banana and plantains are grown both as a staple food and major cash crops for local and export market throughout the tropical and subtropical areas of the world, including India. Many viruses are known to infect banana and plantain, and cause severe damage to the plant. Among banana viruses, *Banana bunchy top* virus (BBTV) and *Banana streak* virus (BSV) are two important DNA viruses that cause significant economic loss to banana growers. These viruses are primarily transmitted through infected planting material. BBTV and *Banana streak Mysore* virus (BSMysV) are transmitted by banana black aphid, *Pentalonia nigronervosa* and mealy bug, *Planococcus citri* respectively, in a persistent manner. BBTV infection can result into 100% yield loss[1]. Significant yield reduction ranging from 7% to 90% has been reported due to BSV[2,3].

BBTV belongs to the genus *Babuvirus* and family Nanoviridae. It has isometric virions measuring 17–20 nm in diameter[4] and its genome consists of at least six circular ssDNA components individually encapsidated in virions[5]. BSV is a para-retrovirus member of the genus *Badnavirus* belonging to the family, Caulimoviridae. It has non-enveloped bacilliform particles measuring 130–150 × 30 nm and contains a circular double-stranded DNA genome of 7.4–8.0 kbp size[6]. Among the BSV species, BSMysV causes severe damage to Mysore subgroup of bananas and the genome of this virus is reported to be integrated into the host genome of banana cultivar 'Mysore' (Syn. Poovan)[7]. Integrated para-retrovirus sequences have been found in the nuclear genome of several plant species, including banana and rice[8,9]. Endogenous para-retrovirus sequences (EPRVs) corresponding to several widespread BSV species are present in the genome of *M. balbisaina*[7]. In fact, due to the widespread occurrence of BSV EPRVs in *Musa* sp. and inter-specific hybrids, BSV has now become a main constraint to *Musa* improvement programmes, germplasm exchange and quality planting materials production using tissue culture[10].

ELISA-based methods lack sensitivity[11] for the detection of BBTV[4,12] and BSMysV[13,14]. Polymerase chain reaction (PCR) is highly sensitive and is employed for the detection of BBTV[15,16]. However, in the case of BSMysV, conventional PCR cannot discriminate EPRVs and their cognate virus[10]. Indeed false positives are common in PCR, which are derived from EPRVs. To avoid such false positives, immuno-capture PCR (IC–PCR) and real-time PCR[8,10] have been applied to detect the episomal forms of a BSV species, *Banana streak Obino L'Ewai* virus (BSOLV). However, because of serological heterogeneity among BSV species, the IC–PCR technique requires a mixture of good-quality antisera for capturing the virus particles. Moreover, polyclonal antiserum is not available for BSMysV. Real-time PCR assays require costly instrument, reagents and may not be economically feasible for routine indexing. Therefore, accurate detection of episomal BSMysV is needed for *Musa* breeding and tissue-culture production of virus-free planting material.

Multiplex PCR-based detection technique has been reported to detect taxonomically different viruses infecting vegetative propagated horticultural and field crops[17,18]. In banana, multiplex IC–PCR has been used to detect three banana viruses, viz. BBTV, *Banana bract mosaic virus* (BBrMV) and *Cucumber mosaic virus* (CMV), except BSV[16]. Therefore, in this study, a simple multiplex reverse transcription–PCR (mRT–PCR) technique was developed and evaluated for detecting two taxonomically different DNA viruses, viz. BSMysV and BBTV.

BSMysV-infected banana plants of cultivars Mysore (Syn. Poovan) and BBTV-infected hill banana (Syn. Virupakshi) plants maintained in the glass house at the National Research Centre for Banana (NRCB), Tiruchirappalli, were used in this study. For validation of mRT–PCR, both infected and healthy samples from banana cv. Mysore were maintained in a permanent experimental field at NRCB. Banana tissue-culture samples obtained from the different commercial tissue-culture laboratories were also used for validating the technique.

Total RNA was extracted from 100 mg BBTV and BSMysV infected and healthy samples using Spectrum Plant total RNA kit (Sigma, USA). Total RNA was resuspended in 100 µl of DEPC-treated water and stored at –86°C until use. cDNA was synthesized by RT using Moloney Murine Leukemia Virus Reverse Transcriptase (MMuLV-RT). The downstream primers for cDNA synthesis were optimized by comparing three primers such as oligo dT, random hexamer primer and virus-specific antisense primer. Fifty nanograms of total RNA was mixed with oligo dT, specific antisense primer or random hexamer. The mixture was incubated at 65°C for 5 min, chilled on ice for 2 min and 7 µl of RT mixture containing 50 mM Tris-HCl, pH 7.3; 75 mM KCl; 10 mM DTT; 2.5 mM $MgCl_2$; 1 mM dNTPs each; 20 U Ribolock RNase inhibitor and 200 U of MMuLV-RT enzyme (MBI Fermentas, UK) was added; the final volume was made up to 20 µl by adding sterile nuclease-free water. Samples were incubated at 42°C for 60 min and subsequently incubated at 70°C for 5 min to inactivate the RT enzyme.

BBTV primers were designed on the basis of published sequence data of BBTV isolate in NCBI GenBank (GenBank accession no. EU140342). The published primer sets for BSMysV[19] and *Musa* sequence-tagged microsatellite site (STMS)[20] primers were used in this study. STMS primers were used as an internal control to check the contamination of DNA in RNA preparation. Details of the primer pairs are given in Table 1.

Simplex RT–PCR was done using each primer pair to evaluate its specificity and to determine optimal PCR conditions using Veriti Thermal Cycler (ABI Applied Biosystems, USA). Initial PCR conditions for each virus are given in Table 1. Gradient PCR was performed using the following parameters: one cycle at 94°C for 1 min, 40 cycles at 94°C for 1 min, 51–59°C for 30 s and 72°C for 1 min, followed by one cycle at 72°C for 10 min. The mRT–PCR was performed by adding a range of template volumes to a PCR master mix containing 10 mM Tris-HCl, pH 7.3, 50 mM KCl; 1.5 mM $MgCl_2$, 0.7 mM each dNTPs, 10 ng each of BBTV and BSMysV primers and 2.5 U *Taq* polymerase (Sigma, USA). The amplified product was electrophoresed in 1.2% agarose gel contain-

Table 1. Primer pairs used for multiplex RT–PCR to detect BBTV and BSMysV

Virus	Primer sequence	Primer position (nucleotide coordinate)[a]	Amplified gene	Product size (bp)	PCR cycling condition	
BBTV-F BBTV-R	5′-ATGGCGCGATATGTGGTATGC-3′ 5′-TCACGTTTTATTCATCTCTGCTTG-3′	1–21 455–479	Partial master rep	479	94°C, 4 min 94°C, 2 min 53°C, 1 min 72°C, 2 min 72°C, 10 min	35 cycles
BSMysV-F BSMysV-R	5′-TAAAAGCACAGCTCAGAACAAACC-3′ 5′-CTCCGTGATTTCTTCGTGGTC-3′	6471–6505 7049–7069	RT/RNAase H region of ORF-III polyprotein	589	94°C, 30 s 94°C, 10 s 64°C, 30 s 72°C, 1 min 72°C, 2 min	30 cycles
AGMI 025[b] AGMI026	5′-TTAAAGGTGGGTTAGCATTAGG-3′ 5′-TTTGATGTCACAATGGTGTTCC-3′	–	STMS	247	94°C, 3 min 94°C, 30 s 57°C, 30 s 72°C, 1 min 72°C, 5 min	35 cycles

[a]Nucleotide coordinate is given with reference to BBTV isolate cv. Virupakshi (GenBank accession no. EU140342).
[b]Published in Lagoda *et al.*[20].

Table 2. Details of banana samples used for validation of mRT–PCR

Location	Type of samples	Number of samples	Number of positive samples		
			BBTV	BSMysV	Dual infection
Research farm, NRCB, Tamil Nadu	Field-grown plants, symptomatic	45	20	15	9
Research farm, NRCB, Tamil Nadu	Field-grown plants, non-symptomatic	15	–[a]	–, 15[b]	–
Coimbatore, Tamil Nadu	Tissue-culture plants, non-symptomatic	48	–	–	–
Kodur, Andhra Pradesh	Tissue-culture plants, non-symptomatic	20	2	–	–
Cochin, Kerala	Tissue-culture plants, non-symptomatic	7	2	2	1
Ahmedabad, Gujarat	Tissue-culture plants, non-symptomatic	14	7	–	–
Jalgaon, Maharashtra	Tissue-culture plants, non-symptomatic	25	7	–	–
Total		174	38	17	10

[a]Virus not detected. [b]All 15 samples were positive for PCR with total DNA as template.

ing 0.5 μg/ml ethidium bromide and photographed. The amplified PCR products were eluted using MinElute Gel Extraction kit (Qiagen, Germany), and cloned into pGEM-T easy vector (Promega) and sequenced at Chromous Biotech Pvt Ltd, Bangalore. The nucleotide sequences obtained were then verified by NCBI–BLAST[21] (www.ncbi.nlm.nih.gov).

In the present study, initially plants were tested for presence of BBTV and BSMysV by PCR. Plants showing mixed infection with BBTV and BSMysV were selected for standardization of mRT–PCR. Total RNA was isolated from plants with mixed infection and cDNA was synthesized using three different primers, viz. random primers, virus-specific antisense primer and oligo dT primers. PCR performed with cDNAs obtained from three different primers showed that the oligo dT primer was not effective for BSMysV; however, it performed better with BBTV. cDNA primed with virus-specific antisense primers resulted in non-specific bands for both the viruses in mRT–PCR (Figure 1), whereas random primer gave reproducible bands for both viruses. No bands were obtained from healthy samples in mRT–PCR. Further, the identity of amplicons was confirmed by sequence analysis with published sequence data available in GenBank. Sequence analysis showed that the amplicons of BBTV (479 bp) and BSMysV (589 bp) matched exactly with the sequence of BBTV (EU140342) and BSMysV (EU140339) respectively.

Further optimization experiments for mRT–PCR were done using random primers as a common primer for cDNA synthesis. Various parameters such as concentration of dNTPs (0.5–2 mM), primer concentration (10–500 ng), annealing temperature (51–59°C) and cDNA template volume (1–5 μl) were taken into consideration for standardization of mRT–PCR. Consistent amplification was obtained in optimized mRT–PCR with 0.5 mM dNTPs, 10 ng of each primer,

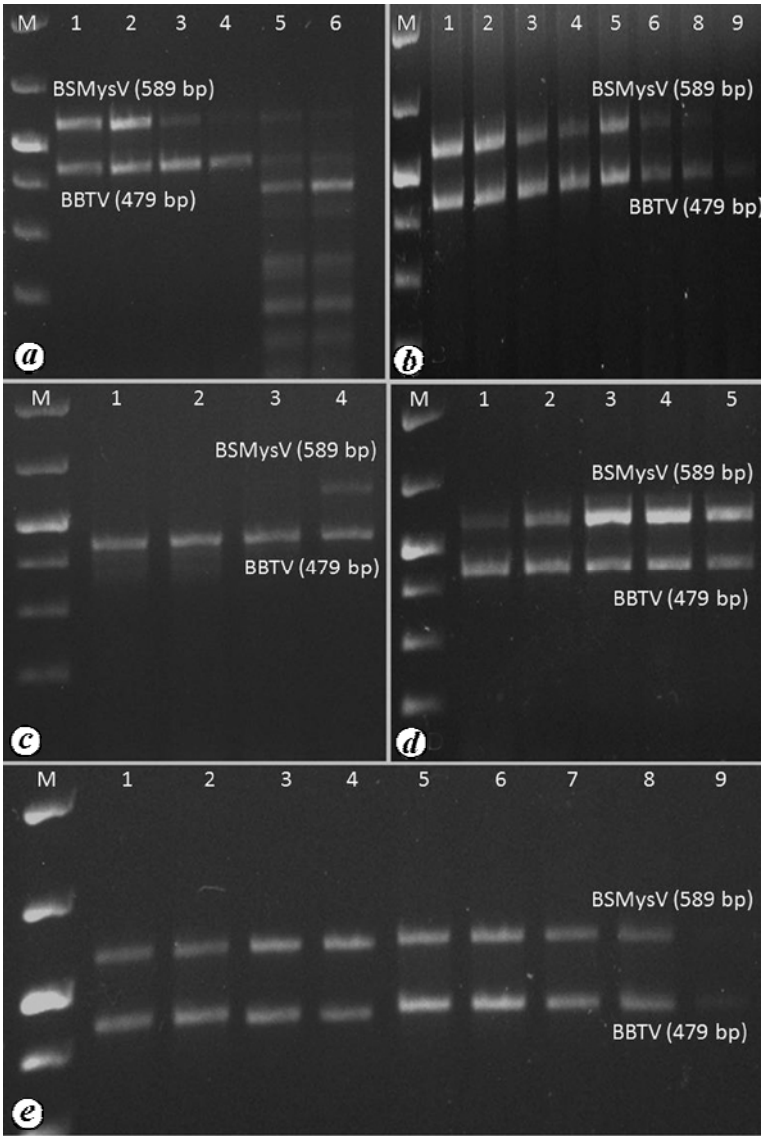

Figure 1. Optimization of mRT–PCR technique for detection of BBTV and BSMysV. ***a***, Agarose gel electrophoresis of mRT–PCR products from BBTV and BSMysV-infected samples. Lanes 1 and 2, cDNA primed with random hexanucleotides; lanes 3 and 4, cDNA primed with oligo dT; lanes 5 and 6, cDNA primed with virus-specific antisense primers. ***b***, Effect of concentration of dNTPs for cDNA synthesis in the RT step of PCR. Lanes 1 and 2, 0.5 mM; lanes 3 and 4, 1 mM; lanes 4 and 5, 1.5 mM; lanes 7 and 8, 2 mM. ***c***, Effect of BBTV and BSMysV primer concentration during PCR assay. Lane 1, 500 ng; lane 2, 250 ng; lane 3, 125 ng; lane 4, 10 ng. ***d***, Optimization of annealing temperature for PCR. The annealing temperature range from 51°C to 59°C is mentioned on the top of each lane. ***e***, Effect of cDNA template volume for PCR. The cDNA volume was increased gradually from 1 to 5 µl with 0.5 µl increments, which are mentioned in the lanes. Lane M, Molecular marker (1 kb ladder). Bands representing the corresponding viral pathogens are indicated along with the size in base pairs (bp).

55°C annealing temperature and 3 µl of cDNA template (Figure 1).

In order to determine the sensitivity level of simplex and mRT–PCR, two-fold serial dilutions in nuclease-free water containing both BBTV and BSMysV cDNAs were tested. In simplex RT–PCR, a distinct band was obtained at 2^{-9} for BBTV and 2^{-10} for BSMysV. But in case of mRT–PCR, 2^{-7} and 2^{-8} were the highest dilutions at which mRT–PCR showed distinct bands for BBTV and BSMysV respectively. However, optimized mRT–PCR conditions enhanced the sensitivity of PCR and detected both the viruses at dilutions of templates up to 2^{-8} (Figure 2). No amplification was observed from healthy samples. These results indicate that mRT–PCR is equally sensitive compared to simplex RT–PCR.

The optimized mRT–PCR for detection of BBTV and BSMysV was validated by using samples collected from experimental field and tissue-culture samples received from different commercial firms located in Maharashtra, Tamil Nadu, Gujarat, Andhra Pradesh and Karnataka.

Of the total 60 samples tested, 44 were positive for virus infection. Of these, 20 were positive for BBTV, 15 were positive for BSMysV and nine samples were positive for combined infection. No amplification was observed in the healthy samples. Out of 114 tissue-culture samples tested, 17 and 2 were positive for BBTV and BSMysV respectively. Only one of the tissue-culture samples was positive for both the virus (Figure 3, Table 2). This result of mRT–PCR was verified by PCR using DNA isolated from the same samples (data not shown). In conventional PCR, the number of samples positive for BBTV was the same as that obtained in mRT–PCR, whereas all 60 field samples were positive for BSMysV. This might be due to the presence of EPRVs in the genomic DNA. Hence, it is clear that mRT–PCR developed in this study can discriminate the virus from that of EPRVs present in the genomic DNA.

Banana bunchy top disease and banana streak disease are two major threats in the production of banana. Due to sensitivity and efficacy, PCR-based diagnostics is preferred for virus indexing of banana. But PCR-based detection of BSMysV is difficult because of the presence of EPRVs in the banana genome. In this study, we have developed the RT–PCR technique to target the transcripts of episomal BSMysV in order to avoid false positives. The strategy adopted was to use RNA extracted from the infected plants as a template for the amplification of both viruses. The first-strand cDNA was initiated by random primer and PCR amplification was done using BBTV and BSMysV-specific primers in mRT–PCR. The absence of DNA in RNA extracts was verified using Musa-specific STMS markers as previously described[10]. Hence, our study showed that mRT–PCR detected only transcripts from the virus and probably not from EPRVs. No amplification was observed in healthy samples. Though RT–PCR was not necessary for BBTV detection when multiplexing was done, the host DNA which contains EPRVs resulted in false positives for BSMysV. In order to avoid such

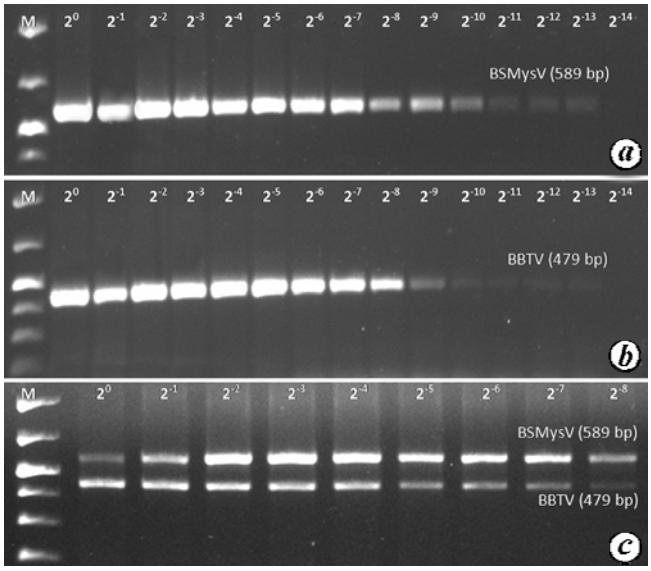

Figure 2. Effect of RNA template dilutions: ***a***, During RT for simplex PCR for BBTV. ***b***, During RT for simplex PCR for BSMysV. ***c***, During mRT–PCR for BBTV and BSMysV. Dilutions of RNA stock are indicated on the top of the each lane. Lane M, Molecular markers (1 kb ladder). Bands representing the corresponding viral pathogens are indicated along with the size in bp.

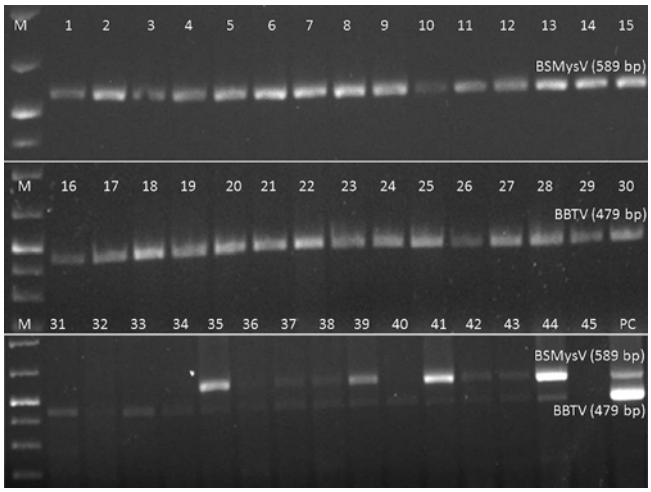

Figure 3. Validation of mRT–PCR for detecting BBTV and BSMysV in 45 leaf samples collected from field-grown plants. Lanes 1–45 are samples; lane PC, Positive control (leaf sample with dual virus infection); lane M, Molecular markers (1 kb ladder). Bands representing the corresponding viral pathogens are indicated along with the size in bp.

false positives, the transcripts of BBTV were targeted in this study. The mRT–PCR technique developed in this study would reduce the cost, labour and time involved in detection. Detection of BSMysV in combination with BBTV by mRT–PCR is expected to find application in detecting the viruses in mixed infections, differentiating episomal BSMysV in *Musa* breeding stocks used in improvement programme and *in vitro* conservation of germplasm. Although this study focused on two banana DNA viruses, the usefulness of mRT–PCR can be extended for the detection of banana RNA viruses in a single assay.

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