World J Microbiol Biotechnol (2013) 29:589–596
DOI 10.1007/s11274-012-1214-z

ORIGINAL PAPER

Development of *Agrobacterium*-mediated transformation of highly valued hill banana cultivar Virupakshi (AAB) for resistance to BBTV disease

Sivalingam Elayabalan · Kalaimughilan Kalaiponmani · Sreeramanan Subramaniam · Ramasamy Selvarajan · Radha Panchanathan · Ramlatha Muthuvelayoutham · Krish K. Kumar · Ponnuswami Balasubramanian

Received: 8 August 2012 / Accepted: 15 November 2012 / Published online: 27 November 2012

Abstract One of the most severe viral diseases of hill banana is caused by banana bunchy top virus (BBTV), a nanovirus transmitted by the aphid *Pentalonia nigronervosa*. In this study, we reported the *Agrobacterium*-mediated transformation on a highly valued hill banana cultivar Virupakshi (AAB) for resistance to BBTV disease. The target of the RNA interference (RNAi) is the *rep* gene, encoded by the BBTV-DNA1. In order to develop RNAi construct targeting the BBTV *rep* gene, the full-length *rep* gene of 870 bp was polymerase chain reaction amplified from BBTV infected hill banana sample DNA, cloned and confirmed by DNA sequencing. The partial *rep* gene fragment was cloned in sense and anti sense orientation in the RNAi intermediate vector, pSTARLING-A. After cloning in pSTARLING-A, the cloned RNAi gene cassette was released by *Not*I enzyme digestion and cloned into the *Not*I site of binary vector, pART27. Two different explants, embryogenic cells and embryogenic cell suspension derived microcalli were used for co-cultivation. Selection was done in presence of 100 mg/L kanamycin. In total, 143 putative transgenic hill banana lines were generated and established in green house condition. The presence of the transgenes was confirmed in the selected putative transgenic hill banana lines by PCR and reverse transcription PCR analyses. Transgenic hill banana plants expressing RNAi-BBTV *rep* were obtained and shown to resist infection by BBTV. The transformed plants are symptomless, and the replication of challenge BBTV almost completely suppressed. Hence, the RNAi mediating resistances were shown to be effective management of BBTV in hill banana.

Keywords Banana bunchy top virus (BBTV) · RNAi-BBTV *rep* · Transgenic hill banana (Virupakshi AAB)

Abbreviations

ECS	Embryogenic cell suspension
RNAi	RNA Interference
GI	Geographical indication
SH	Schenk and Hildebrandt
pEB	Plasmid Elaya Balan
MS	Murashige and Skoog
RT-PCR	Reverse transcription PCR
BBTD	Banana bunchy top disease
THB	Transgenic hill banana
NTHB	Non transformed hill banana
NRCB	National Research Center for Banana
gDNA	Genomic DNA
dpt	Days post transmission
cDNA	Complementary deoxyribonucleic acid

Introduction

Banana crop faces numerous environmental challenges, particularly with fungal and bacterial pathogens as well as the major threatening disease like banana bunchy top virus

S. Elayabalan (✉) · K. Kalaiponmani · R. Panchanathan · R. Muthuvelayoutham · K. K. Kumar · P. Balasubramanian
Department of Plant Molecular Biology and Biotechnology, Centre for Plant Molecular Biology, Tamil Nadu Agricultural University (TNAU), Coimbatore 641 003, Tamil Nadu, India
e-mail: balabiotech@gmail.com

S. Subramaniam
School of Biological Sciences, Universiti Sains Malaysia (USM), Minden Heights, 11800 Georgetown, Penang, Malaysia
e-mail: sreeramanan@gmail.com

R. Selvarajan
National Research Center for Banana (NRCB), Thayanur Post, Tiruchirapalli 620 102, India

(Hu et al. 1996). The problem is aggravated by the limited diversity of banana cultivars around the world. Conventional breeding methods have limited success due to low female fertility, sterility, ploidy levels and poor seed set, besides the process is time consuming. These problems point to the necessity of developing alternate strategies for banana improvement.

In Tamil Nadu (South India), hill bananas (two ecotypes, AAB Pome group namely, *Virupakshi* G.I No: 124 and *Sirumalai* G.I No: 126) were grown at a height of 2,800 to 5,000 feet with well distributed annual rainfall of 1,250–1,500 mm in the lower Palani hill, Sirumalai and Kolli hills (Elayabalan et al. 2008). Hill bananas are known for their special flavor and long shelf life, are unique to the state of Tamil Nadu. Hill bananas are perennial in nature, cultivated along with coffee and pepper also as a multitier system (Elayabalan et al. 2008). However, hill bananas are highly susceptible to banana bunchy top virus (BBTV) disease and its area and production is severely affected due to this viral disease. Currently, there is no strategy available to protect hill bananas against the virus. The improvement of this crop through conventional banana breeding has not been successful. Biotechnological approaches such as tissue culture and genetic transformation are particularly appropriate for this valuable crop.

BBTV genome consists of at least six circular single-stranded DNA components (BBTV DNA-1 to 6) each of 1.1 Kb (Burns et al. 1995). Each of the six DNA components associated with BBTV encodes at least one gene (Beetham et al. 1997). BBTV DNA component 1 (BBTV DNA-1) contains two transcribed ORFs that include replication initiation protein (Rep) (Harding et al. 1991; Hafner et al. 1997), while Wanitchakorn et al. (1997) demonstrated that BBTV DNA-3 encodes the viral coat protein. There are two groups of BBTV, the South Pacific group (isolates from Australia, Burundi, Egypt, Fiji, India, Tonga and Western Samoa) and the Asian group (Vietnam, Philippines and Taiwan), based on sequence analysis of BBTV DNA-1, -3 and -6 (Wanitchakorn et al. 2000). Researchers from Queensland University of Technology (QUT) Australia and University of Hawaii (UH), USA are involved in developing transgenic bananas, particularly on 'Cavendish' banana with resistance to BBTV disease. John Hu and co-workers of University of Hawaii had generated several putative transgenic lines expressing mutated or anti-sense *rep* genes with partial resistance to BBTV (Broth et al. 2009). Some of these banana plants remained symptomless for at least 1 year. James Dale and his group of Queensland University of Technology developed BBTV resistance in banana using a novel approach (Njoroge et al. 2009). In this strategy, virus activated cell death, involves integrating into the host plant a construct encoding a split suicide gene, which is flanked by the target virus intergenic region, which in turn are embedded in introns. The suicide gene is only activated upon infection by the target virus and is only expressed in cells that are infected by the target virus. Activation is by viral *rep* mediated replicative release and circularization during which the suicide gene is reconstituted leading to transcription, processing out of the intergenic region embedded in the intron and finally translation of the suicide gene and cell death. However, both the groups did not use the RNA interference (RNAi) technology for imparting the BBTV resistance.

Many reports have demonstrated that RNAi can be engineered to target viral RNA in plants (Smith et al. 2000; Tenllado et al. 2004). As a proof of the concept that RNAi can be engineered to effectively target DNA virus namely, Mung Bean Yellow Mosaic Virus (MYMV-Vig) was demonstrated by Pooggin et al. (2003). Furthermore, a PTGS-based strategy to control DNA virus replication was demonstrated when plant cells simultaneously transfected with African Cassava Mosaic Virus (ACMV) and with a synthetic siRNA designed to target the AC1 gene of the virus showed a reduction in the accumulation levels of AC1 mRNA by more than 90 % and viral DNA by 70 % compared with controls (Vanitharani et al. 2003). Transgenic cassava expressing the full length AC1 gene (which encodes the replication-associated protein) from ACMV imparted resistance against the virus (Chellappan et al. 2004).

Therefore, it is now well established that both RNA and DNA viruses can be controlled by RNAi approach. The RNA viruses are effectively controlled by silencing the coat protein gene whereas the DNA viruses are effectively controlled by silencing the *rep* gene, which is indispensible for DNA replication of virus. Genetic engineering for viral diseases caused by DNA viruses in several crops resulted in successful viral protection. Hence, an attempt was made to engineer resistance using *Agrobacterium*-mediated transformation for bunchy top disease in hill banana cultivar, *Virupakshi* (AAB) using RNAi technology to silence the *rep* of BBTV.

Materials and methods

Cloning of the BBTV *rep* gene with the polymerase chain reaction (PCR)

Fresh young emerging green leaves with midribs were collected from the infected plants and DNA was isolated using the modified CTAB protocol and stored at −70 °C for further use. Prior to extraction, 100–300 mg of midrib of young hill banana leaves were cut into bits and transferred to a zip lock bag (7 × 9 cm) and 1 mL of extraction buffer (0.2 M EDTA, 1.4 M NaCl, 1 M, CTAB 2 %) was added immediately. The samples were kept 5 min at room

temperature and squeezed by rolling a glass rod over the sample to extract the cell contents. About 500 µL of the cell extract was transferred into an Eppendorf tube, and then 33 µL of 20 % SDS was added into the tube and mixed well. The tube was kept at 65 °C (heating blocks) for 10–12 min and then the tube was centrifuged for 10 min at 12,000 rpm and 450 µL of the supernatant was transferred immediately to a new Eppendorf tube. Then 450 µL of ice cold IPA (Isopropyl alcohol) was added to the supernatant and after mixing, the tubes were kept in ice for 20 min. The tubes were centrifuged for 15 min at 12,000 rpm and supernatant was discarded without disturbing the pellet. The pellet was washed with 500 µL of 70 % ice cold ethanol and centrifuged for 10 min at 12,000 rpm. The supernatant was discarded and the pellet was air dried for 5 min and suspended in 40 µL of 0.1X TE buffer (1 mM Tris HCl pH 8.0 and 0.1 mM EDTA pH 8.0) and kept at 65 °C for 3 min (to suspend the pellet well) and stored at −20 °C. The isolated DNA was checked for its purity by 0.8 % agarose gel electrophoresis and quantified by UV Spectrophotometer. For BBTV *rep* gene specific primer designing, complete nucleotide sequence of several BBTV *rep* genes deposited in NCBI were retrieved. Forward (BBTV-Rep-F: 5′-ACGACAGAATGGCGCGA-3′) and reverse (BBTV-Rep-R: 5′-TCAGCAAGAAACCAACTTTATTC-3′) primers were designed for the amplification of the complete ORF (870 bp) of the *rep* gene after multiple alignment of the Indian isolate of BBTV. PCR amplified BBTV *rep* gene was cloned into T/A cloning vector (pTZ57R/T (MBI Fermentas) and sequenced.

Construction of RNAi vectors using partial 5′ and 3′ end of rep gene

The cloned full-length *rep* gene was used to develop hair pin RNAi cassette targeting the BBTV *rep* gene. The 440 bp of 5′ and 440 bp of 3′ end of the BBTV rep gene were utilized. The partial gene fragment was subcloned in sense and antisense orientation in an intermediate RNAi vector pSTARLING (CSIRO Plant Industry Australia). This vector contains Ubiquitin promoter, Ubi intron, *cre* intron, restriction site for sense and antisense orientation. The cloned hair pin RNAi gene cassette with *cre* intron was released by *Not*I enzyme and cloned into the *Not*I site of binary vector pART27 (CSIRO plant industry, Australia). The pART27 contain *npt*II (kanamycin) gene for plant selection (Fig. 1).

Agrobacterium tumefaciens and plasmids

Agrobacterium tumefaciens strain LBA4404 used in the study. Schematic presentation of the binary vectors used is shown in Fig. 2. The *Agrobacterium* strain LBA4404 (pEB1) was grown in YEP medium for 16–24 h to obtain 1 O.D (Optimal density) culture. The *Agrobacterium* cells pelleted by centrifugation at 4,000 rpm for 10 min and dissolved in equal volume of Schenk and Hildebrandt (SH) basal medium without hormone and the cells were grown for 3 h at 28 °C with 175 rpm shaking.

Transformation and regeneration of transgenic Virupakshi hill banana (AAB) lines

Hill banana embryogenic cell suspension cultures

Embryogenic cell suspensions (ECS) of banana cultivars 'Virupakshi' were initiated and maintained. The 4–6 months old embryogenic calli were used for initiation of cell suspension. About 100 mg of embryogenic calli were transferred to 100 mL conical flasks containing 10 mL of liquid ECS medium containing MS medium with 2,4-D (2 mg/L), IAA (1 mg/L), NAA (1 mg/L), with (45 g/L) sucrose and (20 g/L) maltose, pH 5.3. The flasks were incubated on refrigerated incubator shaker at 100 rpm at 18 ± 1 °C and the cultures were maintained under light/dark cycle of 16/8 h. The cultures were maintained for 2–3 months with periodical replacement (once in 7–10 days) of old medium with fresh medium (Cote et al. 1996) (Fig. 3).

Co-cultivation of ECS, selection and regeneration

ECS of 0.5 mL was co-cultivated by added 10 uL of *Agrobacterium* suspension and kept for 30–60 min at 25 ± 1 °C without shaking. Then ECS containing *Agrobacterium* suspension was diluted by adding 10 mL of Schenk and Hildebrandt (SH) basal medium and incubated for 24 h at 25 ± 1 °C with 100 rpm in dark condition. Then, the ECS was allowed to settle at the bottom and supernatant was discarded with the help of sterile pipette. The ECS was washed twice with SH basal medium containing 250 mg/L of cefotaxime. The ECS cells were then transferred to semisolid medium overlaid with 0.2 µm, 47 mm, grid sterile membrane disc filters (PALL Corporation, USA), and the excess liquid medium was removed with the help of a micropipette. Then the culture was incubated for 4 months at 25 ± 1 °C in dark. After 4 months, induced selected embryos were transferred into regeneration medium containing MS salts with 2.5 mg/L BAP, 1 mg/L GA3, 2.5 mg/L thidiazuron (TDZ), MS vitamins, 100 mg/L glutamine and 30 g/L sucrose 100 mg/L kanamycin and 250 mg/L cefotaxime (Fig. 4). The plantlets with well developed roots were hardened in sterile soil (red soil:sand mixed at 1:2 ratio) in small pots under controlled conditions and then transferred to transgenic greenhouse.

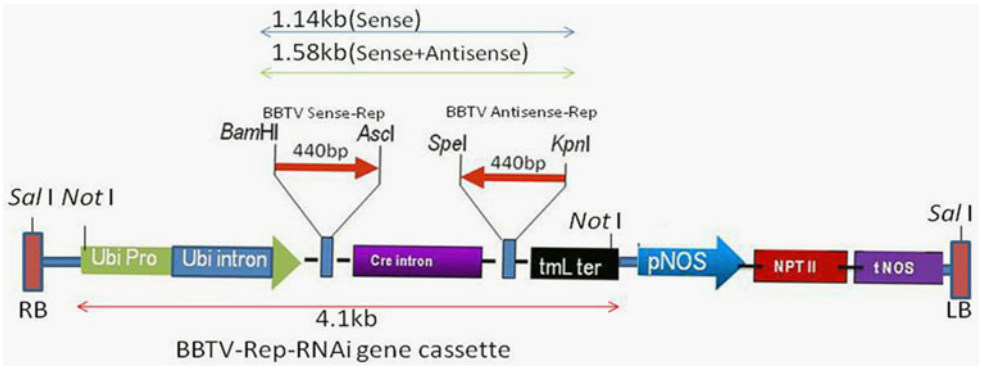

Fig. 1 T-DNA Region of pEB1 showing the BBTV *rep* hpRNAi gene cassette under the control of Ubiquitin Promoter, *cre* intron and tmL terminator

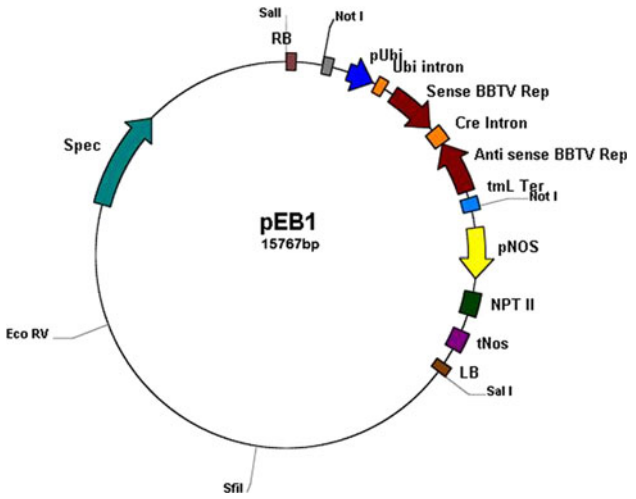

Fig. 2 Binary vector of RNAi-BBTV *rep*

Co-cultivation of ECS derived microcalli

After growing the cells for 3 h at 28 °C, 100 µM acetosyringone was added and used for infecting the 2 months old ECS derived microcalli. Microcalli derived from ECS were taken in a sterile Petri dish and incubated in *Agrobacterium* suspension for 10–15 min followed by blot drying in sterile tissue paper to remove the excess bacterial suspension. Microcalli were transferred to co-cultivation medium and incubated at 25 °C for 3 days. Then the infected microcalli washed three times with sterile water containing 250 mg/L cefotaxime and finally washed with SH basal medium (Fig. 5).

Selection and regeneration of microcalli

Co-cultivated washed microcalli were then transferred to somatic embryo induction medium containing 100 mg/L kanamycin and 250 mg/L cefotaxime. Two sub culturing was done once in 20 days in the fresh medium. The selected microcalli were subcultured for 3–4 cycles. After 2–3 months of subculturing, the microcalli showed the development of somatic embryos. The somatic embryos were then transferred into regeneration medium; containing 100 mg/L kanamycin and 250 mg/L cefotaxime (Fig. 5). The well developed plantlets were hardened in sterile soil (red soil:sand mixed at 1:2 ratio) in small pots under controlled conditions and then transferred to transgenic greenhouse (Fig. 6).

Molecular analysis and bioassay of transformed hill banana

DNA isolation and PCR analysis

DNA was isolated as described above the section of cloning of the BBTV *rep* gene with polymerase chain

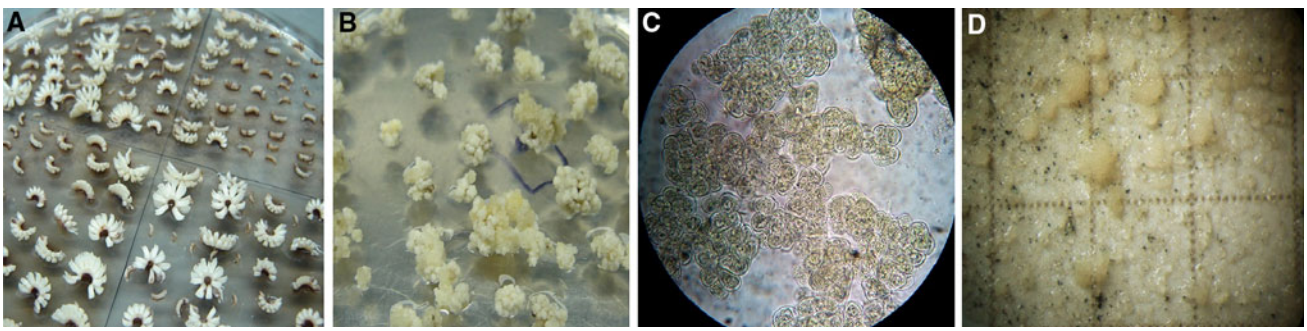

Fig. 3 Initiation of embryogenic cell suspension and microcalli from hill banana immature male flower explants. **a** Immature flower of hill banana; **b** induction of embryogenic calli **c** initiation of ECS in liquid medium and **d** ECS derived microcalli

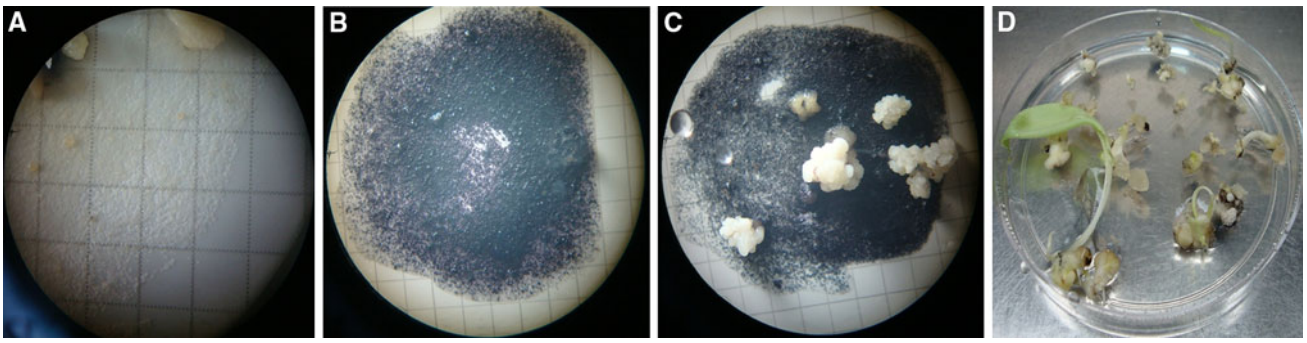

Fig. 4 *Agrobacterium* mediated transformation in hill banana embryogenic cell suspension and regeneration of transformed embryo. **a** Co-culturing of embryogenic cells with *Agrobacterium* suspension; **b** selection of ECS on glass fiber membrane filters with 100 mg/L of kanamycin; **c** induction of embryos from selected ECS and **d** germination of transformed embryos

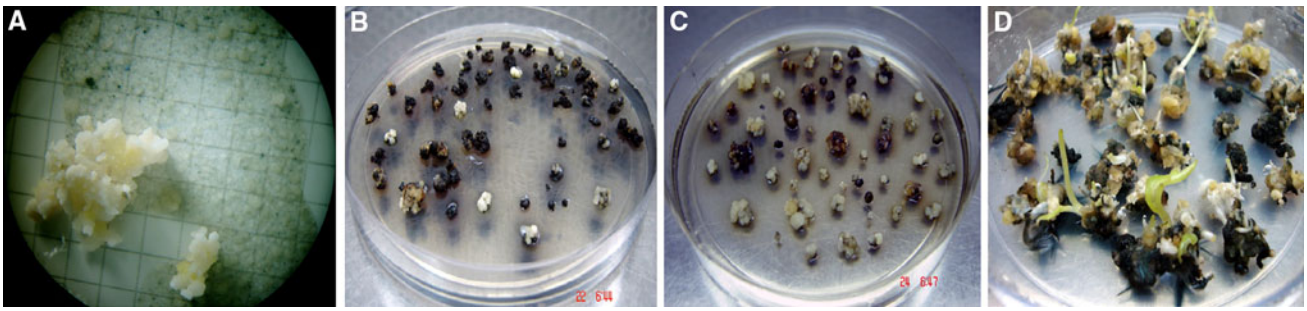

Fig. 5 *Agrobacterium*-mediated transformation in hill banana microcalli and regeneration of transformed embryo. **a** Co-culturing of embryogenic cells derived microcalli with *Agrobacterium* suspension; **b** selection of microcalli with 100 mg/L of kanamycin; **c** induction of somatic embryos from selected microcalli and **d** germination of transformed embryos

Fig. 6 Hardening of transformed hill banana lines in transgenic green house. **a** Primary hardening; **b** 10–15 cm grown plants; **c** secondary hardening in green house

reaction (PCR). The DNA was used as a template for the amplification of *npt*II, *rep* gene and coat protein primer.

Total RNA isolation and RT-PCR analysis

Total RNA was extracted from BBTV infected hill banana, Healthy hill banana and putative transgenic hill banana using SV total RNA isolation kit (Promega, USA). The RNA isolated was converted into cDNA using Revert-Aid^{TM} H minus First Strand cDNA Synthesis Kit (MBI Fermentas, USA). The first strand (cDNA) was used as a template for the amplification of *npt*II, and *actin* gene (used as internal control). The designed primers were used for the amplification of different genes by RT-PCR. Temperature profile used for the PCR amplification is as follows: Initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, synthesis at 72 °C for 1 min and final extension at 72 °C for 10 min. Presence of amplicon was checked through 1.5 % agarose gel electrophoresis.

Virus source, aphid rearing and plant challenges with viruliferous aphids

The BBTV infected banana plants and viruliferous aphid colonies were collected from a orchard at lower Pulaney

Fig. 7 Bioassay for virus challenging through viruliferous banana aphid in transformed hill banana (THB) and non-transformed hill banana (NTHB) under green house condition

region. These BBTV source plants along with viruliferous aphids were kept in an isolated, contained area. To confirm the viruliferous status of aphids PCR analysis was performed using genomic DNA isolated from five random aphid samples before the release of aphids to transmit BBTV in all the BBTD bioassays. In all the BBTD screening to attain maximum transmission of BBTV, five viruliferous aphids were transferred to each transgenic hill banana (THB) and viral free certified by NRCB, non transgenic hill banana (NTHB) having four to five well developed leaves for inoculation access for one-week. At the completion of inoculation-access periods, the in vitro maintained plants were transferred to soil and subsequently all plants were sprayed with insecticide Phosphamidon 2 mL/L (Hu et al. 1996). All the inoculated banana plantlets were grown in an insect-proof contained setup. Aphids were transferred using a water moistened no. 2 camel hair paint-brush. The BBTD symptoms were monitored after 3 and 6 months after inoculation (Fig. 7).

Results and discussion

Agrobacterium-mediated transformation of hill banana

Agrobacterium-mediated transformation of hill banana with developed RNAi constructs for targeting the 5′ end of the *rep* gene was done using embryogenic cells and ECS derived microcalli as starting material for infection. Microcalli was found better for *Agrobacterium* infection than using the ECS for direct coculitvation. The ECS on direct co-cultiivaton resulted in necrosis and cell death, whereas the microcalli were able to tolerate *Agrobacterium i*nfection and resulted in high frequency of transformation. This is in contrast to reports that ECS is the ideal material for *Agrobacterium* infection (May et al. 1995; Engler et al. 2000; Ganapathi et al. 2001; Khanna et al. 2004). Reducing the duration of cocultivation to 2 days and maintaining the cultures at lower temperature (18 °C) resulted in reduced infection injury to microcalli or ECS as noticed by reduced accumulation of phenolics in the subsequent selection stage. Most of the reports used 25 °C during co-cultivation (Khanna et al. 2004). The selection agent, kanamycin (100 mg/L) was used throughout the selection and regeneration of putative transgenic plants. In total, 143 putative transgenic plants were obtained from both type of explants infected with *Agrobacterium* strain LBA4404 (pEB1).

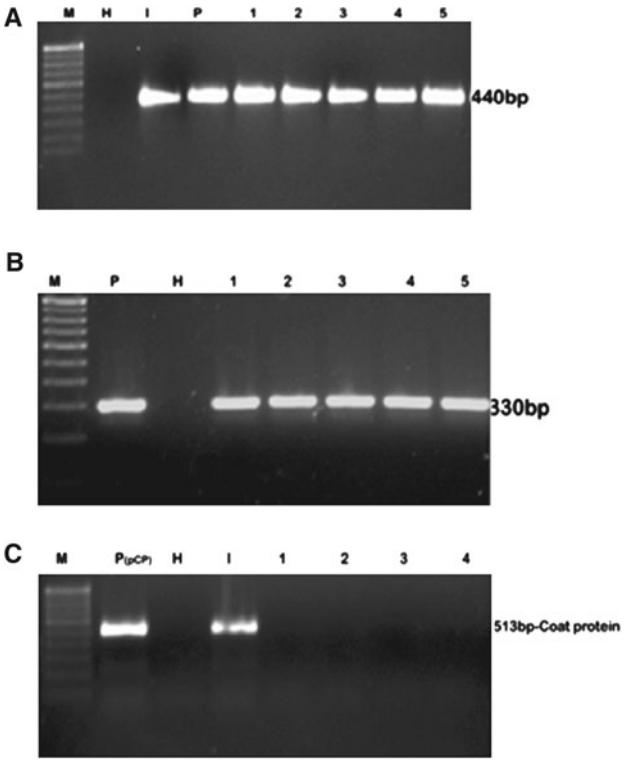

Fig. 8 Molecular analysis of transgenic hill banana through PCR using gene specific primers of *rep* gene (**a**), *npt*II (**b**) and coat protein (**c**). **a** PCR amplification of 440 bp *rep* gene from transgenic hill banana. *Lane M* 100 bp ladder, *Lane H* healthy hill banana plant (negative control), *Lane I* BBTV infected plant, *Lane P* pEB1 (positive control), *Lane 1–5* transgenic hill banana. **b** PCR amplification of 330 bp *npt*II gene from transgenic hill banana. *Lane M* 100 bp ladder, *Lane P* pEB1 (positive control), *Lane H* healthy hill banana plant (negative control), *Lane 1–5* transgenic hill banana. **c** PCR amplification of 513 bp coat protein. *Lane M* 100 bp ladder, *Lane P* pCP (positive control), *Lane H* healthy hill banana plant (negative control), *Lane I* BBTV infected plant, *Lane 1–4* transgenic hill banana

Molecular analysis of the transgenic plants and virus challenged transgenic hill banana plants

Twenty seven plants were analysed through PCR for the presence of the *npt*II gene and *rep* gene. The presence of *rep* gene was confirmed in all the screened putative transformed plants. The amplified product of about 440 bp

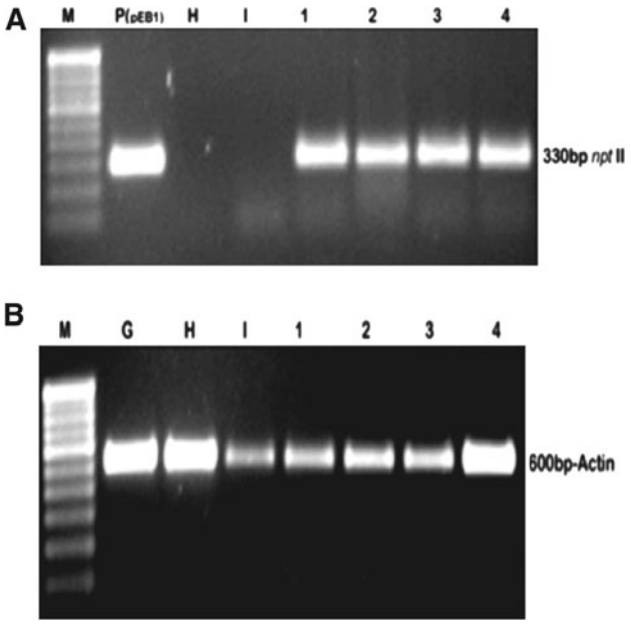

Fig. 9 RT-PCR analysis of RNA isolated from leaf tissue of putative transformed lines using *npt*II (**a**) or actin (**b**) specific primers. **a** RT-PCR amplification of 330 bp *npt*II gene from transgenic hill banana. *Lane M* 100 bp ladder, *Lane P* pEB1 (positive control), *Lane H* healthy hill banana plant (negative control), *Lane I* BBTV infected plant, *Lane 1–4* transgenic hill banana. **b** RT-PCR amplification of 660 bp Actin gene from transgenic hill banana. *Lane M* 100 bp ladder, *Lane G* banana genomic DNA, *Lane H* healthy hill banana plant, *Lane I* BBTV infected plant, *Lane 1–4* transgenic hill banana

was observed from the genomic DNA of all putative transgenic plants tested using *rep* gene specific primers confirming the presence of transgene in all transgenic plants (Fig. 8a). Further to prove that the amplification of *rep* is from the integration of transgene cassette alone and not because of BBTV infection of source plant material. Both *cp*, *rep* and *npt*II genes were also targeted for PCR analysis of transformed hill banana plants. Amplification of *rep*, *npt*II and absence of *cp* in transformed Hill banana plants clearly indicates the integration of transgene in the transformed plants. As there is no *cp* amplification noticed in all the transformed plants screened it is definite that there is no viral infection in the transformed plants and the amplification of *rep* gene is solely due to the integration of transgene and not because of the viral infection (Fig. 8a, c). Even though the male flower buds were collected from hotspot for BBTV infection, the explants were taken from apparently healthy plants and also the explants were taken from the meristematic dome of the male flower.

Further four of these putative transgenic hill banana plants were subjected to RT-PCR analysis using RNA from leaf tissue in order to verify *npt*II transgene expression. The *npt*II transcript amplification of expected fragment size (330 bp) was observed from RNA samples of all tested transgenic plants (Fig. 9a). Specific actin transcript amplification was detected from all plants as an internal control for cDNA synthesis (Fig. 9b). A gDNA control was included in the assay with Actin primers and showed the larger unspliced fragments, indicating DNA contamination was below PCR detection levels in RNA samples (Fig. 9b).

Evaluation of transgenic banana for resistance to BBTD

The transgenic banana plantlets containing the *ihp rep* gene were tested for BBTD resistance by artificial transmission of in vitro plantlets under controlled conditions. Eight PCR-positive transgenic lines (T1–T8) were artificially transmitted with BBTV using insect vector *Pentalonia nigronervosa*. All the tested transgenic lines did not show any symptoms through the duration of the experiment [90 days post transmission (dpt)], indicating that the *ihp rep* gene could provide resistance to BBTV. The control non-transgenic plantlets developed symptoms in about 15 dpt and were completely stunted within 60 dpt (Fig. 10).

Molecular analysis of virus challenged plants

In order to investigate the effect of virus challenge in the transgenic lines, PCR analyses using primers directed against BBTV coat protein, and native Rep were performed using total genomic DNA extracted from the BBTV-inoculated untransformed control and the transgenic plants. BBTV coat protein and native *rep* gDNA sequence were efficiently detected in inoculated untransformed control

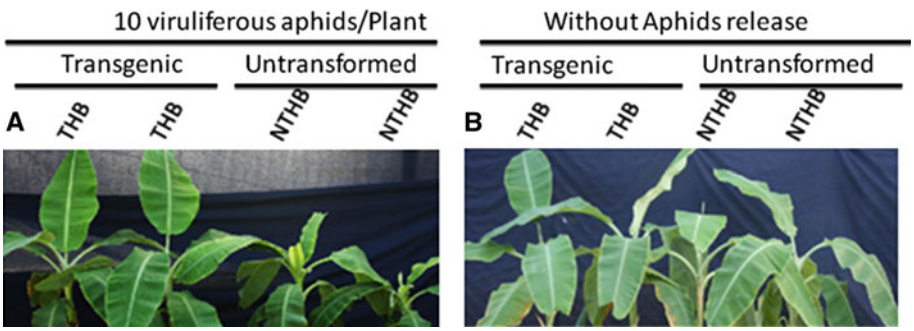

Fig. 10 Virus challenged after 90 days bunchy top virus symptom expression in transformed hill banana (THB) and non-transformed hill banana (NTHB). **a** Transgenic hill banana without symptom and non-transformed hill banana with bunchy top symptom; **b** transgenic hill banana and non-transformed hill banana without bunchy top symptom

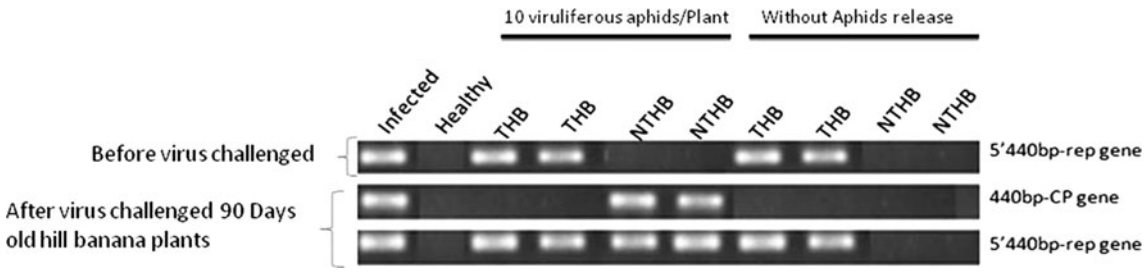

Fig. 11 Molecular analysis for BBTV resistance, before and after virus challenging of transgenic hill banana (THB) and non-transgenic hill banana (NTHB) through PCR after 90 days using gene specific primers of *rep* gene, *npt*II, coat protein and actin internal control

plants, which were showing typical BBTD symptoms. On the other hand, the PCR assay failed to detect any of these gDNAs in the samples obtained from *ihp* BBTV *rep* transformed plants (Fig. 11).

In summary, our results confirm that expression of the BBTV *ihp rep* gene in transgenic hill banana leads to enhanced resistance to BBTD. Transgenic bananas expressing ihp *rep* gene appear to have significant potential to overcome the BBTD, which will boost the available arsenal to combat this epidemic disease and save livelihoods in India. As this transgenic approach has shown resistance against several viral pathogens, it may also provide effective control of other viral diseases of banana such as streak virus in other parts of the world.

Acknowledgments This work was financially supported by the Department of Biotechnology, Government of India. Mr. R. Pavalarajan, Planter and the Tamil Nadu hill banana growers association helped in Virupakshi inflorescence collection from lower Pulney hills.

References

Beetham P, Hafner GJ, Harding RM, Dale JL et al (1997) Two mRNAs are transcribed from Banana bunchy top virus DNA-1. J Gen Virol 78:229–236

Broth W, Perez E, Cheah K, Chen Y, Xie WS, Gaskill D, Khalil S, Hu JS et al (2009) Banana bunchy top virus-resistant transgenic banana plants. Global perspectives on Asian Challenges (September 14–18) Guang Zhou, China. pp 84–85

Burns TM, Harding RM, Dale JL et al (1995) The genome organisation of banana bunchy top virus: analysis of six ssDNA components. J Gen Virol 76(192):1471–1482

Chellappan P, Masona M, Vanitharani R, Taylor N, Fauquet C et al (2004) Broad spectrum resistance to ssDNA viruses associated with transgene induced gene silencing in Cassava. Plant Mol Biol 56:601–611

Cote FX, Domergue R, Monmarson S, Schwendiman J, Teisson C, Escalant JV (1996) Embryogenic cell suspensions from the male flower of *Musa* AAA cv. Grand naine. Physiol Plant 97:285–290

Elayabalan S, Kalaiponmani K, Kumar KK, Angappan K, Balasubramanian P et al (2008) Genetic Engineering of Hill banana ecotypes against BBTV. In: International conference on Banana, Banana and plantain in Africa: Harnessing international partnerships to increase research impact (5–9th October) Mombasa, Kenya, pp 165–166

Engler D, Gutterson N, Nisbet GS et al (2000) Method of genetically transforming banana plants. United States Patent Application, Publication No. 6:133,035

Ganapathi TR, Higgs NS, Kurti PJB, Arntzen CJ, May MD et al (2001) *Agrobacterium*–mediated transformation of embryogenic cell suspensions of the banana cultivar Rasthali (AAB). Plant Cell Rep 20:157–162

Hafner GJ, Harding RM, Dale JL et al (1997) A DNA primer associated with banana bunchy top virus. J Gen Virol 78: 479–486

Harding RM, Burns TM, Dale JL et al (1991) Virus-like particles associated with banana bunchy top disease contain small single-stranded DNA. J Gen Virol 72:225–230

Hu JS, Wang M, Sether D, Xie W, Leonhardt KW et al (1996) Use of polymerase chain reaction (PCR) to study transmission of banana bunchy top virus by the banana aphid (*Pentalonia nigronervosa*). Ann Appl Biol 128:55–64

Khanna H, Becker D, Kleidon J, Dale JL et al (2004) Centrifugation assisted *Agrobacterium tumefaciens*-mediated transformation (CAAT) of embryogenic cell suspensions of banana (*Musa* spp. Cavendish AAA and Lady finger AAB). Mol Breed 14:239–252

May GD, Afza R, Mason HS, Wiecko A, Novak FJ, Arntzen CJ et al (1995) Generation of transgenic banana (*Musa acuminata*) plants via *Agrobacterium*- mediated transformation. Biotechnol J 13: 486–492

Njoroge AM, Geijskes RJ, Harding RM, James AP, Tsao TT, Becker DK, Dale JL et al (2009) Towards transgenic resistance to banana bunchy top virus (BBTV) by expression of defective viral reps. Global perspectives on Asian challenges (September 14–18) Guang Zhou, China, p 164

Pooggin M, Shivaprasad PV, Veluthambi K, Hohn T et al (2003) RNAi targeting of DNA virus in plants. Nat Biotechnology 21:131–132

Smith NA, Singh SP, Wang MB, Stoutjesdijk PA, Green AG, Waterhouse PM et al (2000) Gene expression—total silencing by intron-spliced hairpin RNAs. Nat 407:319–320

Tenllado F, Llave C, Diaz-Ruiz JR et al (2004) RNA interference as a new biotechnological tool for the control of virus diseases in plants. Virus Res 102:85–96

Vanitharani R, Chellappan P, Fauquet CM et al (2003) Short interfering RNA-mediated interference of gene expression and viral DNA accumulation in cultured plant cells. Proc Natl Acad Sci 100:9632–9636

Wanitchakorn R, Harding RM, Dale JL et al (1997) Banana bunchy top virus DNA-3 encodes the viral coat protein. Arch Virol 142:1673–1680

Wanitchakorn R, Hafner GJ, Harding RM, Dale JL et al (2000) Functional analysis of proteins encoded by Banana bunchy top virus DNA A4 to A6. J Virol 81:299–300