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RESEARCH ARTICLE

Construction of BBTV *rep* gene RNAi Vector and Evaluate the Silencing Mechanism through Injection of *Agrobacterium tumefaciens* Transient Expression System in BBTV Infected Hill Banana Plants *cv.Virupakshi* (AAB)

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ABSTRACT

One of the most severe viral diseases of banana worldwide is caused by banana bunchy top virus (BBTV). It is a nanovirus transmitted by the aphids *Pentalonia nigronervosa*. Currently, there are no strategies available in order to completely protect bananas against the BBTV virus. The improvement of banana resistance to BBTV disease through conventional breeding has not been successful and therefore an alternative approach such as genetic engineering system requires producing resistance banana cultivar. In this study, gene isolation and construction of RNAi vector for bunchy top disease management in hill banana was determined. The target of the RNAi is the *Rep* protein encoded by the BBTV-DNA1. It can be concluded that the isolated *rep* gene displayed 100% sequence identity to the BBTV *rep* gene of Indian isolate deposited in the NCBI database. This result shows that BBTV *rep* gene is highly conserved among the different isolates of BBTV that infect the banana in India. Hence, an attempt was made to construct the RNAi vector for BBTV *rep* gene and developed method for evaluate the silencing mechanism through injection of *Agrobacterium* transient expression system in BBTV infected hill banana plants *Virupakshi* (AAB) using RNAi technology to silence and suppress the *rep* gene of BBTV.

Keywords : RNAi-BBTV *rep*, Hill banana, Pathogen-derived resistance.

INTRODUCTION

Hill bananas are known for their special flavour and long shelf life which is unique to the state of Tamil Nadu in India. Hill bananas are perennial in nature which are cultivated along with coffee, pepper and through multitier system. In Tamil Nadu (Southern India), hill bananas (two ecotypes, AAB Pome group) namely *Virupakshi* and *Sirumalai* were grown at a height of 2000 to 5000 feet with well distributed annual rainfall of 1250-1500 mm in the lower Palani, Sirumalai and Kolli hills. However, hill bananas were highly susceptible to banana bunchy top virus (BBTV). BBTV has been the sole cause for reduction of hill banana cultivation from 18,000 ha in 1970s to a mere 2,000 ha at present. Currently, there are no strategies available in order to completely protect hill bananas against the virus and improvement of this crop through conventional breeding has not been successful.

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.,1993), 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.,1997).

For the last five years, two research groups namely Queensland University of technology (QUT) and University of Hawaii (UH) were involved in developing transgenic bananas, particularly on Cavendish type with an attempt to produce resistance variety to BBTV disease. John Hu and co-worker of University of Hawaii reported that several putative transgenic lines expressing mutated or anti-sense *Rep* genes with partial resistance to BBTV (Broth et al., 2009). They reported that some of the transgenic banana plants remained virus free symptom after infected for up to a year. Similarly, James Dale and his group of Queensland University of technology developed BBTV resistance in banana by using a novel approach (Njoroge et al., 2009). In this strategy, virus activated cell death which involves integration into the host plant by construct an encoding a split suicide gene, flanked by the target virus intergenic region in turn is embedded in the introns. The suicide gene is only activated upon infection by the target virus and is only expressed in cells by the similar by the target virus. Activation by viral *Rep* mediated replicative release and circularisation which the suicide gene is reconstituted leading to transcription which will processing out the intergenic region embedded in the intron prior translation of the suicide gene in the final stage. However, both the groups did not use the RNA interference (RNAi) technology for imparting the BBTV resistance in banana.

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, RNAi can be engineered to effectively target DNA virus of Mung Bean Yellow Mosaic Virus (MYMV-Vig) (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. It was shown that reduction in the accumulation levels of AC1 mRNA was achieved by more than 90% and viral DNA by 70% compared with control treatments (Vanitharani et al., 2003). Similarly, 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).

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, engineer resistance to bunchy top disease in hill banana cultivar, *Virupakshi* (AAB*)* was possible by using RNAi technology to silence the *rep* gene of BBTV. Before to generate the transgenic hill banana, to study the mechanism of the RNAi construct in infected hill

banana plants through injection of *Agrobacterium tumefaciens* transient expression system in BBTV infected hill banana plants *Virupakshi* (AAB*)* using RNAi technology.

MATERIALS AND METHODS

Cloning of the BBTV *rep* gene with PCR

Fresh young emerging green leaves with midribs were collected from the infected plants and DNA was isolated using the modified CTAB protocol (Sambrook et al., 1989) and stored at -70°C for further use. Prior to extraction, 100 to 300 mg of midrib of young hill banana leaves were cut into bits and transferred to a zip lock bag (7 cm x 9 cm) and one (1) mL of extraction buffer (0.2 M EDTA, 1.4 M NaCl, 1 M, CTAB 2 %) was added immediately. The samples were kept 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 65ºC (heating blocks) for 10-12 min and then the tube was centrifuged for 10 minutes 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 (1mM Tris-HCl pH 8.0 and 0.1mM EDTA pH 8.0) and kept at 65º C for 3 min (to suspend the pellet well) and stored at -20º 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 into T/A cloning vector (pTZ57R/T (MBI Fermentas) and sequenced.

Construction of RNAi vectors using particle 5' and 3' end of *rep* gene

For the RNAi construct targeting the BBTV *rep* gene was initiated with the full-length *rep* gene 870bp cloned from BBTV infected hill banana sample by designed gene specific primer. Then, the amplified PCR product was cloned and the presence of *rep* gene confirmed by DNA sequencing. The isolated *rep* gene displayed 100% identity to the previously reported BBTV sequences. In order to construct the RNAi vector, 440 bp of 5' and 440bp of the 3'end were utilized. The partial gene fragment was cloned in sense and anti- sense orientation in intermitted RNAi vector pSTARLING (CSIRO plant industry, Australia Fig 2.A). This vector contains Ubiquitin promoter, Ubi intron, Cre intron, restriction site of sense and anti-sense. The cloned RNAi gene cassette was released by *Not* I enzyme digestion and cloned into the *Not* I site of binary vector pART27 (CSIRO plant industry, Australia Fig 2.B). The pART27 contains *npt11* gene to be used a plant selection marker.

Plant materials, preparation of agroinjection suspension and injection method

Field grown BBTV infected hill banana suckers were obtained from an orchard in the Lower Pulneys Hills, located at 2000 to 5000 feet sea level in the Western Ghats of Tamil Nadu. *Agrobacterium tumefaciens* strain LBA4404 used in this 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 hrs to obtain 1 O.D (optimal density) culture. The *Agrobacterium* cells pelleted by centrifugation at 4000 rpm for 10 minutes and dissolved in equal volume agroinjection medium +10g/glucose,200

uM acetosyringone pH 5.5(Sallaud et al.,2003) by vortexing to give an absorbance at 600nm between 0.5 and 0.8. Injection of RNAi construct with help of insulin syringe 12.7mm needle (BD Ultra- Fine needle) into BBTV infected hill banana rhizome the junction between the cortex tissue and terminal growing point (apical meristem) of the rhizome. Sucker is planted in autoclaved sterile pot mixer. After 3-4 weeks, the BBTV recovery symptoms were observed based on the typical symptoms of BBTD (Goddard, 1929). Agroinjection work was carried out in Department of Biotechnology (DBT) Government of India approved Tamil Nadu Agriculture University transgenic green-house condition.

RESULTS

Cloning and characterization of BBTV *rep* gene

Total genomic DNA isolated from virus infected banana leaves was used as a template for PCR amplification of *rep* gene. By using the designed gene specific primers, full length BBTV *Rep* gene of an expected size of 870 bp was obtained via PCR amplification. The PCR amplified products was then cloned into T/A cloning vector, pTZ57R/T (MBIFermetase, USA). Recombinant colonies were identified by blue/white screening and the presence of the insert confirmed by digestion with *Eco*RI and *Hind*III to release the DNA fragment of 870bp. Five recombinant clones were selected and DNA sequencing was carried to determine the DNA sequence. All the five clones displayed no variation in DNA sequence, indicating very high level of sequence similarity. The isolated BBTV *rep* gene showed 100% sequence similarity to the previously reported sequence from Indian BBTV isolate deposited in NCBI database.

Construction of RNAi-*Rep* gene constructs

Cloning of partial BBTV *rep* gene in pSTARLING-A vector

Partial *rep* gene of size 440 bp (1-440 nt region of *rep* gene) was amplified from full length *rep* gene 870bp (Fig. 3a), then cloned into sense and antisense orientation in the pSTARLING-A. Recombinant clones containing both sense and antisense of *rep* gene was identified by the release of 1.58 kb on double digestion with *Bam*HI and *KpnI*. In addition, cloning of either sense (*Bam*HI and *Asc*I) or antisense (*Spe*I and *Kpn*I) of *rep* gene into pSTARLING-A releases 1.14 kb (Fig. 3b, c) fragment. The recombinant clones containing both the sense and antisesense of the *rep* gene was selected and the RNAi-*Rep* gene cassette of size 4.1kb was then released through *NotI* restriction digestion (Fig. 3d). Then, the released fragment was cloned into the *Not*I site of the plant transformation vector (Fig. 3e), pART27 (Fig2.B) and designated as pEB1.The pEB1 binary vector was mobilized into the *Agrobacterium* strain LBA4404 by tri-parental mating method. The presence of pEB1 in *Agrobacterium* strain LBA4404 was confirmed by back transformation into *E.coli* and PCR analysis. Another RNAi vector was constructed utilizing the 440 bp of the 3'end of the BBTV Rep gene (3' of 440bp to 3'of 870bp) and designated as pEB2.

Evaluation of *Agrobacterium* injected pEB1 RNAi construct into BBTV infected hill banana for recovery to BBTD

Agrobacterium injected BBTV infected hill banana plants, the emergence of new leaf the bunchy top virus symptom recovery expression was observed. The leaf number one and two the green streaks were absent and leaf area normally grown as such bunchy top affected leaf and third leaf was the leaf area was expanded and completed green streaks was absent (Fig.4 C). After 35 days the fourth leaf was no bunchy top symptoms and normally grown the plants (Fig.4.D).After 45 days the non-injected hill banana (NIHB) and injected hill banana (IHB**),** the agro injected infected hill banana plant did show the BBTV recovery symptoms through the duration of the experiment 45 days, The control non- injected plantlets developed symptoms were completely stunted and bunchy top symptom (Fig

5.A). The result clearly indicating that the ihpRep gene could provide resistance to BBTV. This result clearly indicated RNAi mechanism was working in transient expression in banana plant.

DISCUSSION

Banana bunchy top virus consists of at least six component of DNA (DNA1 to DNA6) and is transmitted by the aphid, *Pentalonia nigronorvosa.* Out of six DNA component, DNA1 codes for the replication initiation protein (*rep* gene). The *rep* gene is selected for the constructed of RNAi vector as it is reported to be indispensable for the replication of BBTV. *rep* gene is also found to be important for the replication of ssDNA virus that include Geminivirus and nanovirus (includes BBTV, Faba bean necrotic yellow virus [FBNYV]), Milk vetch dwarf virus [MDV] and Subterranean clover stunt virus [SCSV] that causes disease in French bean, Faba bean, Pea). Unlike RNA viruses, the genomes of plant single-stranded DNA viruses do not encode polymerases. Instead, their replication requires interaction between a viral replication-associated protein (Rep) and host polymerases. Rep protein mediates origin recognition and DNA cleavage/ligation to begin and end rolling circle replication (Fontes et al., 1994; Heyraud et al., 1995). In addition to role in replication, Rep protein also interact with host cellular protein (Morilla et al., 2006) and with Gemini virus Ren protein (Settlage et al., 2005).,which induces the cellular genes required for geminivirus DNA accumulation (Selth et al., 2005). These crucial functions in the replication cycle and its multiple interactions make *Rep* an excellent target for DNA virus control by the expression of mutant Rep protein or to express the dsRNA (RNAi) in order to silence the *rep* gene of virus. Gemini viral Rep proteins have been widely exploited to generate resistance in Mung bean, Bean, Cassava, Tabacco, Tomato and Maize.

The John Hu and co-worker of University of Hawaii had generated several putative transgenic Cavendish banana lines expressing mutated or anti-sense *rep* genes with partial resistance to BBTV (Broth et al., 2009). Even though expression of mutated Rep protein provided resistance to DNA virus in plants, it is often associated with negative effect on plant growth and development (Kong et al., 2000). A potential drawback of *Rep* expression in transgenic plants could be the recovery of phenotypically normal plants due to its interaction with pRBR or with other plant proteins that may alter the cell cycle and differentiation programmes (Kong et al., 2000; Shen, 2002).

It is well documented by several reports that RNAi technology is superior compared to antisense, sense or protein mediated resistance technology. Hence in the present study, RNAi approach was followed to engineer resistance in hill banana. The isolated *rep* gene showed 100% sequence identity to the BBTV *rep* gene which is highly conserved among the different isolates of BBTV that infect the banana in India. In order to target the BBTV Rep gene silencing in banana, two different RNAi constructs were made utilizing the 440 bp of 5' end and 440 bp of the 3' end in the binary vector pART27. First the sense and antisense of the *Rep* gene was cloned into the RNAi intermediate vector, pSTARLING-A containing splicable intron.Linking the sense and anti-sense sequences by an intron which is eventually spliced and resulted in the most efficient silencing in plants (Smith et al., 2000; Wesley et al., 2001). Intron containing constructs (ihpRNA) generally gave 90±100% of independent transgenic plants showing silencing. The degree of silencing with these constructs was much greater than that obtained using either co-suppression or anti-sense constructs. Wesley et al. (2001) reported that use of 98 to 853 nt size gene target produced an efficient silencing in a wide range of plant species.

It is now well established that both RNA and DNA viruses can be controlled by RNAi technology. Unlike the RNA virus where only PTGS is mainly used for virus control, the DNA virus control involves both by TGS and PTGS (reviewed by Vanitharanai et al. 2005). Reports on siRNA accumulation in tomato plants infected with the monopartite geminivirus TYLCV (Lucioli et al., 2003) and in cassava plants infected with the bipartite geminivirus ACMV (Chellappan et al., 2004) clearly reflect the role of the RNAi pathway as a natural defence mechanism against these DNA viruses. Therefore, engineer resistance to bunchy top disease in hill banana cultivar, *Virupakshi* (AAB*)* was possible by using RNAi technology to silence the *Rep* gene of BBTV.

CONCLUSION

The present study demonstrates that transient expression of the BBTV ihp *rep* gene in BBTV infected hill banana leads to recovery of BBTD. Results obtained shown that the RNAi can be engineered to effectively target DNA virus. As this transgenic approach has shown resistance against several viral pathogens, it may also provide effective control of other viral diseases of banana.

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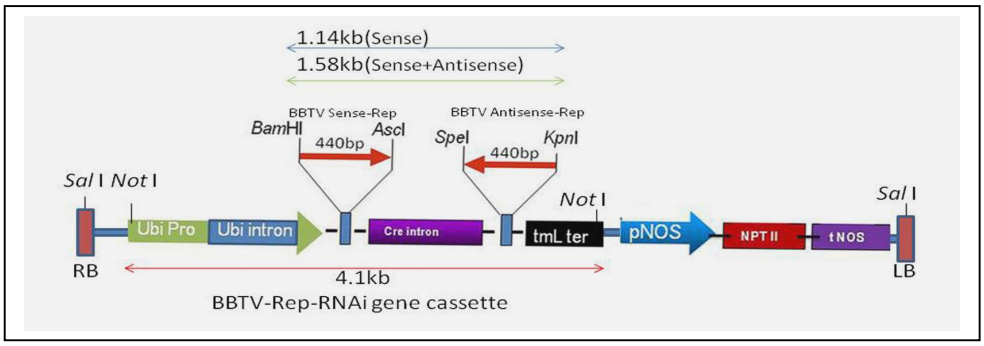

Figure 1: T-DNA region of pEB 1 showing the BBTV rep hpRNAi gene cassette under the control of Ubiquitin Promoter, *Cre* intron and tmL terminator.

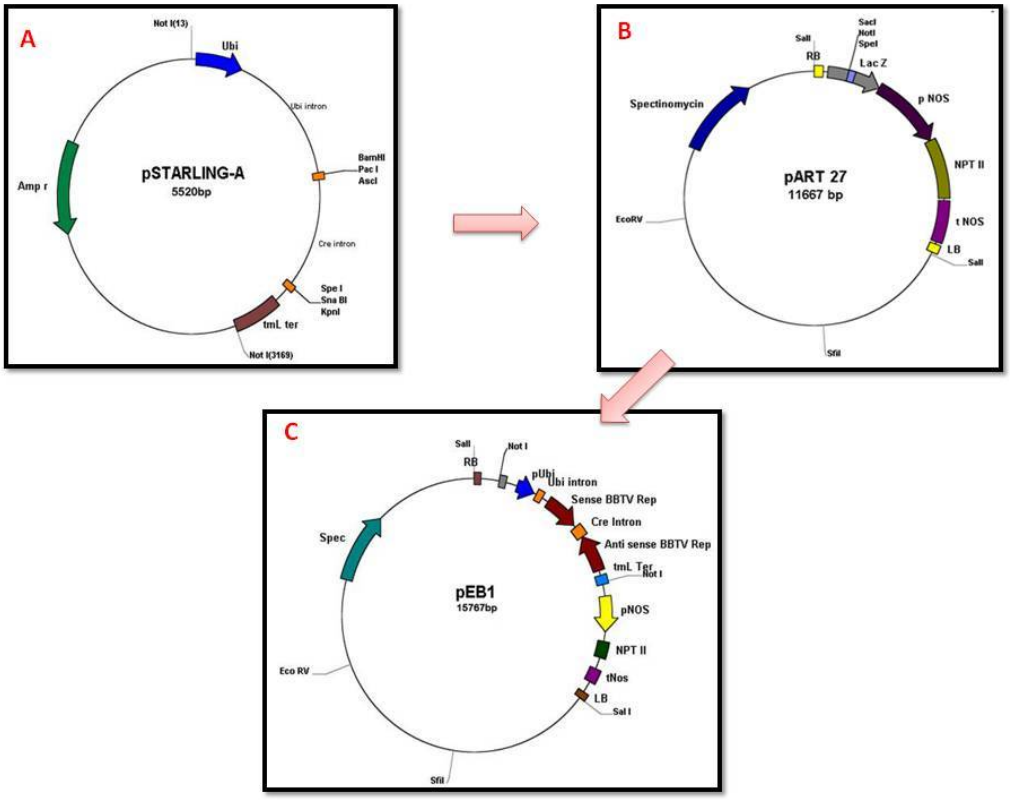

Figure 2 .Steps involving to construction of RNAi vector, by use of intermediated RNAi vector.
(A) Physical map of intermediated RNAi vector - pSTARLING-A ; **(B)** Plant transformation binary vector pART27; **(C)** Binary vector of RNAi-BBTV *rep*.

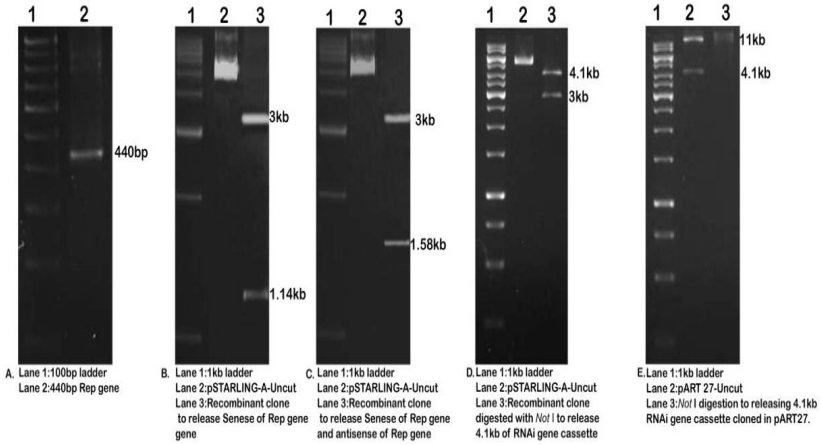

Figure 3. T-DNA region of BBTV *rep* gene RNAi construction

Figure 4 .Transient expression of pEB1 through Agro injection method recovery of BBTD. (A) BBTV infected hill banana plant with typical symptom as plant source of the experiment ; **(B**) Injection of RNAi *rep* gene vector into cortex tissue and apical region of the rhizome region ; **(C** and **D)** Observation made the leaf recovery of BBTV infected hill banana.

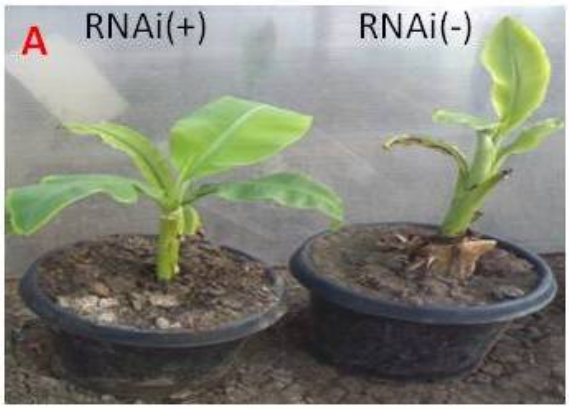

Figure 5. After 45 days BBTV symptom recovery. *Agrobacterium* injected hill banana with BBTV recovery symptom (RNAi +) and non-injected hill banana with bunchy top symptom (RNAi-).