Madras Agric. J., 101 (10-12): 374-378, December 2014
https://doi.org/10.29321/MAJ.10.001218

Agrobacterium-mediated *In planta* Transformation of Hill Banana for Developing Resistance against *Banana Bunchy Top Virus*

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Banana viruses have had a major negative impact on the production of bananas worldwide. Among banana viruses, *Banana Bunchy Top Virus* (BBTV) is one of the most destructive viral diseases affecting Hill banana (AAB) which is known for its special flavor and long shelf life and are unique to the lower Pulney hills of Tamil Nadu, India. In transgenics, Pathogen Derived Resistance (PDR) approach is one of the potential strategies for developing virus resistance in crops which has been proven to be difficult to obtain by conventional breeding. Here we have reported a reproducible and efficient *in planta* transformation method via *Agrobacterium*-mediated genetic transformation in banana. Three-month-old banana suckers were used as explant and *Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector pCAMBIA1301:*bbtv rep* was used in the co-cultivation. The presence and integration of the *gus* and *replicase* genes into the banana genome were confirmed by GUS histochemical assay, polymerase chain reaction and Southern blot hybridization. These results suggest that the *in planta Agrobacterium*-mediated transformation protocol developed in this study for stable integration of *rep* gene of BBTV is efficient and that this transformation system could be widely adopted for routine transformation in banana for transferring genes.

Key words: Banana, Bunchy top, Decortication, Acetosyringone, GUS histochemical assay, *In planta* transformation, *Replicase* gene

Banana is the most popular commercial fruit crop grown all over the world and serves as a staple food in many countries. Banana Bunchy Top Disease (BBTD) caused by Bunchy Top Virus (BBTV) is one of the most destructive diseases of banana in tropical Asia, Australia and the South Pacific (Dietzgen and Thomas, 1991). It is a multi component, single-stranded DNA virus with isometric virions of 18–20 nm in diameter. It retards the growth of infected plants and causes substantial economic losses to banana production (Dale, 1987). BBTV primarily spreads through vegetative propagation and secondarily by banana black aphid vector, *Pentalonia nigronervosa* (Allen, 1978). There are no resistant banana sources to BBTV (Dale, 1987). Hill banana, Virupakshi (ABB) belonging to pome sub group is very famous for its unique flavour, taste, keeping quality and is an ecotype restricted to lower Pulney hills of Tamil Nadu, India and has been registered under GI under PPV& FRA, Government of India (http://www.plantauthority.gov.in/). This variety which was grown in 18,000 ha in 1970's in hills, is now reduced to approximately 2000 ha due to BBTV incidence. In lower Pulney hills, BBTV infection could not be brought down due to rapid spread in the perennial banana by viruliferous aphid vector (*Pentalonia nigronervosa*) which is present round the year in the region. Yield loss due to BBTV is 100 percent if the infection occurs before flower emergence. A loss of 40 crore per annum has been reported in Kerala and the surveys conducted by National Research Centre, Banana during 2007-09 in Andhra Pradesh. In Maharashtra there was a sudden re-emergence of BBTV in 'Grand Naine' banana causing a loss of 50 million US $.

Breeding banana is a complex phenomenon owing to inherent sterility and parthenocarpy in cultivated *Musa* sp. The best option to obtain virus resistant banana is through transgenic approach. Genetic transformation of banana with sequences derived from pathogen is one of the potential strategies for developing virus resistance in banana. Two strategies, namely coat protein- mediated and replicase mediated approaches were most successful in obtaining resistance against target plant viruses. Both strategies have emanated from the concept of "Pathogen Derived Resistance" wherein homology dependent resistance through *replicase* gene has been shown to be robust and widely applicable against several viral pathogens in plants (Prins, 2003; Shekhawat *et al*., 2012).

Although several novel methods for the transformation of plants are available yet presently,

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methods based on the use of the soil bacterium *Agrobacterium tumefaciens* is still preferred in many instances. *Agrobacterium* is capable of transferring any genes to a wide variety of plant species but this can be limited by both host specificity and inability of the bacterium to reach the proper cells in the target tissue. Therefore, a new and efficient *Agrobacterium* based transformation method that overcomes these barriers and enhance DNA transfer is required (Trick and Finer, 1998). In the present study, hill banana has been transformed with *replicase* gene of BBTV through *Agrobacterium* mediated transformation. A new *in planta* method has been attempted for transformation in hill banana with *replicase* gene of BBTV using *Agrobacterium* and decortication method to generate wounds and induce more shoots from transformed cells.

Materials and Methods

Banana cultivar

Healthy virus free suckers of cv. Virupakshi were used for the *Agrobacterium tumefaciens* mediated genetic transformation. The virus indexed BBTV free hill banana plants were collected from research plot of National Research Centre for Banana, Trichy, Tamil Nadu.

Agrobacterium strains and binary vector

Agrobacterium tumefaciens strain LBA 4404 was used in the present investigation. LBA 4404 is an octopine strain with Ach5 chromosomal background carrying pAL4404 as virulence plasmid (Hoekema *et al*., 1983). The plasmid pCAMBIA1301 (CAMBIA, Canberra, Australia), a compact binary vector (11,849 bp), was used. This binary vector contains the â-glucuronidase (*gus*) gene under the control of CaMV 35S promoter, hygromycin resistance gene (*hpt*) driven by the CaMV 35S promoter for plant selection, and a kanamycin resistance gene (*aadA*) which is located outside of the T-DNA regions and only used for bacterial selection. The binary vector pCAMBIA1301 harboring the *replicase* gene was mobilized into *A. tumefaciens*, via electroporation. *A. tumefaciens* strain was maintained on solid LB agar medium supplemented with 10 mg/l of rifampicin and 50 mg/l of kanamycin.

Inoculation and co-cultivation

A single colony of *Agrobacterium* was transferred to 5 ml Luria broth containing 10 mg/l rifampicin and 50 mg/l kanamycin in a vial. The culture was incubated at 28°C in a rotary shaker at 200 rpm for 24 h and was removed at mid-log phase (OD600=0.5) when the cell density was approximately 10_8/ml. The gi was diluted to 1/10 with half-strength MS basal medium. The pseudostem of each virus free hill banana sucker of medium size was cut transversally 2 cm above the collar of the rhizome using a knife disinfected with 2% formol before each operation and the apical meristem was removed at a depth of 4 cm, leaving a 2 cm-diameter cavity in the rhizome; the pseudostem was given sharp cut with crosswise incisions, boring down to the rhizome collar as shown in Fig.1. Three different methods of co-cultivation were followed: *viz*., Injection method, pin prick method and incision made with knife smeared with *Agrobacterium* harbouring the gene. The suckers were co-cultivated with *Agrobacterium* culture having 100mM acetosyringone for 40 min and then thoroughly rinsed and blotted. The rhizomes were then covered with sterilized saw dust and later transferred to a pot containing a mixture of equal parts of sandy loam and poultry dung compost to 5 cm above ground level. Next day 4 ml of benzylaminopurine (BAP) at 40 mg/l was poured on each treated suckers in the cavity left by the removal of the apical meristem. When the new suckers originating from the main sucker (G_2S-second generation) reach a height of 20 to 30 cm, they were dissected again using the same procedure described above, the same quantity of BAP was applied in the cavities and the operation was completed in the same way until third generation suckers (G_3S) were obtained (Macias, 2001). A control was maintained in similar way without using acetosyringone. Each treatment comprised 25 suckers in replicates of three.

GUS histochemical assay

Histochemical assay of GUS was performed according to Jefferson et al. (1987). The banana leaf from the transformed suckers was used for GUS assay. The leaves were incubated overnight at 37_oC in 2 mM X-Gluc (5-Bromo-4-chloro-3-indolyl â-D-glucoronide) in a phosphate buffer (pH 7.0) containing 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1% v/v Triton X-100. The chlorophyll was removed by using 95% ethanol after X-Gluc staining.

Confirmation of transgenic lines using PCR

Total genomic DNA was isolated from leaf tissue by the CTAB method and subjected to PCR using *gus* gene-specific oligonucleotide primer pairs: a forward primer 52 -AGCATCTCTTCAGCGTA AGG-32 and a reverse primer 52 -TGAACAACG AACTG AACTGG-32 with an expected amplicon of 611 bp. Similarly, BBTV *rep* gene-specific oligonucleotide primer pairs: a forward primer 52 -TTGGAT CCATGGCGCG ATATGTGGTA TGC-32 and a reverse primer 52 -TCAGCAAGCAACCAACTTTATTCGA-32 with an expected amplicon of 861 bp were also amplified by PCR. Each 25 ìl PCR reaction mixture contained 10 ng genomic DNA as template, 200 ìM dNTPs, 1.5 mM $MgCl_2$, 15 pM each of forward and reverse primers and 1 unit *Taq* DNA polymerase in 1X reaction buffer. The reaction was carried out in a thermocycler (Eppendorf Master Cycler) at 94°C for 5 min for initial denaturation followed by 40 cycles of

94°C for 1 min, 53°C for 30 s and 72°C for 1 min. A final extension time of 10 min was allowed at 72°C at the end of 40 cycles to allow the completion of synthesis of any incomplete fragments. PCAMBIA1301::BBTV *rep* plasmid and non-transformed plant DNA were used as a positive and negative control, respectively. The reaction product was subjected to electrophoresis in 0.8% agarose gel and DNA bands were visualized under ultraviolet light after staining with ethidium bromide.

Southern blot analysis

Total genomic DNA isolated from leaf tissue of putative transgenic lines by the CTAB method was subjected to southern blot hybridization analysis using standard protocols. Genomic DNA (15 ìg) from PCR positive and non- transformed plantlets was digested with *Hind* III restriction enzyme, resolved on 0.9% agarose gel and blotted on a Hybond N + nylon membrane (Amersham Biosciences, UK) by capillary method and was then subsequently immobilized on the membrane by UV cross linking. Genomic DNA isolated from a non transgenic plant served as a negative control and PCR amplified *rep* gene, served as a positive control in the Southern blot. The membrane was then exposed to DIG labeled probes generated using *rep* gene PCR product as template. Chemiluminescent detection of hybridization signals was performed using PCR DIG probe synthesis kit and chemiluminescent detection Starter Kit II (Roche Applied Science) according to manufacturer's instruction.

Results and Discussion

In the present study, a tissue culture-independent *in planta* transformation protocol was used to develop transformants in banana (Fig. 1). In this method, *Agrobacterium* is targeted to the wounded apical meristem of the banana sucker. Therefore, *Agrobacterium tumefaciens* transfers the gene into the genome of diverse cells which are already destined to develop into specific organs and also the meristematic cells still to be differentiated into plantlets.

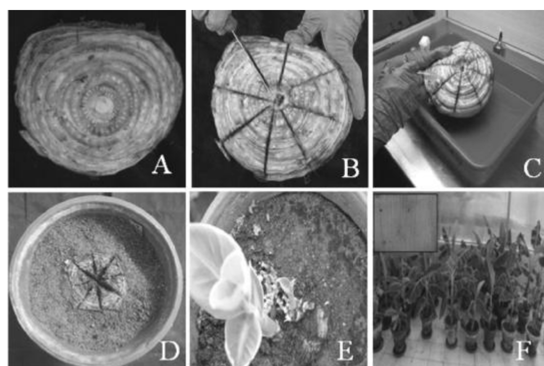

Fig. 1 Steps involved in *in planta* transformation of banana suckers cv. Hill banana (ABB).

A. Banana sucker. **B**. Decorticated banana suckers showing cross-shaped cutting and apical meristem cavity. **C.** Co-cultivation of decorticated sucker by injection method. **D.** Co-cultivated sucker planted in a pot. **E.** Developing buds. **F.** Plantlet transplanted a pot. Inset: Stable *gus* expression in transformed banana leaf).

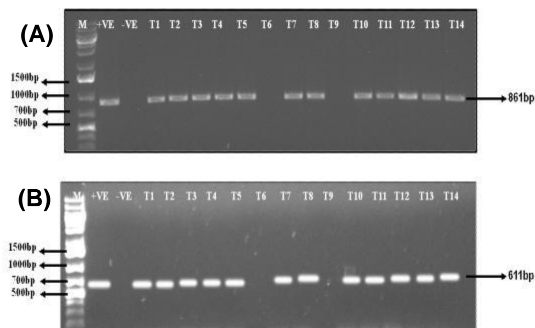

Fig. 2 Molecular analysis of putative transgenics of Banana transformed with pCambia::BBTV*rep* plasmid.

(A) PCR amplification of the genomic DNA showing the amplified product of 861 bp fragment of the *rep* gene from T_0 plants. Lane M = GeneRuler™ 1 kb DNA Ladder; Lane 2 = Positive control (plasmid DNA of the gene construct pCAMBIA:: BBTV*rep* plasmid); Lane 3 = Negative control (control tobacco genomic DNA); Lane 4, 5, 7, 8, 10, 11,12,13 and 14 = Putative transgenic plants. **(B)** PCR amplification of the genomic DNA showing the amplified product of 611 bp fragment of *gus* gene from T_0 plants. Lane 1= GeneRuler™ 1 kb DNA Ladder; Lane 2 = Negative control (control Banana genomic DNA); Lane 3 = Positive control (plasmid DNA of the gene construct pCAMBIA::BBTV*rep* plasmid); Lane 4, 5, 7, 8, 10, 11,13,14,15,16 and 17 = Putative transgenic plants

A total of 225 suckers were decorticated and transformed with *Agrobacterium* harbouring *gus* and *rep* gene Table: 1. The leaves obtained from the transformed and non-transformed suckers were assayed histochemically for GUS expression. Eighty two plants showed GUS expression which clearly indicated the integration of the T- DNA into the banana plant genome. About 54% of the plants exhibited uniform blue coloration indicating that the plantlets probably been regenerated from a single transformed cell. However, 36% of plants showed mosaic appearance and were treated as chimeric for the trait.

Further confirmation of T-DNA integration was done by PCR and Southern hybridization. Total genomic DNA isolated from the GUS positive plants was subjected to PCR using *gus* and *rep* genes specific primer pairs. All produced an expected amplicon of 861 bp (Fig. 2A) and 611 bp (Fig. 2B) respectively , whereas no amplification was observed in non transformed control plants indicating that all putative transformed plants were positive for the transgene. However, screening transgenics by PCR is not foolproof, as reported by

Table 1. Effects of micro wounding in *In planta* co-cultivation

Methods	No. of decorticated suckers	No. of decorticated suckersformed shoots	Gus positive plants(%)	No. of PCR positive plants
Pin prick	75	54	31.8	14
Injection	75	62	61.36	27
Cutting with knife smeared with *Agrobacterium*	75	57	6.81	3

many workers (Barik *et al*., 2005; Hiei *et al*., 1994) due to the possible presence of *Agrobacterium* in the transformed plants. Hence, in order to confirm the stable integration of the transgene, a Southern hybridization was done.

All the PCR positive plants were subjected to southern hybridization to confirm the integration of T-DNA into genomic DNA. Of these, 10 plants (12%) showed a positive reaction confirming stable integration of the *rep* gene (Fig. 3) . All the ten Southern-positive plants produced a single band indicating the presence of single copy of the transgene. The detected bands were above the expected minimum size of the positive control as indicated by its position in the blot. No hybridization signal was observed in the non-transformed plants.

Neither the *rep* gene nor the β- glucuronidase gene (*gusA*) had any apparent effect on the normal development and morphology of the transgenic

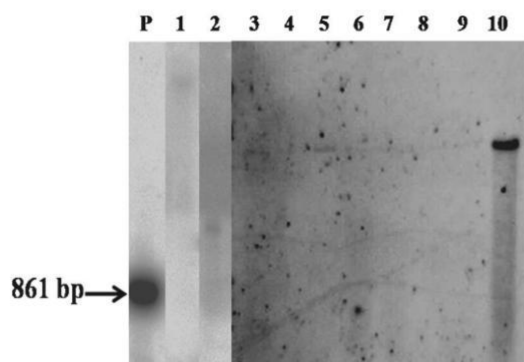

Fig. 3 Southern analysis of putative transgenic banana.

Lane P = Positive control; Lanes 1 to 10 = Putative transgenic plants.

banana plants as all plants maintained in the greenhouse exhibited normal growth and morphology and had a uniform appearance. These plants have to be further tested for their resistance against BBTV. The efficiency obtained by method described in this paper the transformation would allow production of transgenic banana with genes of desirable characters. Similar approach of *in planta* transformation has been successfully attempted in many crops like rice (Supartana *et al*., 2005), buckwheat (Kojima *et al*., 2000), kenaf (Kojima *et al*., 2004), mulberry (*Ping* et al., 2003) and cotton (Keshamma *et al*., 2008).

The simple transformation protocol for hill banana described here does not use tissue culture and offers several potential advantages over shoot tip transformation. We have tested this protocol with other *Musa* cultivars (data not included) and it appears to be cultivar independent. It also opens up new possibilities for improvement of banana cultivars through the introduction of new traits, thus increasing the area that can support the growth of banana and plantain significantly contributing to food security and poverty alleviation.

Acknowledgements

The authors are grateful to the Department of Biotechnology (DBT), New Delhi, for the funding of this project and the Director, NRCB, Tiruchirapalli for encouragement and facilities.

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Received: March 18, 2013; Revised & Accepted: December 29, 2014