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Full Length Research Paper

Rapid and efficient production of transgenic East African Highland Banana (*Musa* spp.) using intercalary meristematic tissues

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East Africa is the largest banana producing and consuming region in Africa. In particular, the East African Highland Banana serves as the major staple crop of countries like Uganda, but production is constrained by a number of serious pests and diseases. Banana breeding is a very difficult and slow process, so genetic engineering offers an alternative approach to improvement. A transformation system using intercalary meristematic tissues was developed using *Agrobacterium* strain EHA105 harboring the binary vector pCAMBIA2301 containing the *gus*A reporter gene and *npt*II as selectable marker. In this paper, a new transformation protocol is described that yields kanamycin-resistant, GUS-expressing banana plants from roughly 10% of the initial explants. The resulting fully-rooted transgenic plants do not appear to be chimeras since they can be stably propagated, GUS activity is observed uniformly throughout the plants including the germline cells of the meristem, and PCR and Southern blots indicate stable integration of the genes into the genome.

Key words: *Agrobacterium*, intercalary meristem, genetic transformation, banana.

INTRODUCTION

Bananas are the fourth most important food crop in the tropical and sub-tropical zones of the world. Annual banana production in the world is estimated at 104 million tons of which less than 10% enters the commercial market, suggesting that the crop is more important as food for local consumption than for export (FAOSTAT, 2004). Most bananas produced in Africa are used as a staple food and a source of cash income for small-holder farmers. In some of the African countries such as Uganda, the daily consumption of banana may exceed 1.6 kg per person (FAOSTAT, 2001), highest in the world. East Africa is the largest banana producing and consuming region in Africa with Uganda being the world's second leading producer with the total production of about 10.5 million tons (FAOSTAT, 2004). More than 85% of the bananas grown in Uganda are comprised of the East African Highland Bananas (*Musa* spp. AAA genotype; EAHBs) including cooking (matooke) and beer (mbidde) types. The EAHBs are special types of banana grown in the Great Lake zone of East Africa.

Banana production is severely limited by various diseases and pests, such as black Sigatoka (*Mycosphaerella fijiensis*), banana *Xanthomonas* wilt (*Xanthomonas campestris* pv. *musacearum*), Fusarium wilt (*Fusarium oxysporum* f. sp. *cubense*), viruses [*Banana bunchy-top virus* (BBTV), genus *Nanavirus* and *Banana streak virus* (BSV), genus *Badnavirus*], weevils and nematodes (Jones, 2000; Tushemereirwe et al., 2004). The use of resistant cultivars is considered the most effective, economical and environmental friendly approach to control diseases and pests. But all EAHBs (AAA-EA) are triploids and therefore difficult to improve through conventional breeding due to sterility of most of the cultivars and long generation times of the life cycle of bananas. Genetic transformation is an alternative option for enhancing production of bananas relative to conven-

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Abbreviations: BAP, 6-benzylaminopurine; EAHBs, East African Highland Bananas; *gus*A, beta-,glucuronidase gene; MS, Murashige and Skoog; *npt*II, neomycin phosphotransferase gene.

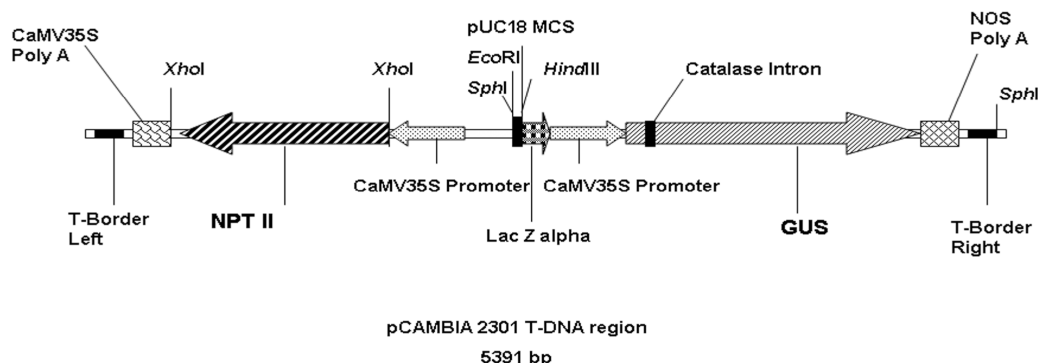

Figure 1. Schematic representation of T-DNA region of binary vector pCAMBIA2301.

tional breeding. Because of the high degree of sterility and clonal mode of propagation, gene flow is a minor issue for this crop, making a transgenic approach even more attractive.

Relative success in the genetic engineering of bananas has been achieved. Protocols have been developed for *Agrobacterium*-mediated transformation as well as microprojectile bombardment of embryogenic cell suspensions (Becker et al., 2000; Ganapathi et al., 2001; Khanna et al., 2004; Sagi et al., 1995). A transformation system using apical meristems from various cultivars of *Musa* has also been established (May et al., 1995; Tripathi et al., 2005). Genetic transformation of the EAHBs has been recalcitrant and not reported as yet.

Currently, most transformation protocols for banana use cell suspensions. Establishing cell suspensions is, however, a lengthy process and is cultivar dependent. At present, the major barrier for transforming the EAHBs appears to be for the limited success in producing embryogenic cell suspension cultures from a wide range of cultivars (Namanya et al., 2004). In this study, we report for the first time a relatively rapid and cultivar-independent *Agrobacterium*- mediated transformation protocol for EAHBs using intercalary meristematic tissues.

MATERIALS AND METHODS

Plant material

Plantlets of the two cultivars 'Mpologoma' and 'Nakitembe' (EAHB-AAA) were regenerated through micro-propagation as described by Tripathi et al. (2003). Established cultures were routinely sub-cultured on fresh semi-solid medium every 3-4 weeks. The regenerated shoots were used for explant preparation for genetic transformation experiments.

Explant preparation and regeneration

Fine cross sections (0.4-0.6 mm thickness) of intercalary meristematic tissues were excised from the corm after removal of the roots and leaves of *in vitro* shoots. Two sections were excised from each corm. The explants were cultured on proliferation medium containing the macro- and micro- mineral salts of MS (Murashige and Skoog, 1962), MS vitamins, myo-inositol (100 mg/l), sucrose (4% w/v), ascorbic acid (100 mg/l), 6-benzylaminopurine (BAP, 5 mg/l), pH - 5.8 and phytagel (2.3 g/l). The medium was autoclaved at 121°C and 103 Kpa for 20 min and poured into 90 mm Petri dishes. Ten explants were incubated on each plate. The cultures were incubated at 26 ± 2°C with a 16 h photoperiod furnished with fluorescent light of 45 µmol $m^{-2}s^{-1}$.

The individual small shoots were transferred to semi-solid medium supplemented with BAP (3 mg/l) and indole acetic acid (IAA, 0.3 mg/l) in culture tubes, for the elongation and maturation of shoots. The elongated shoots were transferred to a rooting medium containing indole 3-butyric acid (IBA, 1 mg/l).

Agrobacterium strains and plasmid

The *Agrobacterium tumefaciens* strain EHA105 was used in this study. The binary vector, pCAMBIA2301 having the neomycin phosphotransferase gene (*npt*II) as selection marker and beta-glucuronidase gene (*gus*A) with a catalase intron as a reporter was obtained from CAMBIA for the transformations (Figure 1). Both the genes were under the regulation of CaMV35S promoter. The vector was transformed into *Agrobacterium* strains according to the modified method of Gynheung (1987). *Agrobacterium* strains harboring the pCAMBIA2301 were grown at 28°C in liquid YEB medium [yeast extract (0.1% w/v), beef extract (0.5% w/v), peptone (0.5% w/v), sucrose (0.5% w/v), $MgSO_4$ (0.04% w/v), pH-7] supplemented with appropriate antibiotics, with shaking (150 rpm) until the $O.D_{600\ nm}$ reached 0.8. The bacterial cells were harvested by centrifugation at 5,000 x g for 10 min at 4°C and re-suspended in 25 ml of antibiotic free pre-induction medium (liquid proliferation medium supplemented with 100 µM acetosyringone). The bacterial suspension was incubated at 28°C for 3 h with shaking at 150 rpm.

Pre-treatment of explants and effect on transformation

The effect of different pre-treatments of explants on the efficiency of *Agrobacterium* to transfer the T-DNA to the explants was compared through transient *gus*A gene expression. Each experiment had three replicates of 40 explants each. The explants were pre-treated in following ways before co-cultivation with *Agrobacterium*.

Effect of micro-wounding

The explants were micro-wounded by bombarding with naked gold particles (BioRad, 1 µm) at 3102.6 kPa at a distance of 10 cm un-

der reduced pressure of -84.65 kPa using Particle delivery system (PDS-1000/He Biolistic, BioRad). The micro-wounded explants were incubated on proliferation medium in the dark for 24 h for recovery, after which these explants were used for co-cultivation with *Agrobacterium* culture.

Effect of heat shock

The explants were suspended in 10 ml of liquid proliferation medium in 50 ml falcon tube and subjected to heat shock for 2 min at 45°C. The supernatant was removed and explants were used for co-cultivation with *Agrobacterium* culture.

Effect of micro-wounding and heat shock together

The explants were micro-wounded as described above and incubated on proliferation medium in the dark for 24 h for recovery. The micro-wounded explants were suspended in liquid proliferation medium and subjected to heat shock for 2 min at 45°C. After treatment the explants were used for co-cultivation with *Agrobacterium* culture.

The pre-treated explants were immersed in *Agrobacterium* suspension in culture flasks and vacuum infiltrated for 2 min under reduced pressure of -84.65 kPa. After the vacuum was released the explants were co-cultivated for 30 min with gentle shaking (30 rpm). Excess bacterial culture was removed after incubation; explants were blotted on tissue papers and co-cultured on co-cultivation medium (proliferation medium containing 100 µM of acetosyringone), for 3 days in darkness. After three days transient expression of the reporter gene in the pre-treated explants was compared with the control explants with out any pre-treatment.

The explants were then transferred to proliferation medium containing 500 mg/l cefotaxime for 7 days with 16 h photoperiod. The control explants were regenerated without any pre-treatment. For optimisation of the pre-treatment, the explants were regenerated on medium without selection agent for comparison of regeneration efficiency with explants without any pre-treatment. The control pre-treated explants were regenerated without agro-infection.

Transformation, selection and regeneration

The explants of two cultivars 'Mpologoma' and 'Nakitembe' were preconditioned on proliferation medium for 24 h in dark. The explants were micro-wounded and recovered on regeneration medium in the dark for 24 h. The micro-wounded explants were subjected to heat shock for 2 min at 45°C and then co-cultivated with *Agrobacterium* for 3 days in the dark on co-cultivation medium after removing the excess bacteria.

The explants were then transferred to proliferation medium containing 500 mg/l cefotaxime for 7 days with 16 h photoperiod. Agro-infected explants were then transferred to the selection medium (proliferation medium containing 100 mg/l kanamycin and 300 mg/l cefotaxime). The cultures were sub-cultured to fresh selection medium every two weeks. The control non-transformed explants were also transferred on selection medium. The putatively transformed shoots were regenerated on selection medium. The meristems were isolated from the regenerated shoots and cultured again on selective medium for second step of selection. Shoots that regenerated after second step of selection were transferred to rooting medium. The rooted plants were assessed by GUS histochemical assay and molecular analysis.

The transgenic plants obtained after two-steps of selection were multiplied by micropropagation on proliferation medium without any selection. The established cultures were routinely sub-cultured on fresh non selective medium every 4 weeks to reveal new shoots or suckers. After six months, the regenerated sucker shoots were excised and transferred to selection medium for 4 weeks. The control non-transformed shoots were also transferred on the selection medium. The shoot tips isolated from the randomly picked kanamycin resistant sucker shoots were checked with GUS histochemical assay for stable expression of transgene.

GUS histochemical assay

The GUS histochemical assay for transient gene expression was performed 3 days after co-cultivation according to the modified procedure of Jefferson (1987), as described by Tripathi et al. (2005) for banana. The leaf segments and roots were excised from kanamycin resistant plants obtained after two-steps of selection and tested for stable expression of *gus*A gene. The leaf segments of various leaves of the same transgenic plant were also tested for GUS expression. The shoot tips were isolated from sucker shoots regenerated on medium without selection, and tested for stable expression of *gus*A gene.

DNA isolation and PCR analysis

Genomic DNA was isolated from putative transformed plantlets obtained after two-steps of selection, using a modified CTAB (Hexadecyltrimethylammonium bromide) extraction method for *Musa* described by Gawel and Jarret (1991). PCR with *gus*A gene specific primers was used to confirm presence or absence of transgene into the plant genome. The primer sequences were 5' TTTAACTATGCCGGGATCCATCGC 3' and 5' CCAGTCGAGCATCTCTTCAGCGTA 3'. A 25 µl PCR reaction mixture contained 1.5 mM $MgSO_4$, 1 X reaction buffer, 0.2 mM nucleotide mix, 1 µM primers, 1 Unit of Taq DNA Polymerase and 1 µg of template DNA. The initial denaturation of DNA was done at 94°C for 5 min, followed by 35 cycles of amplification as denaturing at 94°C (1 min), annealing at 55°C for 1 min and extension at 72°C for 1 min. The final extension was at 72°C for 7 min. PCR products were visualized by electrophoresis on 0.8% agarose gel with ethidium bromide staining.

Southern hybridization

Genomic DNA (20 µg) from plants regenerated after two-steps of selection were restricted with *Hind*III. The *Hin*dIII restriction enzyme has one cleavage site in the binary vector. The restricted DNA was resolved on 0.8% (w/v) agarose gel and blotted onto "Zetaprobe" nylon membrane (according to the manual provided by BioRad). The blots were hybridized with DIG labeled *gus*A probes generated using PCR DIG probe synthesis kit. Hybridization and detection of the probe was carried out using DIG Luminescent Detection kit for Nucleic Acids (Roche Diagnostics) according to manufacturer's instructions.

Statistical analysis

All the experiments were replicated three times with 40 explants each. The means and standard errors were calculated and analyzed using SAS/STAT Software V8.

RESULTS

Regeneration from intercalary meristematic tissues

Fine cross sections (0.4-0.6 mm thickness) of intercalary meristematic tissues were excised from the corm after

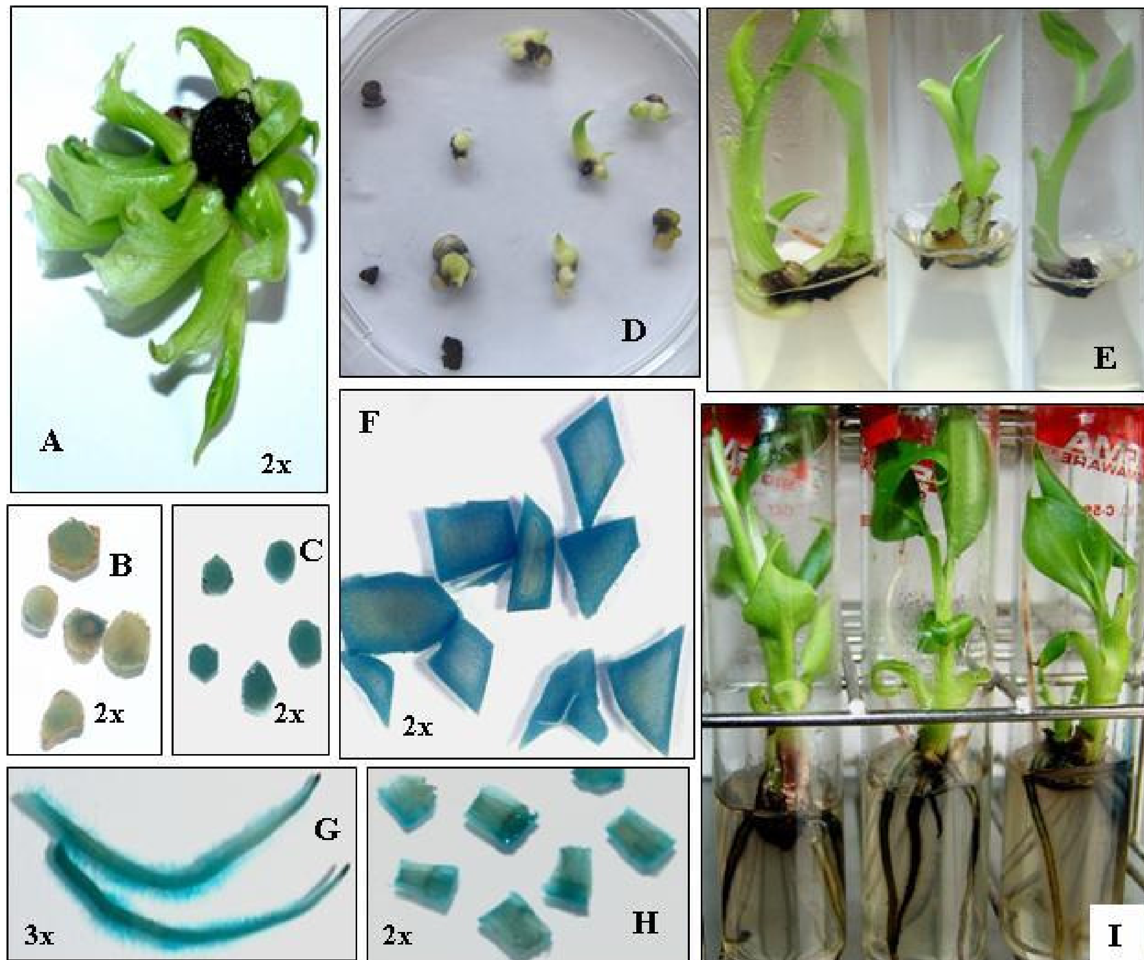

Figure 2. Transformation and regeneration of intercalary meristematic tissues of East African Highland Banana using *Agrobacterium tumefaciens* strain EHA105 harboring pCAMBIA2301 binary vector. **A:** Cluster of multiple shoots from non-transformed explant after 4 weeks of culture; **B-C:** Transient expression of *gus*A gene in explants 3 days after co-cultivation (B) explants without pre-treatment, (C) pretreated explants; **D:** Shoot buds developed from explants on selection medium 4 weeks after co-cultivation; **E:** Shoots regenerated on selection medium in 8 weeks; **F-H:** Stable expression of *gus*A gene in (F) leaf segments, (G) roots excised from transgenic plants obtained after two-steps of selection, (H) shoot tips isolated from sucker shoots of transgenic plants; **I:** Rooted transgenic plants obtained after two-steps of selection.

removal of the roots and leaves of *in vitro* shoots. All of the explants placed on the proliferation medium turned green, and induction of clusters of multiple shoot buds was observed in about 95% of explants within 2 weeks. Within 4 - 5 weeks, about 12–14 shoots were obtained from each explant (Figure 2A). Individual shoot separated from the clusters of multiple shoots elongated and matured in 2 weeks on elongation medium, and rooted plantlets were obtained 2 weeks after transferring the mature shoots to rooting medium.

Effect of pre-treatment of explants on transformation

Different pre-treatments of the explants showed significant effects on the efficiency of *Agrobacterium* to transfer the T-DNA to the explants, as determined by assessment of transient expression of the beta-glucuronidase (*gus*A) gene. Transient expression was the highest in explants which were micro-wounded via microprojectile bombardment with naked gold particles followed by heat shock prior to agro-infection (Table 1, last column). About 76% of the explants that were pre-treated with micro-wounding and heat shock appeared blue in the histochemical assay. In contrast only 42% of agro-infected explants showed transient GUS expression without any pre-treatment. Micro-wounding resulted in 68% of the plants showing GUS expression. The GUS expression, as judged by production of the blue coloration, was light and in sectors in the Agro-infected explants that were not pretreated (Figure 2B), whereas the color was intense and uniform blue in explants pre-treated with micro-

Table 1. Transient expression and regeneration efficiency of pre-treated explants of East African Highland Banana cultivar Mpologoma, 3 days after co-cultivation with *Agrobacterium tumefaciens* EHA105 harboring pCAMBIA2301.

Treatment	No. of explants regenerated	Mean (±SE) regeneration efficiency%	Mean (±SE) no. of shoots	No. of explants showing blue stain	Mean (±SE) efficiency of transient GUS expression %
No treatment	38.0±0.6	94.8±1.6	12.0±2.2	00	00
Co-cultivation without any pre-treatment	19.7±0.3	49.2±0.8	7.0±0.6	16. 7±0.7	41.7±1.6
Control with heat shock	37.7±0.3	94.2±0.8	15.3±1.8	00	00
Co-cultivation after heat shock	28.7±1.2	71.7±3.0	12.7±2.9	20.7±1.8	51.7±4.4
Control with micro-wounding	37.7± 0.8	92.5±1.4	12.0±1.2	00	00
Co-cultivation after micro-wounding	20.0±0.8	50.0±1.4	6.3±0.3	29.3±1.8	68.3±9.3
Control with micro-wounding and heat shock	37.3±1.2	93. 3±3.0	16.3±1.5	00	00
Co-cultivation after micro-wounding and heat shock	28.3± 2.3	70.8±5.8	12.1±1.1	30.3±1.7	75.8± 4.2

40 explants were used for each treatment and mean was calculated from data of three replicate experiments.

Table 2. Transformation efficiency of East African Highland Bananas using intercalary meristematic tissues with *Agrobacterium tumefaciens* EHA105 harboring pCAMBIA2301.

Cultivar	No. of explants transformed	No. of shoots regenerated after 1st step of selection	No. of transgenic plants recovered after two-steps of selection	Transformation frequency (%)
Mpologoma	240[a]	34	25	10.4
Nakitembe	120[b]	17	12	10

Transformation efficiency was calculated based on number of transgenic lines obtained from total number of explants co-cultivated with *Agrobacterium*.
[a]240 explants were transformed in six experiments with 40 explants each.
[b]120 explants were transformed in three experiments with 40 explants each.

wounding followed by heat shock (Figure 2C). No blue coloration was observed in control pre-treated explants.

Pre-treatment of explants also showed substantial effect on regeneration after co-cultivation with *Agrobacterium* (Table 1). Micro-wounding of explants did not significantly effect the regeneration. Necrosis which effected regeneration was observed in the explants after co-cultivation, but was less severe in explants pretreated with heat shock than those explants without treatment. *Agrobacterium* infection decreased regeneration to 49% in comparison to control non-transformed explants (94%), but the heat shock treatment increased the regeneration efficiency to about 71% in Agro-infected explants. The number of shoots development was also increased in explants after heat shock treatment (Table 1). Therefore, explants were pre-treated with micro-wounding followed by heat shock prior to agro-infection in all the transformation experiments.

Transformation, selection and regeneration

The neomycin phosphotransferase gene (*npt*II) gene was used as selection marker and kanamycin (100 mg/l) was used in the medium as the selection agent for stable transformation. The concentration of kanamycin used effectively killed non-transformed explants, but allowed growth of transformants (Figure 2D), although at a slower rate than occurs on proliferation medium lacking kanamycin. Thus, the time to obtain transgenic shoots (4-5 cm; Figure 2E) was lengthened from six weeks without selection to eight weeks in the presence of kanamycin. Multiple shoots (4-6) were obtained from each explant on the selection medium. The control non-transformed explants did not develop any shoots on selection medium. The meristems isolated from the shoots obtained on selection medium were subjected to a second step of selection. Shoots were regenerated from about 70% of meristems on second step of selection in 7-8 weeks (Table 2). These regenerated shoots were assumed to be transgenic and all the shoots were rooted in 2 weeks (Figure 2I). The well-rooted transgenic plants after two-steps of selection were obtained in about 6 months. These resulting plants were used for the studies described below on expression and integration of transgenes in the plant genome.

In total, 25 stable transgenic lines were produced from 240 explants of 'Mpologoma' and 12 transgenic lines from 120 explants of 'Nakitembe' leading to a transformation efficiency with the intercalary meristematic tissues of about 10% for both cultivars (Table 2). Transformation efficiency was determined by calculating the number of

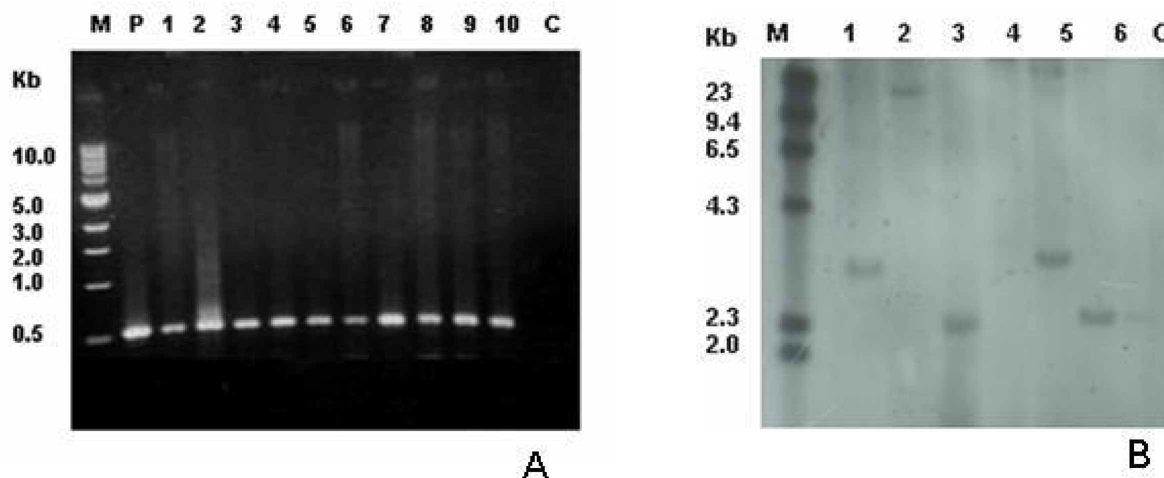

Figure 3. Molecular analysis of transgenic plants. **A:** PCR analysis of transgenic plants amplifying a 500bp internal fragment of *gus*A gene. Lanes 1 - 10, transgenic plants; M, molecular marker; P, plasmid pCAMBIA2301; and C, control non-transformed plants. **B:** Southern hybridization with genomic DNA of six transgenic plants obtained in individual events, using *gus*A probe. Lanes 1 – 6, *Hind*III restricted genomic DNA of transgenic plants; M, molecular marker; C, genomic DNA of control non-transformed plant.

transgenic lines obtained from total number of explants co-cultivated with *Agrobacterium*. All the plants regeneratated from one explant were considered to be siblings and counted as one.

The transgenic plants obtained after two-steps of selection were multiplied by micro-propagation on proliferation medium without any selection, and about 6-8 shoots were obtained from each transgenic plant in 7-8 weeks. The clusters of shoots were separated, and transferred to fresh non-selective medium every 4 weeks to reveal new side shoots (sucker shoots). When these were again subjected to selection on kanamycin, all the shoots stayed green and were elongated after culturing on selection medium for 4 weeks, whereas no control plants survived. The shoot tips were isolated from these kanamycin resistant sucker shoots and were checked for expression of GUS through histochemical assay.

GUS histochemical assay

Transient GUS expression assay three days after co-cultivation of explants showed uniform blue coloration confirming transient expression of the reporter gene in all the surface cells (Figure 2C). A uniform blue coloration was observed in all the leaf segments and roots of the transgenic plants obtained after two-steps of selection, confirming stable expression of *gus*A gene through out the plant indicating uniform transformation (Figure 2F, G). The leaf segments from various leaves of the same transgenic plant also showed uniform blue coloration indicating uniform transformation of the plant. The uniform blue staining was observed in all the shoot tips of the sucker shoots obtained from transgenic plants on medium with no selection (Figure 2H). Blue coloration in shoot tips of the plants confirmed the expression of *gus*A gene in these tissues and suggests germ line cell transformation and stable transfer of genes to suckers. No blue coloration was observed in leaves, roots and shoot tips of control non-transformed plants.

Molecular analysis of transgenic plants

The integration of the transgene into the genome of transgenic plants obtained after two-steps of selection was confirmed by PCR and Southern blot analysis. PCR was performed using *gus*A primers and the amplified fragments of approximately 500 bp corresponding to the amplified internal fragment of *gus*A gene were detected (Figure 3A). The amplified products were observed in all the plants tested, confirming the presence of transgenes and no plant escapes. No amplified product was observed in case of non-transformed plant.

Southern blot analysis of six randomly selected transformed plants of individual events digested with *Hind*III, hybridized with *gus*A probe confirmed the integration of *gus*A gene in the plant genome (Figure 3B). All the plants tested showed different pattern of integration. No signal was observed in the non-transformed control.

DISCUSSION

Genetic transformation procedure described in this study offers distinctive advantages over previous methods for banana in that it is rapid, simple, efficient and cultivar-independent. Transgenic plants were obtained with two

cultivars of EAHB, using this procedure. Most of the previous transformation protocols for banana were based on using cell suspension cultures. The development of cell suspensions is time consuming and cultivar-specific. It takes about 2 years to produce a cell suspension in banana from callus induction to rooted plant regeneration (Ganapathi et al., 2001). In contrast, this protocol allowed us to obtain rooted plants in 6-9 months. Transformation systems using apical meristem and corm slices have been reported (May et al., 1995; Tripathi et al., 2005) but the transformation efficiency was low and often led to chimeras. In the study reported here, intercalary meristematic tissues were chosen as the target for transformation because they have the potential to regenerate plants from many different cultivars, unlike somatic embryogenesis which is restricted to only a few cultivars, and indeed, transformation efficiency was greater with intercalary meristematic tissues than with apical meristems.

The development of the induced shoots from meristematic tissues was similar to that of the sucker bud development. Apical dominance was removed by cutting off the apex, and the intercalary meristems in the axil of the leaf sheath were activated by plant growth regulators in the culture media as also observed by Devlin and Witham (1983). Previous histological analysis of the vegetative growth of suckers has shown that they originate independently from the intercalary meristems that form at the base of each leaf (Fisher, 1978). Okole and Schulz (1996) have observed that shoots developed from the non-green proliferating parenchyma cells below the epidermis outside the central vascular region.

Hernandez et al. (1999) has reported chemotactic movement and attachment of *A. tumefaciens* to wounded tissues of banana. Microprojectile bombardment is reported to be an effective method of wounding tissues to promote *Agrobacterium*-mediated transformation (Bidney et al., 1992). We observed a significant increase in transformation efficiency measured as transient expression of reporter gene, in explants micro-wounded by microprojectile bombardment with naked gold particle prior to co-cultivation with *A. tumefaciens*. This improvement in transformation efficiency can be attributed to the initiation of active cell division upon wounding (Sangwan et al., 1992), the improved binding of *Agrobacterium* to the newly synthesized cell wall at the wound sites (Binns, 1991) and the production of *vir*-inducing compounds by the metabolically active cells (Stachel et al., 1985). In this study, the wounded explants were preconditioned in dark for 24 h to maximize the biochemical micro-wounding response before co-cultivation. Sunilkumar et al. (1999) also reported that the production of *vir* gene inducers by the explant during the preconditioning period is an important factor that contributes to increased transformation efficiency.

In the present study, we observed that the explants were very sensitive to co-cultivation with *Agrobacterium* and turned necrotic within three days in culture after Agro-infection, but necrosis could be overcome by pre-treating the explant with heat shock prior to co-cultivation and this also increased transformation efficiency. Hansen and Durham (2000) have also reported that co-cultivation of wheat and maize tissues with *Agrobacterium* resulted in necrosis due to programmed cell death, and heat shock had prevented this response. Similar results were also observed with banana embryogenic cell suspension cultures (Khanna et al., 2004).

The differences between infection efficiencies of the different virulence of *Agrobacterium* strains are known to exist. Previous studies in our laboratory showed that EHA105 strain was the best strain for genetic transformation of bananas (Tripathi et al., 2005) and, for this reason, was used in this study as well. EHA105 is derivative of super virulent strain EHA101 (Hood et al., 1993). The *Agrobacterium* culture was pre-induced by adding acetosyringone in the medium. It has been reported that pre-induction of *Agrobacterium* increased transformation efficiency in banana (Khanna et al., 2004).

The transformation of meristematic cells may result in chimeric plants when only one or a few cells receive T-DNA. To overcome this problem, explants were pre-treated and vacuum infiltrated to allow *Agrobacterium* to penetrate the tissue. The number of cells transformed per explant was high and measured 100% as can be seen in Figure 2C. Specifically, the GUS expression was uniform across the surface of the explant that was pre-treated before co-cultivation. In contrast, the number of cells transformed in explants without treatment was small as evidenced by the scattered isolation of transgenic sectors (Figure 2B).

From past experience, we realized it was very important to assess whether these plants were fully transformed or represented chimeras. To avoid obtaining chimeric plants, we used small pieces of meristematic tissues that were repeatedly screened for kanamycin resistance. The fine sections of explants were micro-wounded to expose the intercalary meristematic tissues to *Agrobacterium*. To obtain uniformly transformed plants we performed two-steps of selection and regeneration to avoid regeneration of any non-transformed cells. The plants obtained after two-steps selection and regeneration were confirmed as pure transgenic cell lines by polymerase chain reaction (PCR), Southern blot analysis and GUS histochemical assays from different tissues. The uniform blue staining of leaf segments from various leaves of the same transgenic plant and roots of the transgenic plants obtained after two-step of selection, confirmed the stable transformation (Figures 2F, G). To further confirm the stable transformation, transgenic plants were micro-propagated on medium without any selection and the sucker shoots (new side shoots) obtained were selected on kanamycin. Survival of all shoots on kanamycin confirmed the stable transfer of transgene to suckers, which are the seed materials for vegetatively

propagated crops. The expression of *gus*A gene in shoot tips isolated from the sucker shoots obtained on medium without any selection, confirmed the stable transformation of germ line cells and inheritance of gene to suckers through vegetative propagation (Figure 2H). We have not observed any chimeric plants in lab conditions after propagating through tissue culture but further studies to confirm the stable uniform expression of transgene in the field grown plants and in the successive suckers needed.

In conclusion, a rapid and efficient transformation protocol based on direct shoot regeneration of *Agrobacterium* infected intercalary meristematic tissues was developed. This system avoids the formation of callus and cell suspension cultures that can cause somaclonal variations, but uses organogenesis that allows recovery of regenerated plants in a short period of time. This procedure offers several potential advantages over the use of embryogenic cell suspensions as it allows rapid production of banana transgenic plants without involving time consuming development of cell cultures and is cultivar independent. This study has opened new frontiers for genetic manipulation of bananas for disease and pest resistance, using a rapid and cultivar-independent transformation system. The use of appropriate gene constructs may allow the production constraints of pests and diseases to be addressed in a sustainable and environmental friendly manner and thus contribute significantly to food security and poverty alleviation in Africa.

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