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Full Length Research Paper

Agrobacterium-mediated transformation of plantain (*Musa* spp.) cultivar Agbagba

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An *Agrobacterium*- mediated plant transformation system was developed for the production of transgenic plantain [*Musa* spp. cultivar Agbagba (AAB)]. Apical shoot tips were transformed using *Agrobacterium* strain EHA105 with the binary vector pCAMBIA 1201, having the hygromycin resistance gene as a selection marker and GUS-INT as a reporter gene. Transient expression of the β-glucuronidase (*uid* A) gene was achieved in transformed apical shoot tips. The hygromycin resistant shoots were regenerated 4 to 5 weeks after co-cultivation of explants with *Agrobacterium*. The two step selection procedure allowed the regeneration of shoots which were uniformly transformed. The integration of the *uid* A gene was confirmed by polymerase chain reaction (PCR) and Southern blot analysis. In this study, transformation based on regeneration from apical shoot tips has been demonstrated. This process does not incorporate steps using disorganized cell cultures but uses micropropagation, which has the important advantage that it allows regeneration of homogeneous populations of plants in a short period of time. This study shows the enormous potential for genetic manipulation of *Musa* species for disease and pest resistance, as well as abiotic factors, using a rapid and non-species specific transformation and regeneration system.

Key words: Plantain, *Musa, Agrobacterium*-mediated transformation, apical shoot tip.

INTRODUCTION

Plantains and bananas (*Musa* spp.) are staple food for rural and urban consumers in the humid tropics and are an important source of income. World *Musa* production is currently about 104 million tones annually (FAOSTAT, 2004). Many pests and diseases have significantly affected *Musa* cultivation. Black Sigatoka (*Mycospha-erella fijiensis*), wilt (*Fusarium oxysporum* f. sp. *cubense*), bacterial wilt (*Xanthomonas campestris* pv. *Musacea-rum)*, viruses [*Banana bunchy-top virus* (BBTV), genus *Nanavirus* and *Banana streak virus* (BSV), genus *Badnavirus*] and nematodes cause significant crop losses worldwide (Jones, 2000; Tushemereirwe et al., 2003). Long generation times, various levels of ploidy, sterility of most edible cultivars and the lack of genetic variability have hampered the development of disease-resistant *Musa* by conventional breeding.

Genetic transformation has become an important tool for crop improvement. Relative success in genetic engineering of bananas and plantains has been achieved recently to enable the transfer of foreign genes into plant cells. Genetic transformation using microprojectile bombardment of embryogenic cell suspensions is now routine (Becker et al., 2000; Cote et al., 1996; Sagi et al., 1995) . However, *Agrobacterium*-mediated transformation offers remarkable advantages over direct gene transfer methodologies. It reduces the copy numbers of the transgene, potentially leading to fewer problems with transgene co-suppression and instability (Gheysen et al., 1998; Hansen and Wright, 1999; Shibata and Liu, 2000). The protocol has been developed for *Agrobacterium*-mediated transformation of embryogenic cell suspensions of the banana cultivars (Ganapathi et al., 2001; Khanna et al., 2004) . At present most of the transformation protocols use cell suspension cultures.

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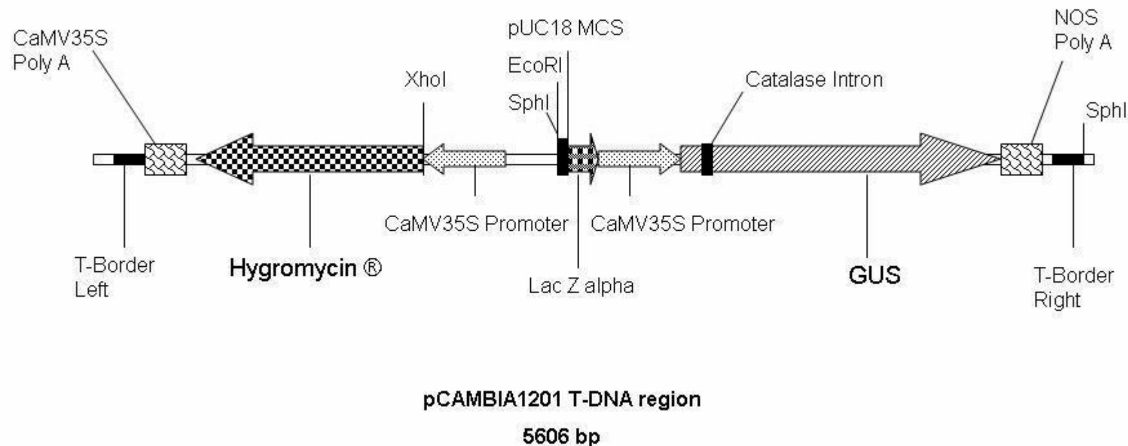

Figure 1. Schematic representation of T-DNA region of binary vector pCAMBIA 1201.

However, establishing cell suspension is lengthy process and cultivars dependent. There is only one report of transformation using shoot tips from the *Musa* CV Grand Nain (May et al., 1995) . Therefore, this study was conducted in order to develop an efficient transformation system to augment and facilitate breeding of disease-resistant banana and plantain for sub-Saharan Africa.

MATERIALS AND METHODS

Plant material

The plantlets of the plantain cultivar Agbagba (AAB) were regenerated through micropropagation using apical shoot tips as described by Tripathi et al . (2003). The apical shoot tips with 2 to 3 leaf primordia (approx. 5 mm) were excised from the suckers of cultivar Agbagba and cultured on regeneration medium. The medium contained the macro- and micromineral salts of MS (Murashige and Skoog, 1962), iron chelate, thiamine (1 mg/l), myo-inositol (100 mg/l), sucrose (4%, w/v), ascorbic acid (100 mg/l), supplemented with 6-benzylaminopurine (BAP, 5 mg/l). The pH of the medium was adjusted to 5.8 and the medium was solidified with 0.2% (w/v) gelrite. The medium was dispensed into culture tubes and autoclaved at 121°C and 103 Kpa for 20 min.

The shoot tip cultures were incubated at 26 ± 2°C with a 16 h photoperiod furnished with fluorescent tube providing light of 3000 lux for 4 weeks. The shoot tips were first cultured in liquid medium on a gyratory shaker at 100 rev/min, and then transferred to a semi-solid regeneration medium after 3 weeks. Established cultures were routinely sub-cultured on fresh semi-solid medium every 3 to 4 weeks. For the elongation and maturation of shoots, individual shoots were transferred to semi-solid medium supplemented with BAP (3 mg/l) and indole acetic acid (IAA, 0.3 mg/l). After 3 weeks, the elongated shoots were transferred to a rooting medium containing indole 3-butyric acid (IBA, 1 mg/l). Single shoot was transferred to root induction medium and incubated in the culture room. The rooted plants were transferred to soil in the pot and hardened in the screen house.

Explant preparation

Apical shoot tips (approximately 2 mm) were isolated from *in vitro* regenerated shoots, bisected longitudinally and cultured on MS medium supplemented with BAP (5 mg/l) for 24 h in the dark. The explants were microwounded by bombarding with naked gold particle at 450 psi at a distance of 10 cm under reduced pressure of –25 in.Hg using a BioRad Gene Gun. The microwounded explants were incubated on regeneration medium in the dark for 24 h for recovery, after which these explants were co-cultivated with *Agrobacterium* culture.

Agrobacterium strains and plasmids

The *Agrobacterium tumefaciens* strains EHA105, LBA4404, C58 and GV2260 were used in the study. The binary vector pCAMBIA 1201 having the hygromycin resistance gene as selection marker (Figure 1) and *uid* A gene encoding for β-glucuronidase (GUS) with a catalase intron as a reporter was used for the transformations. The vector was transformed into *Agrobacterium* strains according to the modified method of Gynheung (1987). A single colony of the *Agrobacterium* strain was inoculated in 25 ml of YEB (0.1% yeast extract, 0.5% beef extract, 0.5% peptone, 0.5% sucrose, 0.04% $MgSO_4$) liquid with appropriate antibiotics and grown at (28°C) till OD_{600nm} = 0.5. The cells were sedimented at 5000 g for 5 min and pellet was re-suspended in 10 ml of 0.15 M $CaCl_2$. The bacteria cells were centrifuged again at 5000 g for 5 min and re-suspended in 0.5 ml of ice-cold 20 mM $CaCl_2$. The cells (0.2 ml) were transferred to a 1.5-ml eppendorf tube and 1 µg of plasmid pCAMBIA 1201 DNA was added, mixed and incubated on ice for 30 min. The mixture was freezed in liquid N_2 for one min, and then thawed at 37°C water bath. The medium (1 ml) was added to the mixture and incubated at 28°C for 2 to 4 h with gentle shaking. The cells were collected by centrifuging at 5000 g for 5 min and re-suspended in 0.1 ml of YEB liquid. The bacteria cells were then spreaded on YEB plates (YEB medium containing 1.5% agar) with appropriate antibiotics and incubated at 28°C for 2 to 3 days. The resultant antibiotic resistant colonies were checked for the presence of vector. The engineered strains of *Agrobacterium* were maintained and used for plant transformations.

The cultures of *Agrobacterium* strains harboring the pCAMBIA 1201 were inoculated in 25 ml of liquid medium supplemented with appropriate antibiotics and grown at 28°C for 48 h. About 1 ml of inoculum from the 48 h grown culture was used to inoculate 25 ml of fresh medium and the culture was grown further at 28°C till the $O.D_{600nm}$ reached 0.8. The bacteria cells were harvested at 5,000 g for 10 min at 4°C and re-suspended in 25 ml of antibioitic free regeneration medium supplemented with 100 µM acetosyringone.

Inoculation and co-cultivation of explants

The efficiency of various strains of *Agrobacterium* to transfer the T-DNA to the explants was compared through transient *uid* A gene expression. Each experiment had three replicates. The explants were co-cultivated with culture of *Agrobacterium* strains having pCAMBIA 1201 for 30 min with gentle shaking. Acetosyringone (100 μM) was added during the co-cultivation. After 30 min the liquid culture was removed and explants were blotted on tissue papers and co-cultured on regeneration medium containing acetosyringone (100 μM) for 3 days in dark. After three days transient expression of reporter gene in the explants was compared through GUS histochemical assay. The explants were then transferred to regeneration medium containing cefotaxime (500 mg/l) for 7 days with 16 h photoperiod.

Selection and regeneration

Agrobacterium infected explants were transferred to the selection medium (regeneration medium containing 25 mg/l hygromycin and 300 mg/l cefotaxime). The cultures were transferred to fresh selection medium every two weeks. The putatively transformed shoots regenerated on selection medium were transferred to rooting medium. The rooted plants were assessed by GUS histochemical assay and molecular analysis.

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). The explants were washed in 70% ethanol followed by incubation in fixation solution (0.3% v/v formaldehyde, 10 mM MES, pH 5.6, 0.3 M mannitol) for 30 to 45 min at room temperature. The explants were vacuum infiltrated for 2 min for proper fixation and extensively washed with 50 mM phosphate buffer (pH 7.0). The fixed explants were incubated with the substrate solution (1 mM X-gluc, 50 mM sodium-phosphate (pH 7.0), 5 mM potassium ferricyanide, 5 mM ferrocyanide, 10 mM EDTA, 50 mM Ascorbic acid), vacuum infiltrated for 2 min and incubated at 37°C for 20 to 24 h. Chlorophyll was removed by immersing the explants in solution of methanol and glacial acetic acid (3:1) for 3 to 4 h followed by dehydration in a series of ethanol (50, 70, and 95%). All plants putatively transformed were tested for GUS expression.

DNA isolation and PCR analysis

Genomic DNA was isolated from putative transformed plantlets using a rapid CTAB (hexadecyltrimethylammonium bromide) extraction method (Stewart and Via, 1993). PCR with GUS gene specific primers was used to confirm presence or absence of transgene into the plant genome. The primer sequences were 5'TTTAACTATGCCGGGATCCATCGC3' and 5' CCAGTCGAGC-ATCTCTTCAGCGTA3'. A 25 μl PCR reaction mixture contained 1.5 mM MgSO4, 1X reaction buffer, 0.2 mM nucleotide mix, 1 μM primers, 1 unit of Taq DNA Polymerase and 1 μg of template DNA. The reaction mixture was subjected to an initial denaturation step of 94°C for 5 min, followed by 35 cycles of 94°C for 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 checked on 0.8% agarose gel.

Southern hybridization

The plant genomic DNA samples were restricted with EcoRI, 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 ^{32}P-labeled SphI fragment of pCAMBIA 1201 containing the CaMV35S promoter and GUS (*uid*A) gene region as a probe. The membrane was washed and exposed to X-ray film (Kodak) at -80°C using an intensifying screen.

RESULTS AND DISCUSSION

Regeneration of plants

All the isolated shoot tips placed on the medium turned green and cluster of multiple shoots were obtained within 4 weeks. About 10 to 12 shoots were obtained from each explant. The shoots were subculture on fresh medium every 3 to 4 weeks. A single shoot tip produced about 125 to 144 shoots within 8 weeks. Through repeated subculturing of proliferating shoots, an open-ended system can be maintained.

The individual shoots were separated from the clusters of shoots and cultured in a medium containing BAP (3 mg/l) and IAA (0.3 mg/l) for further development and maturation. The developed shoots were then transferred to rooting medium and adventitious roots were developed in 2 weeks. The rooted plantlets were transferred to soil in screen house.

Agrobacterium-mediated transformation

Transformation of plants by *Agrobacterium*-mediated DNA transfer is the most commonly used phenomenon in accomplishing plant gene transfer. Protocols have been developed for efficient *Agrobacterium*-mediated transformation in both dicotyledonous and monocotyledonous plants, including a large number of crop species. For some time monocotyledons were considered to be insensitive to *Agrobacterium* infection. However, in recent years several reports on *Agrobacterium*-mediated transformation in a number of monocotyledonous plant species including rice (Hiei et al., 1994; Rashid et al., 1996), barley (Tingay et al., 1997), maize (Ishida et al., 1996) and wheat (Cheng et al., 1997) have been reported. Strains containing super virulent plasmids have facilitated transformation of some recalcitrant monocotyledonous plants. It is believed that the factor that limits transformation success in monocotyledonous plants is not only the transfer and integration of T-DNA into the plant genome but also the plant regeneration. Often the regeneration rates are poor with monocotyledonous plants and this is further reduced under selection during transformation.

Success in genetic engineering of bananas and plantains has been achieved recently to enable the transfer of foreign genes into plant cells. *Musa* spp. was generally regarded as recalcitrant for *Agrobacterium*-mediated transformation. Hernandez (1999) has reported chemotactic movement and attachment of *A. tumefaciens*

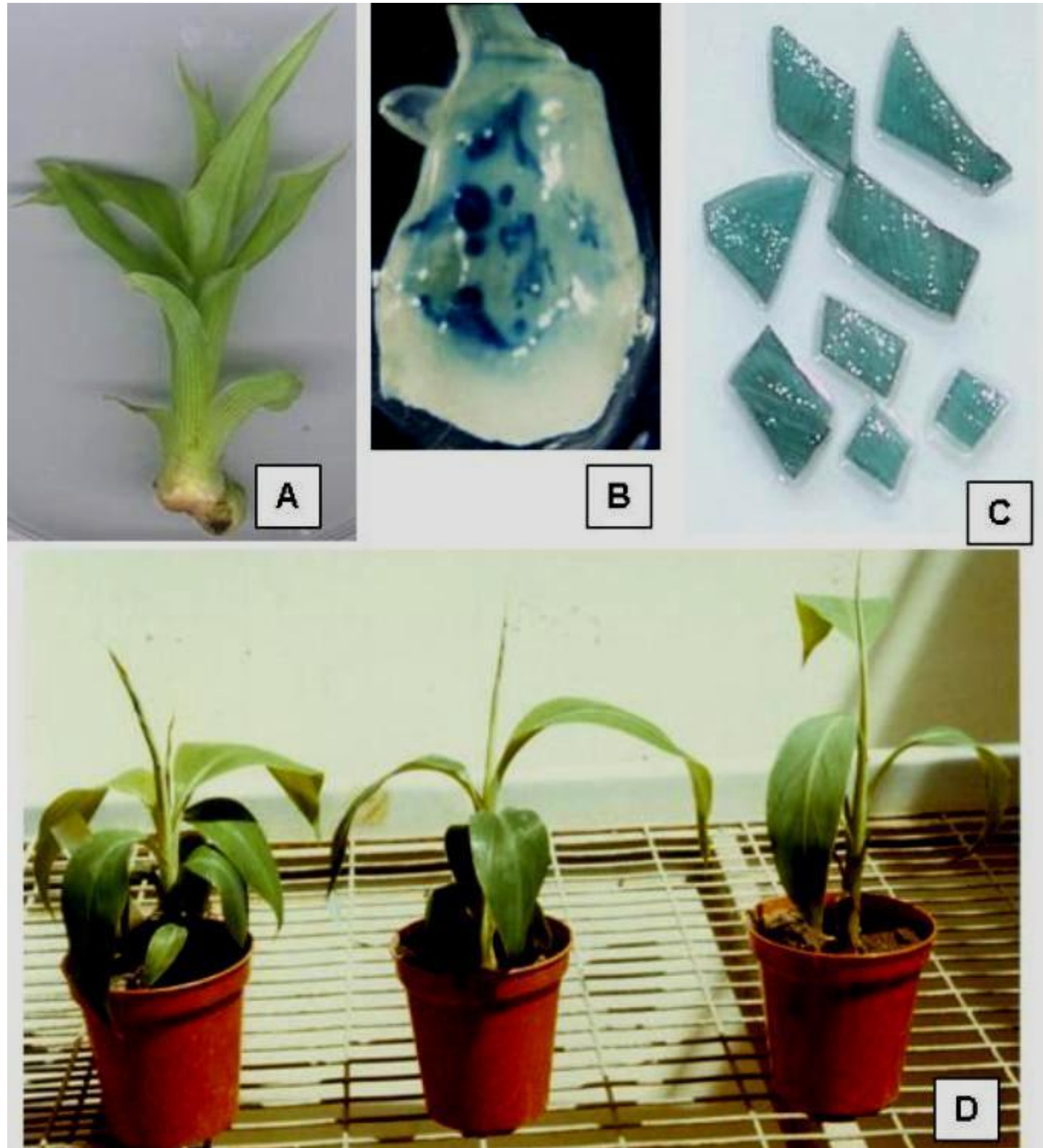

Figure 2. A. Transgenic shoot regenerated on selection medium; B. Transient expression of uid A gene, 48 h after co-cultivation; C. Stable expression of uid A gene in the leaves of the transgenic plants; D. Transgenic plants in containment house.

to wounded tissues of banana. Therefore, we have microwounded the explants before agro-infection. The wounded explants were incubated in dark for 24 h to maximize the biochemical wounding response before co-cultivation with *Agrobacterium* in the presence of acetosyringone.

The differences between infection efficiencies of the different virulence of *Agrobacterium* strains are known to exist. To compare the ability of various *Agrobacterium* strain to infect and transfer the T-DNA into the plantain explants, pCAMBIA 1201 with the *uid* A reporter gene used. The plasmid pCAMBIA 1201 has been used successfully for transformation of tobacco and rice via particle bombardment and *Agrobacterium*-mediated methods (Hajdukiewicz et al., 1994; Hiei et al., 1994). Transient expression of the *uid* A gene driven by the CaMV35S promotor was used to confirm the transfer of transgene from super virulent strain EHA105 to an ordinary strain LBA4404.

Transient *uid* A gene expression, i.e., the development of blue foci after staining, was observed in 60 to 70% of the explants co-cultivated with EHA105 (1201) (Figure 2B). The explants co-cultivated with other strains showed expression of *uid* A gene in only 40 to 45% (C58 and LBA4404) or 20 to 25% (GV2260). The recovery of stable transformants mainly depends on the regenerative

competence of the target tissues and their recovery after co-cultivation. The regeneration of explants after co-cultivation with various *Agrobacterium* strains was also compared. The explants co-cultivated with EHA105 regenerated at an efficiency of about 98% whereas with other strains the efficiency was 70% (LBA4404 and C58) and 60% with GV2260. The comparison of *uid* A gene expression and evaluation of regeneration efficiency after co-cultivation by various strains indicated that EHA105 was the best strain for transformation of apical shoot tips of the plantain cultivar Agbagba.

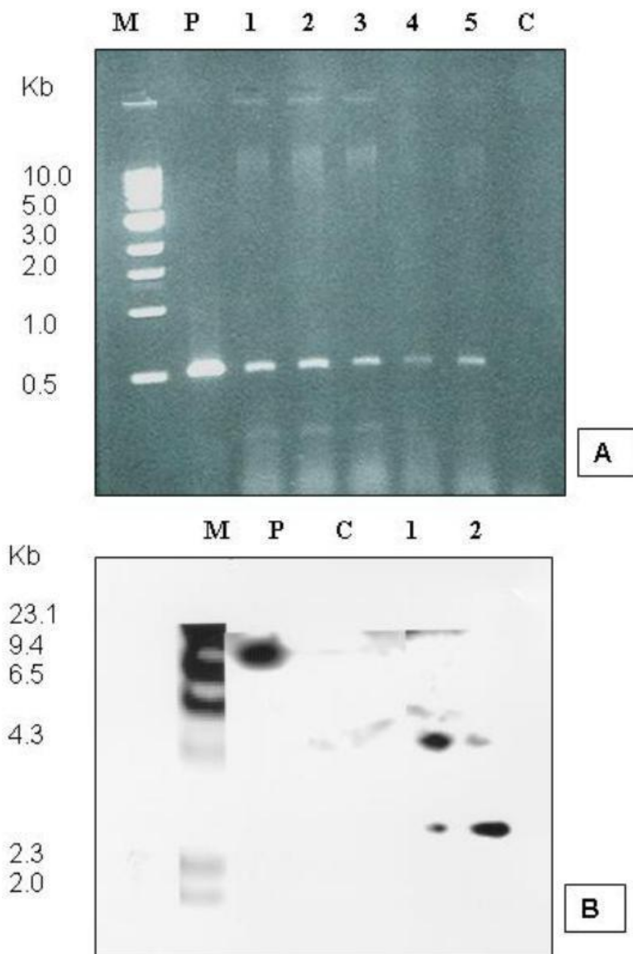

Figure 3. A. PCR analysis amplifying a 500 bp internal fragment of *uid* A gene. Lane 1, molecular marker; P, plasmid pCAMBIA1201; 1- 5, transgenic plants; C, untransformed plants. **B.** Southern blot analysis using the genomic DNA of two transgenic plants. (lanes 1 and 2); P, plasmid pCAMBIA 1201; C, untransformed plants.

Selection and regeneration of transgenic plants

The agro- infected explants were regenerated on hygromycin selective medium. After 5-6 weeks putative transgenic shoots were obtained from the transformed explants. The control untransformed explants did not develop shoots formation on selection medium. All the shoots were transferred to the rooting medium and roots were formed in 2 weeks (Figure 2A). The leaves from all the regenerated plants were tested by GUS histochemical assay for stable expression of the reporter gene. Blue coloration was observed in the transformed leaves (Figure 2C).

This transformation procedure may result in the development of chimeric plants since multiple cells are involved in shoot development and only a proportion of them may be transformed. However, the recovery of transformed cells and tissues can be enhanced by tissue culture manipulation and optimizing selection procedures. To get uniformly transformed plant we have performed two steps of selection and regeneration. The apical meristem was isolated from all the regenerated putative transformed plants. These meristems were regenerated on selection medium containing hygromycin. Not all the meristem isolated from putative transformants had regenerated on selection medium. The hygromycin resistant plants regenerated on 2nd step of selection were used to test the expression of reporter gene in all the leaves. The uniform blue coloration was observed in all the leaves segments of the plant (Figure 2C). This confirms that the regenerated plants after second step of selection were uniformly transformed.

Molecular analysis of transformants

The integration of the transgene into the plant genome was confirmed by PCR and Southern blot analysis. PCR was performed using *uid* A primers and the amplified fragments approx. 500 bp corresponding to the amplified internal fragment of *uid* 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 untransformed plant.

Southern blot analysis was performed with EcoRI digested genomic DNA of 2 transformed plants. The hybridization signals were observed at 4.5 kb and 3 kb, confirming the stable integration of *uid* A gene into the plant genome (Figure 3B, lanes 1 and 2). The same pattern was observed in both the plants because they were regenerated from the same explants through micropropagation. Positive control plasmid pCAMBIA 1201 DNA also generated the hybridization signal (Figure 3B, lane P) . No signal was observed in the untransformed control (Figure 3B, lane C).

CONCLUSIONS

In this study, we have shown that transformation based on regeneration from apical shoot meristems can be used

to transform *Musa* species. This process does not incorporate steps using disorganized cell cultures, but uses micropropagation techniques that allow 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 for rapid transformation of *Musa* species without time consuming development of cell cultures. We have tested this protocol with several *Musa* cultivars with different ploidy (data not included) and it appears to be cultivar independent.

This study shows the enormous potential for genetic manipulation of *Musa* species for disease and pest resistance, as well as abiotic factors using a rapid and non-species specific transformation and regeneration system. The use of appropriate constructs may, after screening and biosafety concerns have been addressed, allow the production constraints of nematodes, fungi, bacteria and virus infection to be addressed in a sustainable and environmentally friendly manner. It may also be possible to incorporate other characteristics such as drought tolerance, thus increasing the area that can support the growth of banana and plantain and thus contributing significantly to food security and poverty alleviation.

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REFERENCES

Becker DK, Dugdale B, Smith MK, Harding RM, Dale JL (2000). Genetic transformation of Cavendish banana (*Musa* spp. AAA group) cv. Grand Nain via microprojectile bombardment. Plant Cell Rep. 19: 229-234.

Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW, Wan Y (1997). Genetic transformation of wheat mediated by Agrobacterium tumefaciens. Plant Physiol. 115: 971-980.

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 Nain. Physiol. Plant 97: 285-290.

FAOSTAT Agriculture Data (2004) http://apps.fao.org.

Ganapathi TR, Higgs NS, Balint-Kurti PJ, Arntzen CJ, May GD, Van Eck JM (2001). Agrobacterium-mediated transformation of the embryogenic cell suspensions of the banana cultivars Rasthali (AAB). Plant Cell Rep. 20: 157–162.

Gheysen G, Angenon G, Montague MV (1998). Agrobacterium-mediated plant transformation: a scientifically intriguing story with significant application. In: Lindsey K (ed) Transgenic plant research. Harwood Academic Press, Netherlands. pp. 1-33.

Gynheung (1987). Methods in Enzymol. 153: 292-305.

Hajdukiewicz P, Svab Z, Maliga P (1994). The small versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. Plant Mol. Biol. 25: 989-994.

Hansen G, Wright MS (1999). Recent advances in transformation of plants. Trends Plant Sci. 4: 226-231.

Hernandez JBP, Remy S, Sauco VG, Swennen R, Sagi L (1999). Chemotactic movement and attachment of *Agrobacterium tumefaciens* to banana cells and tissues. J. Plant Physiol. 155: 245-250.

Hiei Y, Ohta S, Komari T, Kumashiro T (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant J 6: 271-282.

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996). High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nature Biotechnol. 14: 745-750.

Jefferson RA (1987). Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. 5: 387-405.

Jones DR (2000). Diseases of Banana, Abacá and Enset.. CAB International. p. 544.

Khanna H, Becker D, Kleidon J, Dale J (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, Rownak A, Mason H, Wiecko A, Novak FJ, Arntzen CJ (1995). Generation of transgenic banana (*Musa acuminata*) plants via Agrobacterium-mediated transformation. Bio/Technology 13: 486-492.

Murashige T, Skoog, F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15 : 473-497.

Rashid H, Yokoi K, Toriyama K, Hinata K (1996). Transgenic plant production mediated by Agrobacterium in Indica rice. Plant Cell Rep 15: 727-730.

Sagi L, Panis B, Remy S, Schoofs H, De Smet K, Swennen R, Cammus B (1995). Genetic transformation of banana (*Musa* spp.) via particle bombardment. Bio/Technol. 13:481-485.

Shibata D, Liu YG (2000). *Agrobacterium*-mediated plant transformation with large DNA fragments. Trends plant Sci. 5: 354-357.

Stewart CN, Via LE (1993). A rapid CTAB DNA isolation technique for RAPD finger printing and other PCR application. BioTechniques 14: 748-750.

Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thomton S, Brettel R (1997). *Agrobacterium tumefaciens*-mediated barley trans-formation. Plant J. 11:1369-1376.

Tripathi L, Tripathi JN, Oso RT, Hughes Jd'A, Keese P (2003). Regeneration and transient gene expression of African *Musa* species with diverse genomic constitution and ploidy levels. Tropical Agr. 80:182-187.

Tushemereirwe WK, Kangire A, Smith J, Ssekiwoko F, Nakyanzi M, Kataama D, Musiitwa C, Karyeija R (2003). An out break of Banana bacterial wilt in Uganda. InfoMusa 12: 2-6.