Full Length Research Paper

Agrobacterium mediated transformation of banana (Musa sp.) cv. Sukali Ndiizi (ABB) with a modified Carica papaya cystatin (CpCYS) gene

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Conventional banana breeding for pest and disease resistance is a very difficult and slow process due to the limited sources of resistance, sterility of cultivated banana varieties, high polyploidy levels, long cropping cycle and the lack of rapid screening methods. Molecular breeding using the transgenic approach with candidate genes such as cystatins offers an alternative method to banana improvement. Cystatin proteins inhibit the activity of cysteine proteases responsible for the breakdown of dietary proteins in the gut of many pests including nematodes resulting in protein deficiency. In this study, the papaya cystatin gene was introduced into the banana genome. Embryogenic cell suspension (ECS) cultures of the banana cultivar Sukali Ndiizi (ABB) were used as explants material for the successful transformation of banana. The Carica papaya cystatin gene (CpCYS-Mut89) previously modified to improve its inhibitory potential against banana pests was introduced into this cultivar using Agrobacterium tumefaciens, strain LBA4404 and the gus reporter gene was used to observe successful transformation process. We report the successful protocol for routine transformation of this cultivar, which was completed in six months with plant regeneration observed at a frequency of 23%. An additional four months was required to multiply the regenerant lines in order to have at least 20 plants per line for downstream challenging studies. Putatively transgenic plants were analyzed by PCR using hpt and CpCYS-Mut89 specific primers to confirm the presence of transgenes. Out of 28 selected lines, 27 were positive for both hpt and CpCYS-Mut89 transgenes giving 96.4% transformation efficiency. Five lines were then selected on the basis of putative PCR positives and a Southern blot analysis gave hybridization signals with 1 to 4 copy number integration patterns characteristic of Agrobacterium mediated transformation. These results confirm stable gene integration in East African banana cultivar cv. Sukali Ndiizi (genome group ABB) through an efficient Agrobacterium-mediated transformation protocol described for routine use in future improvement of this crop with genes of economic importance.

Key words: Cystatins, banana, Agrobacterium mediated transformation, southern blot.

INTRODUCTION

Banana is one of the most important staple food crops in the tropics and a source of income to millions of poor subsistence farmers. It is the developing world's fourth most important food crop after rice, wheat and maize (Bioversity International, 2006). In Uganda, it is one of the most important staple crops contributing about 30% of the total food consumption and 14% total crop value (Kalyebara et al., 2005). About 24% of the agricultural households are engaged in banana production. Banana being a year round fruiting crop ensures food security at

household level, providing food to more than 70% of Uganda's population on a regular basis. Banana is primarily grown for subsistence needs and any surplus for sale to local markets.

Two major pests affect banana productivity through damage to the stem and root system. These are the banana weevil (*Cosmopolites sordidus*) (Gold et al., 2001) and parasitic nematodes of various species (Speijer et al., 1998). Banana weevil and nematodes are heavily responsible for the decline and disappearance of highland bananas from their traditional growing zones in East Africa (Gold et al., 1999). Several migratory endoparasitic nematodes including, *Pratylenchus goodeyi, Radopholus similis* and *Helicotylenchus multicinctus* have been reported to attack bananas in Uganda leading to serious crop losses (Gold et al., 1994). *R. similis*, the burrowing nematode is reported to be the most damaging, with a life cycle of 20 to 25 days. *R. similis* feeds and reproduces within living corm and root tissue, affecting the root cortex leading to necrosis before death (Sarah et al., 1996). Tissue damage impairs water and nutrient uptake and causes poor plant anchorage leading to plant toppling, crop losses and reduced bunch weight (Speijer et al., 1998).

Conventional banana breeding for pest and disease resistance is a difficult and slow process due to the limited sources of resistance, sterility of cultivated banana varieties, high polyploidy levels, long cropping cycle and the lack of rapid screening methods. Biotechnology tools such as genetic engineering can offer potentially effective and sustainable solution to the problem of controlling banana parasitic nematodes and banana weevils by transferring important genes into the banana genome without altering the original characteristics of a given cultivar. *Agrobacterium*- mediated transformation is one of the methods of gene transfer that has been successful in the production of transgenic banana of other internationally important cultivars (Khanna et al., 2004; Ghosh et al., 2009). It is a well-known fact that banana as in many monocotyledonous plants cultivar or variety recalcitrance to tissue culture and Agro-bacterium mediated genetic transformation. Therefore the objective of this work was to develop an efficient agrobacterium mediated transformation protocol for routine genetic improvement of this popular east African Cultivar based on recently developed embryogenic cell suspension cultures.

MATERIALS AND METHODS

The study was carried out at the National Agricultural Research Laboratories Kawanda, one of the research institutes of the National Agricultural Research Organization, in the tissue culture laboratory, biotechnology laboratory and green house. Kawanda

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(0°25'N, 32°32'E) is 13 km north of Kampala city and 1,195 m above sea level. The climate is tropical with bimodal rainfall. Embryogenic cell suspensions (ECSs) of banana cv. Sukali Ndiizi (AAB) developed in the tissue culture laboratory at National Agricultural Research Laboratories were used. ECS were sub-cultured and maintained as previously described (Cote et al., 1996). The *Agrobacterium* strain LBA4404 was used. The transformation vector was designed using pCAMBIA1300 binary vector obtained from CAMBIA. The papaya cystatin gene (*Cp*CYS-Mut89), a donation from the laboratory at University of Pretoria was cloned within the multiple cloning sites (MCS) under the double enhancer version of Cauliflower Mosaic Virus (CaMV) 35S promoter and CaMV 35S terminator. The pCAMBIA vector back-bone sequence also constituted kanamycin resistance gene (*npt11*) for bacterial selection and hygromycin B phosphotransferase (*hpt*) resistance gene as the plant selectable marker gene. The *hpt* gene was driven by the CaMV 35S promoter (Figure 1). In addition, ECS were transformed with the binary vector pGreen constituting the *gusA* gene under the control of the maize poly-ubiquitin promoter as the positive control. Successful transformation was monitored by transient histochemical Gus assay.

Agrobacterium- mediated transformation of ECSs of the banana cv. Sukali Ndiizi

Embryogenic cell suspensions (ECSs) from male flowers of banana cultivar Sukali Ndizi were transformed using the Centrifugation Assisted *Agrobacterium*-mediated Transformation system (CAAT) as previously reported by Khanna et al.(2004). To increase the competence of ECSs and therefore transformation efficiency, they were refreshed with new media five days prior to transformation. Prior to transformation, a settled cell volume (SCV) of 1.25 ml ECSs in 50 ml of liquid MA2 were cultured for five days to induce fast cell division and subsequently increase their competence for *Agrobacterium* infection. Liquid MA2 consisted of standard MS salts and vitamins (Murashige and Skoog, 1962), 4.1 µM biotin, 4.5 µM 2, 4-D, 680 µM glutamine, 100 mg/l malt extract, 20 mg/L ascorbic acid, 45 g/L sucrose and the pH was adjusted to 5.3 before autoclaving. A 0.5 ml five day-old aliquot settled cell volume of banana ECSs were re-suspended in 10 ml activated *Agrobacterium* suspension, adjusted to 0.6 (OD$_{600nm}$). The mixture of ECS and Agrobacterium were incubated in the dark at 22°C for 5 days. Infected ECS were then washed in liquid MA2 containing a broad spectrum antibiotic cefotaxime and then transferred onto selective embryo formation media (MA3) comprising 3.2 g/L SH salts, standard MS vitamins, 4.1 µM biotin, 100 mg/L malt extract, 680 µM glutamine, 230 mg/L proline, 100 mg/L myo-inositol, 60 mg/L citric acid, 40 mg/L ascorbic acid, 10 g/L PVP 10, 400 mg/L L-cysteine, 1.1 µM NAA, 0.2 µM zeatin, 0.5 µM kinetin, 0.7 µM 2-ip, 45 g/L sucrose, 10 g/L lactose and 2.3 g/L phytagel).

Histochemical Gus assay

Five days after co-cultivation of ECS with the *A. tumefaciens* harbouring pGreen binary vector with *gusA* gene encoding the β-glucuronidase (GUS) enzyme, transformed ECS of the banana cv. Sukali Ndiizi were assayed for transient expression of the *gusA* reporter gene. Three samples of 0.02 ml SCV were incubated in a Gus assay substrate constituting 100 mM sodium phosphate (pH 7.0), 50 mM ascorbate, 0.1% tritonX-100, 0.4 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) according to Jefferson (1987). Sterile sheets of filter paper were laid in sterile 15 cm diameter Petri dishes and 1 ml of X-Gluc staining solution was placed at the center of each sterile filter paper. Transformed cells plated on 50 µM nylon mesh, were transferred onto the wet filter
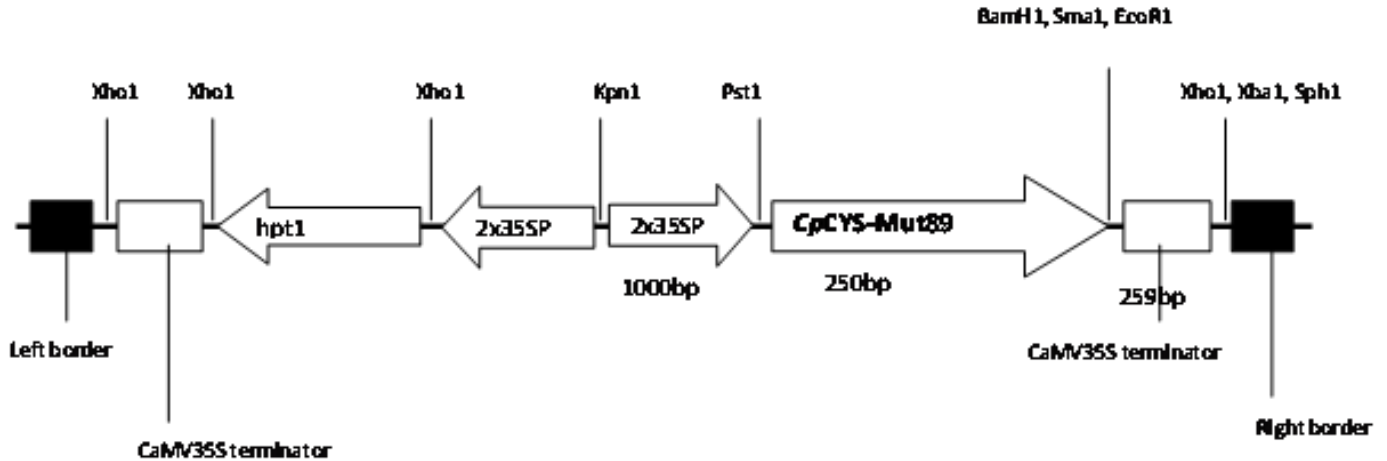
Figure 1. Schematic representation of the T-DNA for pCAM35S-CysMut89.

papers and stained with additional 200 µL of X-Gluc staining solution. The Petri dishes were sealed with cling film and incubated at 37°C overnight for expression of β-glucuronidase (*gusA)* gene. The numbers of blue foci were then observed under a stereo microscope and photographed using a digital camera. Histochemical GUS staining of leaves, stem and roots of greenhouse transgenic plants two months after weaning (~30 cm tall) was also performed for detection of stable *gus* expression in mature tissues. To prevent interference of visual analysis and photographing from chlorophyll and other pigments, the stained materials were soaked and washed in a solution containing acetic: ethanol (1:3).

Selection and regeneration of transgenic banana plants

Infected ECS were washed with liquid MA2 medium containing a broad spectrum antibiotic cefotaxime (300 µg/µL) and drained. Transformed ECS were then transferred to selective semisolid MA3 medium with 300 µg/µL cefotaxime to kill off bacteria and 50 µg/µL hygromycin to select transformed cells. Transformants maintained selective semisolid MA3 medium with subsequent subculture every two weeks until embryos were obtained. After three months of incubation in the dark at 26°C, grayish-white masses of cell clusters appeared. The cell clusters were individually transferred onto selective semisolid RD1 media consisting of (standard MS salts and vitamins (Murashige and Skoog, 1962), 20 mg/L ascorbic acid, 100 mg/L myo-inositol, 30 g/L sucrose, pH 5.3 and 2.3 g/L phytagel) with 300 µg/µL cefotaxime and 50 µg/µL hygromycin for further embryo initiation, until good embryos were obtained. The embryos were maintained on RD1 for for months. Single embryos were then transferred onto selective semisolid MA4 media constituting standard MS salts supplemented with Morel vitamins (Morel and Wetmore, 1951), 0.22 µM 6-benzylaminopurine (6-BAP), 1.14 µM IAA, 30 g sucrose, pH 5.8 and 2.3 g phytagel (which is embryo germination media according to Namanya et al. (2004)) with 300 µg/µL cefotaxime and 50 µg/µL hygromycin. After one month, the selection pressure was removed by transferring the regenerated shoots onto anti-biotic free semisolid proliferation media containing standard MS salts and vitamins, 20 mg/L ascorbic acid, 30 g/L sucrose, 5 mg/L 6-BAP, pH 5.8 and 2.3 g/L phytagel for shoot multiplication, with subculture of 2 weeks interval. The banana shoots were then transferred onto proliferation media with half strength of 6-BAP after obtaining at least four clones per plantlet to maintain single shoots. After ten months, 57 rooted lines of

approximately 5 cm in height were weaned and placed in a high humidity chamber at 27°C in the biosafety level II green house. After a month, the shade was removed and plants transferred to pots. The ECS transformed with the *gusA* gene went through the same selection and regeneration process but with 50 µg/µl geneticin for plant selection.

DNA isolation and PCR analysis

Genomic DNA of papaya cystatin transformed and non transformed, two month old green house banana plants was extracted from cigar leaf tissue using the modified cetyltrimethylammonium bromide (CTAB) protocol (Gawel and Jarret, 1991). DNA was analyzed using PCR to detect presence of the hygromycin B phosphotransferase (*hpt*) selectable marker and the papaya cystatin gene (*Cp*CYS-Mut89). The PCR reaction contained 1.0 mM MgCl$_2$ for *Cp*CYS-Mut89 primers and 1.5 mM MgCl$_2$ for *hpt* primers, 0.4 µM of each of the primer pairs, 1× PCR buffer, 0.24 mM dNTPs and 0.02Unit Taq/reaction (25 µl). The primers CYS_For (5′-GGA ATT GTG ATC GGT GGT TT-3′), CYS_Rev (5′- CGA GCT AAT GAG CAT CAC CA-3′), HYG-F (5′-CTA TCG GCG AGT ACT TCT ACA CAG-3′) and HYG-R (5′-CCC ATG TGT ATC ACT GGC AAA C-3′) were designed using an online primer designing software (Primer3, version 0.4.0, http://frodo.wi.mit.edu) as forward (For) and reverse (Rev) for *Cp*CYS-Mut89 gene, and as forward (F) and reverse (R) for *hpt* gene. The reaction mixture was subjected to an initial denaturation step of 94°C for 3 min followed by 35 cycles of 94°C for 30 s; annealing temperatures of 60°C for *hpt* and 61°C for *Cp*CYS-Mut89 for 1 min; 72°C for 1 min and a final extension step of 72°C for 8 min.

Southern blotting analysis

Genomic DNA putatively transformed lines was restricted with 100 units of EcoRI (New England Biolabs). EcoRI 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 the positively charged nylon membrane (Boehringer Mannheim) by the method of Southern (1975). The blots were hybridized with DIG labeled *Cp*CYS-Mut89 probes generated using PCR DIG probe synthesis kit (Roche). Hybridization and detection of the probe was done using the DIG luminescent detection kit for nucleic acids (Roche
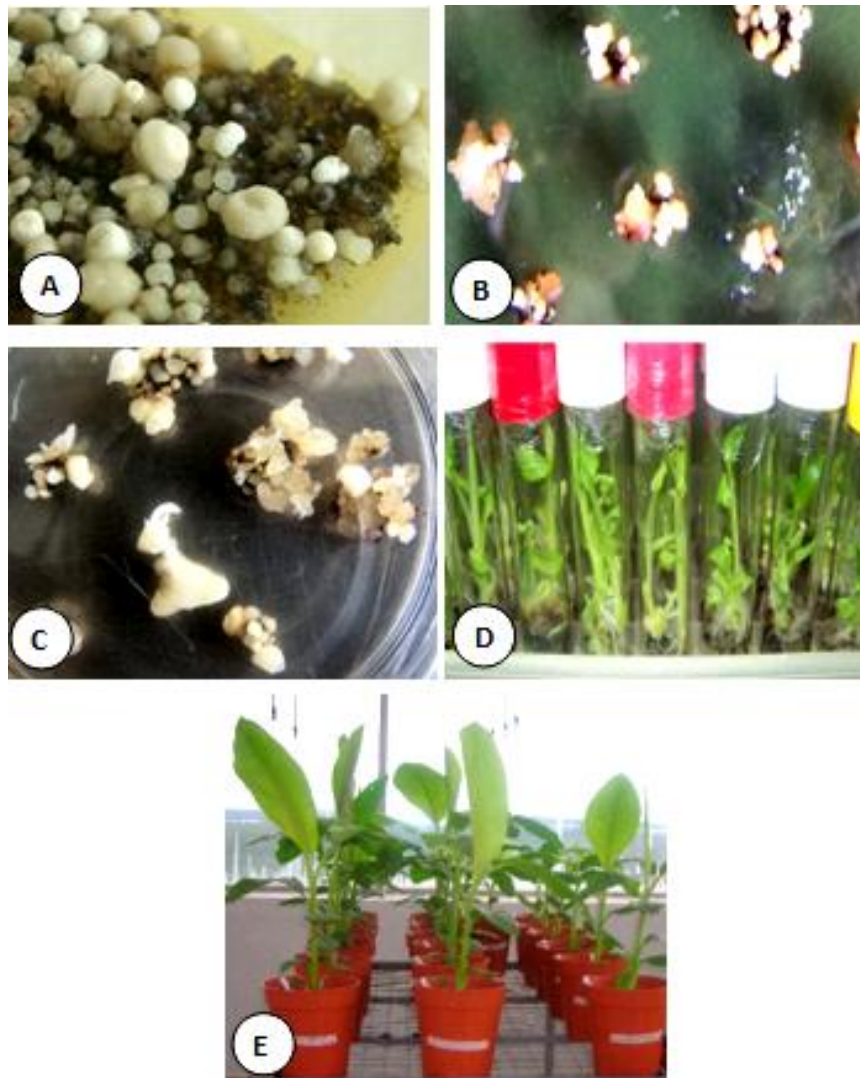
Figure 2. Transformation and regeneration of transgenic bananas. **A.** White clusters of Ndiizi cells with black cells in the background show that the black cells were non-transformed and thus died due to the toxicity of the hygromycin antibiotic in the selection MA3 media. **B.** Mature embryos on selection embryo initiation media (RD1) 2 months after transferring the white clusters to RD1 with some embryos germinating into shoots on selection MA4 1 month after transferring them onto MA4. **D.** Multiple shoots regenerated on non-selection proliferation media **E.** Transgenic lines in the green house.

Diagnostics) according to manufacturer's instructions. The hybridization signal was detected by autoradiography.

RESULTS

Agrobacterium mediated transformation of the banana cv. Sukali Ndiizi

Readily available ECSs of the banana cultivar Ndiizi were transformed with *Agrobacterium* confirmed to carry the binary vector with *Cp*CYS-Mut89 gene. Distinct whitish cell clusters were observed three months after transfer to selective MA3, whereas untransformed cells showed necrosis (Figure 2A). The cell clusters were then transferred onto selective semisolid RD1 media with subsequent sub culturing every two weeks to allow embryo initiation. Embryos were observed on selective RD1 after two months (Figure 2B). Mature antibiotic resistant embryos were then transferred onto selective semisolid MA4 media and embryos were observed germinating into shoots after one month (Figure 2C). The 57 hygromycin resistant shoots obtained were multiplied on proliferation media without any selection and about 3 to 8 shoots per plant were obtained in two months (Figure 2D). All plants regenerated from one cell clump were
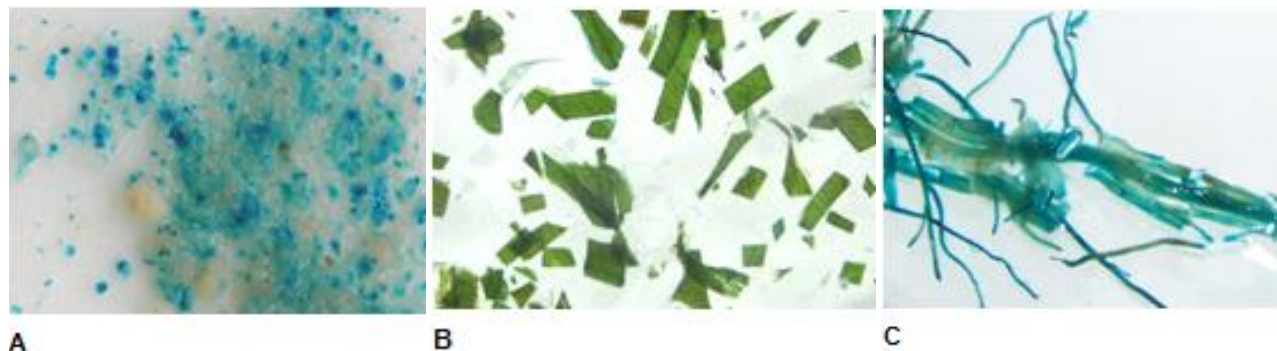
Figure 3. Histochemical assay for transient and stable expression of the *gusA* gene in *Agrobacterium*- transformed ECSs and mature tissues from 2 month old greenhouse grown plantlets of banana cv. Sukali Ndiizi after overnight incubation at 37°C. *Gus* expression in *gus* transformed embryogenic cells (A), leaf tissues (B); root tissues (C).
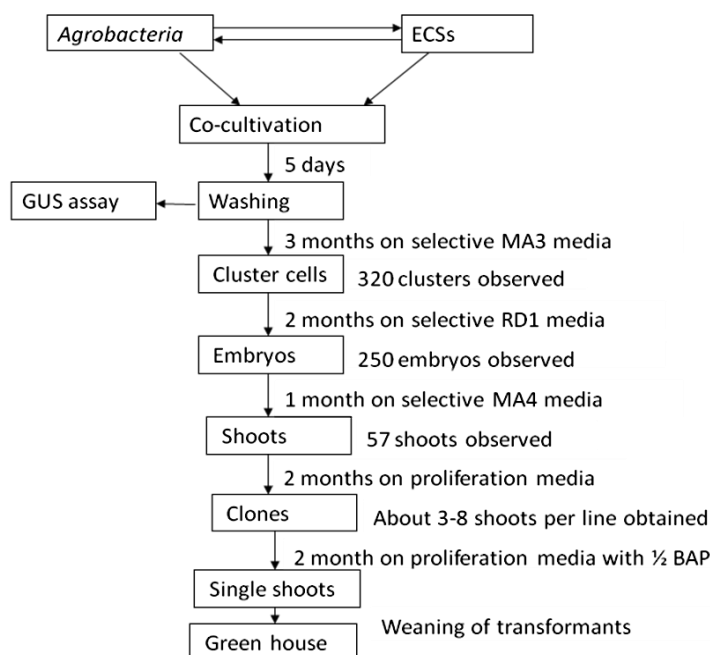
Figure 4. Schematic representation of the transformation of banana cultivar Sukali Ndiizi, selection and regeneration process indicating the time intervals between stages and number of cell clusters, embryos and shoots obtained, before transfer to a biosafety level-II greenhouse.

considered to be clones and counted as one line. A summary of the whole transformation, selection and regeneration process is represented in the schematic diagram (Figure 4).

Histochemical GUS assay on the transformed ECSs of the banana cv. Sukali Ndiizi

The samples of transformed ECSs were histochemically stained for the expression of the *gusA* gene. Samples of leaves, stems and roots were also collected from two

month old greenhouse grown transgenic plants and histochemically stained. The dark blue spots show that Ndizi cells and mature tissues took up the reporter gene (Figure 3). Positive results from the histochemical *gus* expression assay indicate that there was successful insertion of the target gene into banana using the *gus* expressing cells and mature tissues as an indicator.

Molecular analysis of transgenic plants

The presence and integration of the transgene in the
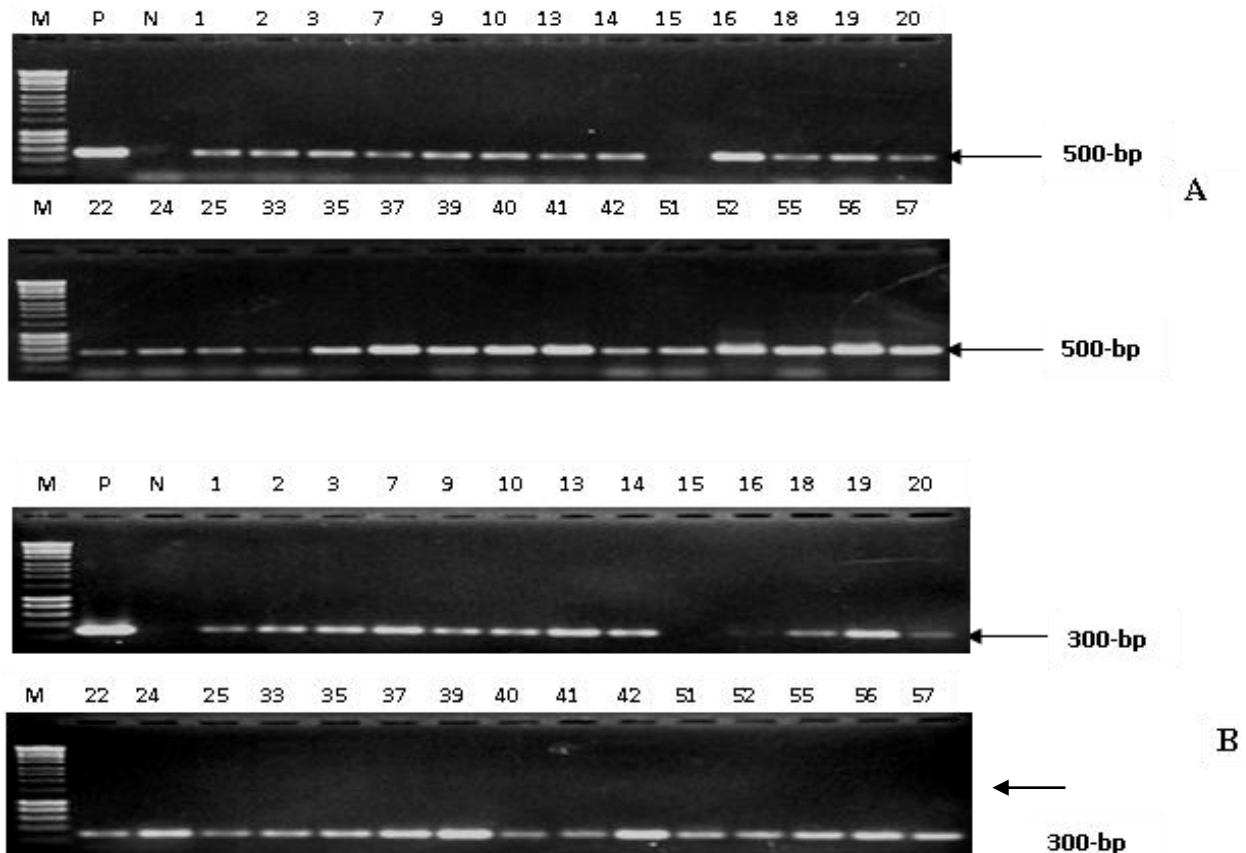
Figure 5. A, PCR analysis of the putatively transformed banana plants amplifying a 300 bp internal fragment of the cystatin gene. **B,** PCR analysis of the putatively transformed banana plants amplifying a 500 bp internal fragment of the hygromycin gene. M, DNA sizing marker (Hyperladder1); P, plasmid;   Lanes 1-57, putatively transformed plants.

genome of transgenic plants was confirmed by PCR and Southern blot analysis. PCR was carried out with *hpt* and *Cp*CYS-Mut89 specific primers and amplified products of about 500 bp (Figure 5A) and 300 bp corresponding to an internal fragment of the *hpt* and *Cp*CYS-Mut89 gene were obtained (Figure 5B). Amplified products were observed in 27 out of the 28 selected transformed lines. Line 15 neither amplified for *hpt* primers nor *Cp*CYS-Mut89 specific primers, indicating that it was an escape. No amplified product was observed in case of the non-transformed plant (negative control). Southern blot analysis of 5 randomly selected transgenic banana plants of individual events digested with EcoRI and hybridized with *Cp*CYS-Mut89 probe confirmed integration of *Cp*CYS-Mut89 gene in the banana genome. Different banding patterns were obtained indicating independent transgenic events. The non-transformed plant did not show any signal (Figure 6).

DISCUSSION

An efficient transformation system using appropriate

initial explants is a key in producing transgenic plants (Ghosh et al., 2009). The histochemical Gus assay was performed to assess the success of insertion of the *gusA* gene into the embryogenic cells using *Agrobacterium*-mediated transformation. High expression of *gusA* gene in ECSs indicates competence of the embryogenic cells to *Agrobacterium*-mediated transfer. Positive results were also obtained from histochemical staining of leaves, stems and roots indicating stable *gus* expression in mature tissues. High transient and stable *gus* expression in ECSs and mature tissues of banana has also been observed by Khanna et al. (2004) in Cavendish and Lady finger and Huang et al. (2007) in *Musa acuminate* cv. Mas respectively.

Agrobacterium-mediated transformation has been successfully used to obtain transgenic banana plants using different initial explants. May et al. (1995) used apical meristems of the cultivar Grand Naine as initial explants to obtain transgenic plants. Intercalary meristematic tissues of East African highland bananas have also been used as initial explants for transformation (Tripathi et al., 2008). In this study, embryogenic cell suspensions (ECSs) of the banana cv. Sukali Ndiizi were
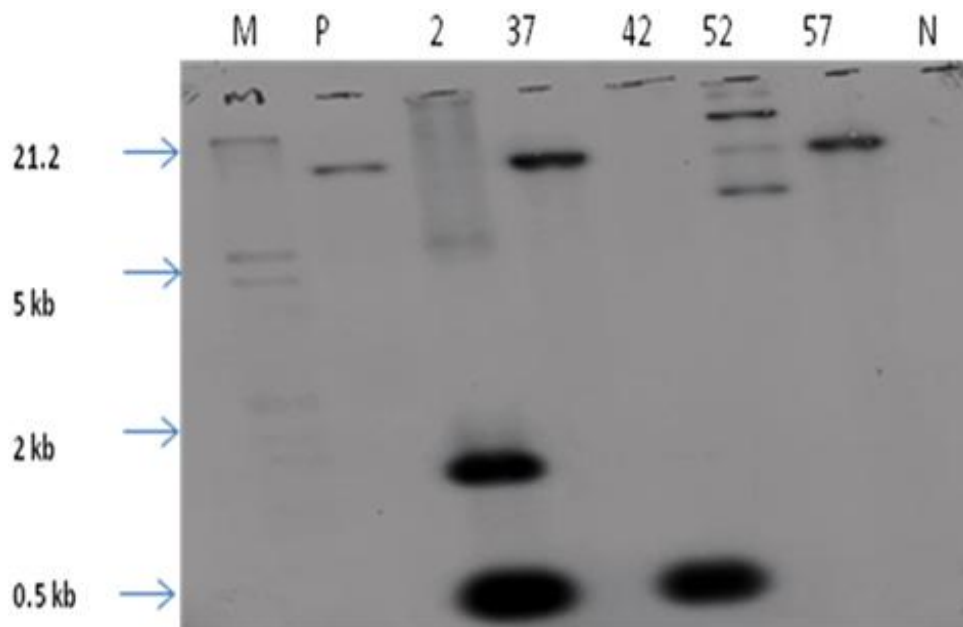
Figure 6. Southern blot analysis of five randomly selected transgenic banana lines.

transformed using *Agrobacterium* strain LBA4404. This method is less tedious and faster for the cultivars for which formation of ECS is possible because repeated rounds of selection are not necessary (Ghosh et al., 2009). Other methods of transformation as electroporation (Sagi et al., 1994), and biolistics have been used for banana transformation (Becker et al., 2000). However, *Agrobacterium*-mediated transformation is the most preferred due to the high transformation frequencies (Khanna et al., 2004) with fewer transgene copy numbers and significantly higher transient and stable gene expression compared to biolistics (Arinaitwe, 2008).

Agrobacterium was pre-induced with a surfactant, pluronic F68 (Cheng et al., 1997) to aid in the transformation of banana cells (Khanna et al., 2004). The co-cultivation process was done at 22°C for 5 days as compared to 3 days by Khanna et al. (2004) because overgrowth occurs at higher temperature leading to the death of banana cells. Acetosyringone was included in pre-induction media (TMA1) and co-cultivation media to enhance transfer of the T-DNA, a compound that enhances chemotactic movement and attachment of *Agrobacterium tumefaciens* to wounded tissues of banana cultivars (Hernández et al., 1999). Heat shocking of banana cells prior to transformation was done to minimize cell death response resulting in recovery of a large number transformed plants following *Agrobacterium* infection. Centrifugation was done to increase contact of *Agrobacterium* with host cells (Khanna et al., 2004) hence increased transient gene expression. Post co-cultivation wash of ECSs in liquid recovery media removed *Agrobacterium* colonies sticking to ECSs and

infected cells recover from infection after-effects like phenolics before being transferred to selection media. After three subcultures *Agrobacterium* contamination was completely eliminated and cells proliferated more vigorously. After three weeks of *Agrobacterium*-mediated transformation, ECS turned brown due to necrosis and massive death of non-transformed embryogenic cells on selective MA3 media also occurred. One month later, numerous whitish cell clumps appeared on the surface with dead cells in the background. Similar results were obtained by Arinaitwe (2008) and Ghosh et al. (2009). One of the critical steps after transformation is the selection procedure used for the recovery of transgenic shoots (Joersbo, 2001). This is because embryogenic cell colonies obtained during the selection procedure must survive the stress induced by the combination of media and antibiotics used to kill both *Agrobacterium* and untransformed plant cells (Arinaitwe, 2008). In this study, *hygromycin B phosphotransferase* (*hpt*) was used as a selectable marker and hygromycin antibiotic (50 µg/µl) was used as a selection agent for stable integration. Hygromycin is a preferred selection for easily scoreable phenotype and faster inhibition response of explants (Sreeramanan et al., 2006). Sreeramanan et al. (2006) further reported that hygromycin inhibited growth of non-transformed banana buds at lower concentrations.

Distinct cell clusters were observed on selective MA3 after 3 months and distinct embryos were observed on RD1 after 2 months. Embryos were able to germinate on MA4 after 1 month. Embryos from a cell clump were assumed to have resulted from the same transformation event and all plants from such a cluster were counted as one line. Although, *Agrobacterium*-mediated transforma-

tion is a good transformation system, not all the transformed ECSs developed into embryos and not all embryos developed into shoots. This is because of the differences in embryogenic competences among the ECSs. Variation in embryogenicity depends on accumulation of auxin in embryogenesis competent protodermal and subprotodermal explants cells as a result of auxin treatment, expression of embryogenesis controlling gene especially the genes related to auxin signaling as LEC2 (Kurezyńska et al., 2007; Ledwoń and Gaj, 2009), cell division and cytological changes in pre-embryogenic cells including formation of thick cell wall and cutin deposition, and symplasmic isolation of embryogenic cells (Kurezyńska et al., 2007).

The PCR technique was used to confirm successful introduction of the transgene into the banana genome. However, presence of residual *Agrobacterium* in transgenic plants leads to false positives making conventional PCR unconvincing unless verified by stable integration of the transgene in the plant genome (Chen et al., 2006; Ghosh et al., 2009). To minimize this problem, putatively transformed plants were maintained on antibiotic free media for four months and then transferred to greenhouse and maintained in the green house for at least two months before samples were collected for PCR analysis.

Stable integration of the papaya cystatin gene was further confirmed by Southern blot analysis. The mobility of the bands differed in most transgenic lines, verifying that the plants were the result of independent transformation events which are probably randomly located on the genome. Simple integration patterns have been reported in banana with different genes using *Agrobacterium*-mediated transformation (Khanna et al., 2004; Arinaitwe, 2008; Ghosh et al., 2009). Lines 37 and 56 shows similar integration profiles indicating that they were most likely clones from one transformation event and hence derived from a single transformed embryogenic cell. Southern blot analysis with the modified papaya cystatin (*Cp*CYS-Mut89) probes gave hybridization patterns in the transformed and not the untransformed control suggesting that there is no existence of highly complementary papaya cystatin sequences in banana or the probe design was efficient enough not to pick them up. The negative signals in the untransformed controls confirm that the observed signals truly indicate the integration of the transgene. This was further supported by the positive signal from the vector construct pCAMCYSMUT89, implying that the probes correctly detected the integration events.

Conclusion

Bananas are important staple foods, which are difficult to breed due to the high sterility of commercial cultivars. This crop being perennial in nature, suffers numerous pests such as the banana weevil and parasitic

nematodes, and hence the need to generate transgenic bananas with novel genes such as cystatins to confer elevated resistance against the banana weevil and banana parasitic nematodes. In this study, ECSs of the banana cultivar Sukali Ndiizi were successively transformed with the modified papaya cystatin (*Cp*CYS-Mut89*)* using the *Agrobacterium*- mediated transformation method. When transgenic plants are obtained, the earliest essential step is molecular characterization. Many primary transformants must be analyzed due to the random insertion of new DNA into the plant genome, often leading to a generation of plants with multiple transgene copies integrated into one or more chromosomal locations. The number of copies of the exogenous gene inserted into the genome is one of the reasons for reported transgenic silence (Weng et al., 2004). Most plants with one or two integration events yield high-level expression of the exogenous gene, whereas lower and unstable transgene expression and transgene silencing has been associated with high numbers of copies (Flavell, 1994; Kooter et al., 1999; Iyer et al., 2000). In this study, a total of 57 transgenic lines were generated. PCR analysis of the selected transgenic lines of Sukali Ndiizi shows 96.4% presence of the *Cp*CYS-Mut89 gene. Southern blot analysis of the putative positives confirmed stable integration of the *Cp*CYS-Mut89 gene into the banana genome. The transgenic lines show one to four integration patterns, which are characteristic of *Agrobacterium*- mediated transformation. The integration patterns were also generally random, implying different transformation events in the generated lines.

Other cystatins like the rice cystatin (Oryzacystatin-I) have been integrated into many plant species to provide protection against plant parasitic nematodes and coleopterans. These include potato (Atkinson, 1996), rice (Vain et al., 1998), Cavendish banana (Atkinson et al., 2004) for nematodes and potato (Lecardonnel et al., 1999) for coleopterans. In all these cases, increased protection to nematode and coleopteran damage was observed. In the current study, we introduced a cystatin from *Carica papaya* (Song *et al.*, 1995) that was later modified to improve its inhibition capacity by Kiggundu (2008) into the banana cultivar Sukali Ndiizi in an approach to protect banana against the banana weevil and banana parasitic nematodes.

REFERENCES

Arinaitwe G (2008). An improved *Agrobacterium*- mediated transforma-

tion method for banana and plantain (*Musa spp*). PhD Thesis, Katholieke Universiteit Leuven, Belgium.

Atkinson HJ (1996). Prospects for the control of potato cyst nematodes. J. R. Agric. Soc. Engl. 157:66-76.

Atkinson HJ, Grimwood S, Johnston K, Green J (2004). Prototype demonstration of transgenic resistance to the nematode *Radopholus similis* conferred on banana by a cystatin. Transgenic Res. 13:135-142.

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.

Bioversity International (2006). Socio-economic importance of bananas. Global Musa Genomics Consortium. www.musagenomics.org.

Chen Y, Lu L, Deng W, Yang X, McAvoy R, Zhao D, Pei Y, Luo K, Duan H, Smith W, Thammina C, Zheng X, Ellis D, Li Y (2006). *In vitro* regeneration and *Agrobacterium*-mediated genetic transformation of *Euonymus alatus*. Plant Cell Rep. 25:1043-1051.

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

Cote FX, Domergue M, Monmarson R, Schwendiman SJ, Tiesson C, Escalant JV (1996). Embryogenic cell suspensions from male flowers of Musa AAA cv. Grand naine. Plan. Physiol. 97:285-290.

Flavell RB (1994). Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc. Nat. Acad. Sci. USA. 91:3490-3496

Gawel NJ, Jarret R (1991) A modified CTAB DNA extraction procedure for *Musa* and *Ipomea*. Plant Mol. Biol. Rep. 9:262-266.

Ghosh A, Ganapathi TR, Nath P, Bapat VA (2009) Establishment of embryogenic cell suspension cultures and *Agrobacterium*-mediated transformation in an important Cavendish banana cv. Robusta (AAA) Plant Cell Tiss. Org. 97:131-139.

Gold CS, Karamura EB, Kiggundu A, Bagamba F, Abera AMK (1999). Geographic shifts in highland cooking banana (*Musa spp.*, group AAA-EA) production in Uganda. Int. J. Sust. Dev. World 6:45-56

Gold CS, Pena JE, Karamura EB (2001). Biology and integrated pest management for the banana weevil, *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae). Int. Pest Management Rev. 6:79-155.

Gold CS, Speijer P, Karamura E, Tushemereirwe W, Kashaija I (1994). Survey methodologies for banana weevil and nematode damage assessment in Uganda. Afr. Crop Sci. J. 2:309-321.

Hernández JBP, Remy S, Saúco VG, Swennen R, Sági L (1999). Chemotactic movement and attachment of *Agrobacterium tumefaciens* to banana cells and tissues. J. Plant Physiology, 155:245-250.

Huang X, Huang XL, Xiao W, Zhao JT, Dai XM, Chen YF, Li XJ (2007). Highly efficient *Agrobacterium*-mediated transformation of embryogenic cell suspensions of *Musa acuminata* cv. Mas (AA) via liquid co-cultivation system. Plant Cell Rep. 26:1755-1762

Iyer LM, Kumpatla SP, Chandrasekharan MB, Hall TC (2000). Transgene silencing in monocots. Plant Mol. Biol. 43:323-346.

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

Joersbo M (2001). Advances in the selection of transgenic plants using non-antibiotic marker genes. Physiol. Plant. 111:269-272.

Kalyebara R, Wood S, Abodi P (2005). The potential economic benefits of improved banana productivity in Uganda: an industry scale analysis. *IFRI BRIEF 9.*

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.

Kiggundu, A. 2008. Engineering plant cysteine protease inhibitors for the transgenic control of banana weevil, *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae) and other coleopteran insects in transgenic plants. PhD Thesis, University of Pretoria South Africa.

Kooter JM, Matzke TA, Meyer P (1999). Listening to the silent genes: transgene silencing, gene regulation, and pathogen control. Trends Plant Sci. 4:340-347.

Kurezyńska UE, Malgorzata GD, Ujezak A, Mazur E (2007). Histological analysis of direct somatic embryogenesis in *Arabidopsis thaliana* (L.) Heynh. Planta. 226:619-628.

Lecardonnel A, Chauvin L, Jouanin LO, Beaujean A, Prevost G, Sangwan-Novrreel B (1999). Effect of rice cystatin I expression in transgenic potato on Colorado potato beetle larvae. Plant Sci. 140:71-79.

Ledwoń, A, Gaj DM (2009). LEAFY COTYLEDON2 gene expression and auxin treatment in relation to embryogenic capacity of *Arabidopsis* somatic cells. Plant Cell Rep. 28:1677-1688.

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

Morel G, Wetmore RH (1951). Tissue culture of monocotyledons. Am. J. Bot. 38:138-140.

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

Namanya P, Magambo SMJ, Mutumba G, Tushemereirwe W (2004). Somatic Embryogenesis from immature male inflorescences of East African Highland Banana cv. Nakyetengu. Afr. Crop Sci. J. 12:43-49.

Sarah JL, Pinochet J, Stanton J (1996). The burrowing nematode of bananas, *Radopholus similis* cobb. *Musa* Pest Fact Sheet No. 1. INIBAP, Montpelier, France.

Song I, Taylor M, Baker K, Bateman RC (1995). Inhibition of cysteine proteinases by *Carica papaya* cystatin produced in *Escherichia coli*. Gene 162:221-224.

Southern EM (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Speijer PR, Mudiope J, Ssango F, Adipala E (1998). Nematode damage and densities at different plant growth stages of East African highland banana (*Musa* AAA), cv. Mbwazirume. Afr. Crop Sci. J. 6:283-292.

Sreeramanan S, Maziah M, Abdullah MP, Rosli NM, Xavier R (2006). Potential selectable marker for genetic transformation in banana. Biotechnol. 5:189-197.

Tripathi L, Tripathi JN, Tushemereirwe KW (2008). Rapid and efficient production of transgenic East African Highland Banana (*Musa spp.*) using intercalary meristematic tissues. Afr. J. Biotechnol. 7:1438-1445.

Vain P, Worland B, Clarke MC, Richard G, Beavis M, Lin H, Kohli A, Leech M, Snape J, Christon P, Atkinson HJ (1998). Expression of an engineered cysteine proteinase inhibitor (Oryzacystastin-ID86) for nematode resistance in transgenic rice plants. Theor. Appl. Genet. 96:266-271.

Weng H, Pan A, Yang L, Zhang C, Liu Z, Zhang D (2004). Estimating the number of transgene copies in transgenic rapeseed by Real-Time PCR assay with *HMG I/Y* as an endogenous reference gene. Plant Mol. Biol. Rep. 22:289-300.