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TRANSIENT EXPRESSION OF β-GLUCURONIDASE IN RECALCITRANT UGANDAN SWEETPOTATO AND PUTATIVE TRANSFORMATION WITH TWO *CRY* GENES

A. SEFASI, G. SSEMAKULA[1], M. GHISLAIN[2], K. PRENTICE[2], A. KIGGUNDU[3], R. MWANGA[2] and S.B. MUKASA
School of Agricultural Sciences, College of Agricultural and Environmental Sciences, Makerere University, P. O. Box 7062, Kampala, Uganda
[1]National Crops Resources Research Institute, Box 7084 Kampala, Uganda
[2]International Potato Center, P. O. Box 1558, Lima 12, Peru
[3]National Agricultural Research Laboratories Institute, Kawanda, P. O. Box 7065 Kampala, Uganda
Corresponding author: sbmukasa@caes.mak.ac.ug, sbmukasa@yahoo.com

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ABSTRACT

Sweetpotato (*Ipomoea batatas* Lam.) has high potential to contain hunger, malnutrition and poverty in sub-Saharan Africa (SSA), since it gives early yield with few inputs. However, productivity of the crop in Africa is very low due to various challenges, such as severe viral diseases and increasing attacks by sweetpotato weevils, *Cylas puncticollis* and *C. brunneus*. Effective resistance to weevils has not been identified in the sweetpotato gene pool. On the other hand, the weevil-resistance genes, *cry7Aa1* and *cry3Ca1* were assembled into a plasmid vector for use in genetic transformation of African sweetpotato cultivars. The parameters for efficient transfer of these genes and the conditions for *de novo* regeneration optimised in preliminary studies were used in the genetic transformation of Ugandan landrace 'Kyebandula' with *Agrobacterium tumefaciens* EHA 105 harbouring the plasmid pCIP84, which contains *cry7Aa1*, *cry3Ca1* and *nptII* in its T-DNA. Fifty-four percent of the explants formed adventitious buds. With a mean of 7 buds formed per explant, 6.0% explants formed shoots with a mean of one shoot per explant for those explants that formed shoots on medium containing 50 mg L^{-1} kanamycin as a selection agent. PCR analysis using primers for *cry7Aa1* showed that the transformation efficiency could be as high as 2%. These data highlight the potential of genetic transformation in transferring resistance genes and pave way for enhancement of food security through production of adapted sweetpotato weevil resistant cultivars.

Key Words: *Agrobacterium tumefaciens*, β-glucuronidase, *Ipomoea batatas*

RÉSUMÉ

La patate douce (*Ipomoea batatas* Lam.) a un potentiel de contenir la faim, la malnutrition et la pauvreté en Afrique Sub Saharienne étant donné sa précocité et son grand rendement avec peu d'intrants. Par ailleurs, sa productivité est trop basse due aux diverses contraintes liées aux maladies virales et attaques sans cesse croissante des charançons *Cylaspuncticollis* and *C. brunneus*. Une résistance efficace au charançon n'a pas encore été identifiée dans la collection des gènes de la patate douce. D'autre part, les gènes de résistance aux charançons, *cry7Aa1* et *cry3Ca1* étaient assemblées un vecteur de plasmide pour utilisation dans la transformation génétique des cultivars de patate douces africaines. Les paramètres pour transfert d'efficacité de ces gènes et les conditions de régénération *de novo* optimisées dans des études préliminaires étaient utilisées dans la transformation génétique du landrace ougandais 'Kyebandula'avec l'*Agrobacterium tumefaciens* EHA 105 portant le plasmide pCIP84, contenant les *cry7Aa1*, *cry3Ca1* et *nptII* dans son T-ADN. Cinquante quatre pourcent des explants ont formé des bourgeons adventices. Avec une moyenne de 7 bourgeons par explant, 6.0% d'explants ont formé des tiges avec une moyenne d'une tige par explant pour ces explants qui ont formé des tiges sur le media contenant 50 mg L^{-1} de kanamycine comme agent de sélection. L'analyse par PCR utilisant des primers pour *cry7Aa1* a montré que la

transformation efficace pourrait être aussi élevée que 2%. Ces données soulignent le potentiel de transformation génétique dans le transfert des gènes de résistance et un moyen d'améliorer la sécurité alimentaire à travers la production des cultivars de résistance adaptée aux charançons de la patate douce.

Mots Clés: *Agrobacterium tumefaciens*, β-glucuronidase, *Ipomoea batatas*

INTRODUCTION

Plant transformation through *Agrobacterium* species is the most commonly used system since the transferred DNA has minimal rearrangement and there are only few transgene copies inserted into the plant genome (Choi *et al.,* 2007; Xing *et al.,* 2007). However, the process of transfer of foreign genes using *Agrobacterium* and integration into plant genomes is complex (Valentine, 2003). There are so many variables that influence *Agrobacterium*–mediated transformation, and no evidence of obvious combinations of these variables exists for broad application across plant species and cultivars (Xing *et al.,* 2007; González *et al.,* 2008). In addition, sweetpotato (*Ipomoea batatas* Lam.) is considered recalcitrant to genetic transformation, as different cultivars respond differently to transformation conditions (Zang *et al.,* 2009; Yang *et al.,* 2011). African cultivars have been reported to be particularly difficult to transform (Luo *et al.,* 2006; Tovar *et al.,* 2009).

Reporter genes have been used to develop transformation systems for cultivars that were not known to be amenable to genetic transformation (Xing *et al.,* 2007; Yu *et al.,* 2007). The common reporter genes that have been used to detect gene expression and protein localisation *in vitro* include chloramphenicol acetyl transferase (CAT), green fluorescent protein (GFP) (Lawton *et al.,* 2000), luciferase (LUC) and â-glucuronidase (GUS) (Xing *et al.,* 2008). Although the *gfp* has been used in some cases (Lawton *et al.,* 2000), the GUS gene remains the most common reporter for sweetpotato transformation (Song *et al.,* 2004; Yu *et al.,* 2007; Yang *et al.,* 2011). The histochemical procedure used to demonstrate GUS activity in transformed plant tissue is very powerful even for resolving differences in gene expression between individual cell and cell types within tissues (Jefferson *et al.,* 1987).

The factors that are critical for successful genetic transformation include appropriate selective antibiotics, concentration of acetosyringone, duration of co-cultivation with *Agrobacterium*, *Agrobacterium* concentration, light/dark treatment during co-culture, wounding of explants, infiltration of *Agrobacterium*, agitation during *Agrobacterium* infection, preculture and use of low temperature (Xing *et al.,* 2007; Yu *et al.,* 2007; González *et al.,* 2008). The aim of this study was to develop an *Agrobacterium*-mediated transformation system for a Ugandan sweetpotato landrace 'Kyebandula' based on transient expression of β-glucuronidase (GUS).

MATERIALS AND METHODS

Plant material. The Ugandan landrace Kyebandula was used in the optimisation of plant transformation conditions. Stem cuttings containing five to eight nodes were taken from the sreenhouse to the laboratory; washed for fifteen minutes with flowing tap water and submerged in 70% ethanol for two minutes. Thereafter, the vines were immersed for twenty minutes in 39% (v/v) commercial JIK, containing 3.85% sodium hypochlorite (NaOCl) and 0.03% Tween 20®. After surface sterilisation, the vines were transferred to sterile water and rinsed three times. The vines were then each cut into one or two nodes and established on sweetpotato propagation medium *in vitro*. The cultures were used to supply whole leaf, stem internode pieces, petioles and primary root explants for co-culture with *Agrobacterium* at the National Agricultural Research Laboratories (NARL), Kawanda, Uganda.

Bacterial strain and plasmid for transient expression. *Agrobacterium tumefaciens* strain EHA105 (rifampicine resistant) harbouring the

plasmid pCAMBIA1305.1 was used to transform of cv. Kyebandula explants at NARL. The pCAMBIA1305.1 plasmid contained the â-glucuronidase (GUS) gene driven by the constitutive CaMV (cauliflower mosaic virus) 35S promoter (Fig. 1). The transfer DNA (T-DNA) containing the GUS gene also had the hygromycin resistance gene under the double CaMV 35S promoter. *Agrobacterium* from a liquid stock was streaked on solid LB medium in plates and left to grow for 3 days at 28 °C in dark. The composition of LB medium was 10 g L^{-1} bacto-tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} sodium chloride and 15 g L^{-1} bacterialogical agar. The following antibiotics were added after the media was cooled; 100 mg L^{-1} Kanamycin, 25 mg L^{-1} rifampicin and 250 mg L^{-1} carbenicillin. A single colony of the *A. tumefaciens* from the three-day-old cultures was picked and grown overnight in 25 ml liquid LB medium on a shaker at 200 rpm and at a temperature of 28°C. The *Agrobacterium* cells were then centrifuged at 13,000 rpm for 10 min and the pellets were resuspended in liquid co-culture medium. The co-culture medium was composed of MS components, 30 g L^{-1} sucrose, 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 100 µM acetosyringone. The cultures were shaken at 70 rpm for 1 hour, before being used for infection of explants.

Histochemical localisation of gus activity. Explants were immersed in liquid co-culture medium with various *Agrobacterium* optical density (OD) concentrations (0.4, 0.6, 0.8, 1.0) at 600 nm for 1 hr, while shaking at 45 rpm. The explants were then removed from the liquid infection medium and blotted on filter-paper, before placing on solid co-culture medium for 3 days in dark. The second experiment investigated the effect of co-culture duration on transformation frequency. This was conducted by immersing explants in co-culture medium with *Agrobacterium* OD concentration of 1.0 at 600 nm before placing the explants on solid co-culture medium for various days (1, 2, 3, 4, and 5 days) depending on the experiment. This was followed by a third procedure in which explants infected with *Agrobacterium* at an OD concentration of 1.0 at 600 nm were placed on solid co-culture medium overlaid with filter paper; while another group of explants was placed on medium without filter paper on it. The co-culture duration for this procedure was 4 days at 23 °C in dark. The transformation frequency was determined by screening for transient GUS expression using histochemical localisation of GUS activity after co-culture.

Following co-cultivation, the explants were harvested for GUS staining, using histochemical GUS assay as described by Jefferson *et al.* (1987). The explants were washed in 70% ethanol for 2 minutes before incubation in fixation solution for 45 mins at room temperature. The fixation solution was made by mixing 0.3% (v/v) formaldehyde, 10 mM MES (pH 5.6) and 0.3 M mannitol. This was followed by washing the fixed explants with 50 mM phosphate buffer (pH 7.0) before incubation in substrate solution at 37 °C for 24 hours. The substrate solution was made by mixing 50 mM sodium phosphate (pH 7.0), 5 mM potassium ferricyanide, 5 mM ferrocyanide, 10 mM EDTA, 50 mM ascorbic acid and 1 mM 5-bromo-4-chloro-3-indolyl-D-glucuronide (X-Gluc).

Chlorophyll was removed by immersing the explants in a solution of methanol and glacial acetic acid (3:1) for 4 hours. The explants were

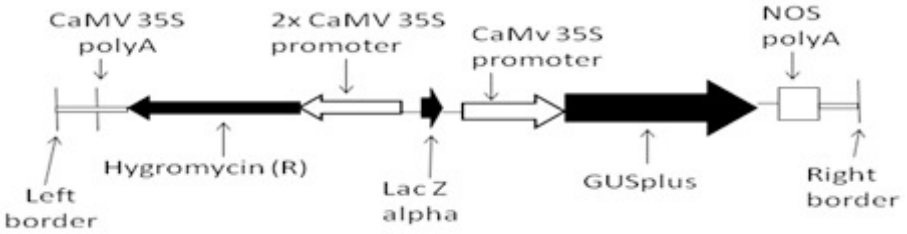

Figure 1. Schematic representation of T-DNA region of the plasmid construct pCAMBIA1305.1 used to transform of cv. Kyebandula explants to investigate transient expression of the GUS gene.

then dehydrated in a series of ethanol (50, 70, and 95%). The explants were then examined for expression of blue spots and photographed.

Each of the three experiments was carried out with 20 explants and repeated three times. Non-transformed explants were used as controls. This was achieved by placing the explants in a liquid co-culture medium without *Agrobacterium,* followed by treating the transformed explants in subsequent stages. Data on frequency of explants showing GUS activity was transformed using the arcsine square root before analysis to stabilise the variance. Statistical analysis for the effect of the investigated parameters on GUS activity was performed using general linear model of analysis of variance (ANOVA) at 5% level of significance. The significance between mean values was tested using the least significance difference (LSD) method at the 5% level.

Sensitivity of non-transformed explants to kanamycin. The sensitivity of untransformed explants to kanamycin was investigated by adding various concentrations of kanamycin (0, 25, 50, 75, 100 and 125 mg L^{-1}) to callus induction medium. This was conducted in order to determine the optimal concentration for kanamycin for killing non-transformed plant cells. The callus induction medium (CIM) was MS medium with 30 g L^{-1} sucrose, 3 g L^{-1} phytagel, 1.0 mg L^{-1}2,4-D, 250 mg L^{-1} cefotaxime and the different concentrations of kanamycin. The cultures were placed in dark at 28 °C and the CIM was refreshed every 2 weeks. The survival of explants and formation of callus was assessed after 8 weeks in culture.

Transformation with weevil-resistance genes. The plasmid vector bearing the weevil-resistance genes was referred to as pCIP84. The plasmid vector was transformed into the *Agrobacterium tumefaciens* hypervirulent strain EHA105 (kanamycin resistant) through electroporation at the International Potato Centre (CIP), Lima, Peru (Kreuze *et al.,* 2009; Tovar *et al.,* 2009). This plasmid has a pCAMBIA2305.1 backbone. It has two weevil resistance genes, *ß-Amy:cry7Aa1* and *gSPOA1:cry3Ca1* with the *nptII* selectable marker gene in its transferred DNA (T-DNA) (Fig. 2).

PCR confirmation of weevil-resistance genes in plasmid pCIP84. The presence of *cry7Aa1* and *cry3Ca1* genes in the plasmid was confirmed upon receiving it at NARL. The *Agrobacterium* harbouring the plasmid pCIP84 was grown as described above, with Kanamycin (50 mg L^{-1}) added as the only antibiotic. Plasmid DNA was extracted from *Agrobacterium* cells using a plasmid extraction kit provided by Promega. A cleared lysate was achieved by pouring the *Agrobacterium* culture into a 2 ml tube and centrifugation at 13,000 rpm for 5 minutes. The available volume (20 ml) of the *Agrobacterium* culture could not fit into the 2 ml tube at once. Therefore, only 1.5 ml of the *Agrobacterium* culture was added to the 2 ml tube at a time.

Another 1.5 ml was added to the same tube after centrifugation of the bacterial cells and removal of the suspension. These were centrifuged as above and the process was repeated until all the *Agrobacterium* cells from the 20 ml culture were collected in the 2 ml tube. The pellet was then re-suspended in 250 µL cell suspension solution. The rest of the DNA extraction process was conducted according to the instructions in the Promega kit.

The plasmid DNA was used in PCR to confirm integrity of *cry7Aa1* and *cry3Ca1* genes before embarking on transformation of sweetpotato at NARL. Equal amounts of 100 ng of total DNA were amplified in 25 µL reactions using specific primers for the *cry7Aa1* gene: 5'-ACAACTCATCACCATACCAAAC-3' forward

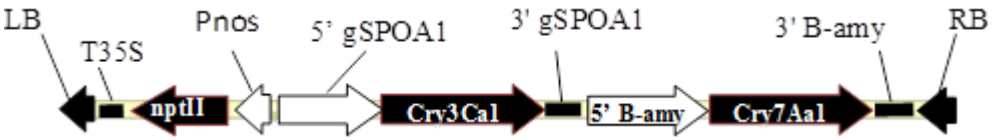

Figure 2. Schematic representation of T-DNA region of plasmid vector pCIP84 used in transformation of cv. Kyebandula explants with *cry* genes.

and 5'-AAGAGCAAGATGCAAGTTTG-3' reverse primers. These primers were expected to give products of 608 bp size. The specific primers for *cry3Ca1* gene were: 5'-CACCTATAG TAAAACCATTGGACAC-3' forward and 5'-TGCATGAAAGCCTTAAGAGG-3' reverse. These primers were expected to give fragments of 530 bp size. PCR amplifications for both *cry7Aa1* and *cry3Ca1* genes were performed with an initial denaturation at 93 °C for 2 min, followed by 35 cycles at 93 °C for 15 sec, 55 °C for 30 sec, 72 °C for 2 min, and final extension at 72 °C for 7 min. PCR products were separated by electrophoresis on a 1.0% (w/v) agarose gel for 45 min at a voltage of 80. The gel picture for the three separate tubes containing the same plasmid received from CIP, Lima, is shown in Figure 3.

Genetic transformation of sweetpotato with weevil-resistance genes. A single colony of *A. tumefaciens* strain EHA105: pCIP84 was cultured in 2 ml of LB liquid containing 50 mg L^{-1} Kanamycin for 48 hr under conditions described above. A total of 20 µl of this culture were inoculated into 20 ml of fresh LB liquid (as above) and grown as above to an OD_{600} of 1.0. The culture was centrifuged at 2000 g for 10 min at 4 °C. The bacterial pellet was re-suspended in an equal volume of bud induction medium (BIM) and incubated for 1 hr at 28 °C with rotary shaking at 100 rpm. BIM was composed of MS basal salts, sucrose (30 g l^{-1}), myo-inositol (0.1 g l^{-1}) and sweetpotato vitamin stock (1 ml l^{-1}). The media was adjusted to pH 5.8 before autoclaving as above. TDZ (4.0 µM) and acetosyringone (100 µM) were supplemented to the medium after autoclaving. Freshly harvested *in vitro* stem pieces (0.6 – 1.0 cm) from internode positions 2, 3 and 4 of cv. Kyebandula were incubated in the bacterial suspension with rotary shaking at 100 rpm for 20 min at 28 °C, and then blotted dry on sterile filter paper. Three plant transformation experiments were conducted with each having 100 explants.

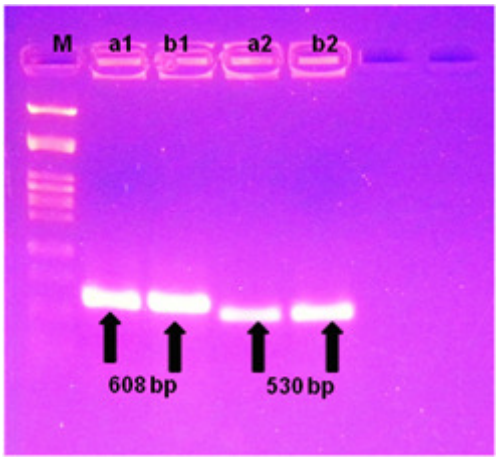

Figure 3. PCR amplification fragments of *cry7Aa1* and *cry3Ca1* from DNA sequences contained in the T-DNA of the pCIP84 vector construct. Two culture tubes were supplied from CIP-Lima, containing *Agrobacterium* EHA105 with the pCIP84 vector construct. Lane M: DNA marker. Lanes a1 and a2 are *cry7Aa1* and *cry3Ca1*, respectively, from one culture tube while Lane b1 and b2 are *cry7Aa1* and *cry3Ca1,* respectively, from another culture tube.

The explants were placed on sterilised filter paper, overlaid on solid BIM. Solid BIM was made by adding 3 g L^{-1} of phytagel to liquid BIM and autoclaving as above. Co-cultivation was carried out for 4 days at 23 °C in the dark. After co-cultivation, the explants were washed in antibiotic wash medium (liquid BIM with 500 mg L^{-1} cefotaxime) for 10 min, rinsed three times in sterile water, and then blotted dry on sterile filter paper. The explants were then placed on solid BIM with 4.0 µM TDZ, 250 mg L^{-1} cefotaxime and 100 mg L^{-1} Kanamycin for 3 days. They were placed horizontally on the medium and partially pressed into the medium.

The explants were then transferred to bud elongation medium (BEM) comprised of MS salts (4.3 g L^{-1}), sucrose (30 g L^{-1}), myo-inositol (0.1 g L^{-1}), phytagel (3 g L^{-1}), 0.25 µM NAA, 250 mg L^{-1} cefotaxime and 100 mg L^{-1} kanamycin. The petri-dishes containing the cultures were placed in dark for 4 weeks at 28 °C before transfer to 16 hr photoperiod under same temperature. The cultures were transferred to fresh bud elongation medium every 4 weeks. Regeneration of the putative transformed plants was achieved through adventitious shoot formation on the same medium within 12 weeks.

DNA extraction and PCR analysis of putatively transgenic plants. DNA from ten putatively transformed plants, was used in initial analysis of the transformed plants. Four grammes of plant tissue was ground in liquid nitrogen, added to 20

mL of CTAB extraction buffer (100 mM Tris-HCl, 25 mM EDTA, 1.4 M NaCl and 2% CTAB) with 200 µL of 2-mercaptoethanol in a 50-mL polyethylene tube and incubated at 65°C in a water bath for 40 min, with occasional opening of the tubes to reduce build-up of pressure. An equal volume of chloroform/isoamyl alcohol (24:1) was mixed with the extract by inverting the tubes for 5 min and spun at 16,000 g for 5 min. The upper phase was transferred to a new 50-mL tube and 0.6 volumes of isopropanol (-20 °C) was added. DNA was precipitated by placing tubes at -20 °C for 10 min and spun at 16,000 g for 5 min. The supernatant was discarded. The pellet was washed twice with 75% ethanol and dried. The pellet was then dissolved in 30 µL nuclease-free water and digested with 1 µL of 10 mg mL^{-1} RNase for 15 min at 37 °C. The PCR conditions for *cry7Aa1* were similar to those used for analysis of integrity of the pCIP84 vectors (above). It is preferred that transgenic plants are not contaminated with the *Agrobacterium* used in genetic transformation. This is to ensure that the PCR results of the *cry* genes are not due to bacteria presence on plant tissue, but due to transformation of the plants. This step is also important to minimise future biosafety concerns.

The presence of *Agrobacterium* in the putatively transformed plants was detected by PCR using primers for *virD2* gene which is found in the *Agrobacterium,* but is not in the T-DNA of the plasmid pCIP84. The primers for *virD2* are: 5'-ATGCCCGATCGCGCTCAAGT-3' forward and 5'-CCTGACCCAAACATCTCGGCT-3' reverse. PCR conditions for *virD2,* gene were: initial denaturation at 94 °C for 2 min; followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and final extension at 72 °C for 10 min. The PCR products were separated by electrophoresis on a 1.0 % (w/v) agarose gel for 45 minutes at a voltage of 80. The expected fragment size from the PCR was 338 bp.

Experimental design and data analysis. All experiments were laid out in a completely randomised design. Ten explants were used in each experiment replicated three times. This gave a total of 30 explants for each experiment. The duration of each experiment was specific for the particular treatment (above). Frequencies were calculated by expressing the number of explants showing transient GUS expression or surviving on media (for kanamycin sensitivity), as a percent of the total number of explants investigated. The frequency data were transformed using the arcsine square root before analysis to stabilise the variance. Statistical analyses were done using Analysis of variance (ANOVA) and means were compared using the least significant difference (LSD) test at $P \leq 0.05$ level.

RESULTS

Effect of concentration of *Agrobacterium*. Transformation frequency was determined through the number of explants showing transient GUS expression using histochemical localisation of GUS activity. GUS staining after 3 days co-culture showed that *Agrobacterium* concentration at an OD of 1.0 at 600 nm gave the highest transformation frequency for all the sweetpotato organs, namely primary roots, petioles, whole leaves and stem internode pieces (Fig. 4). This concentration of *Agrobacterium* was significantly ($P < 0.001$) better than the rest in improving transformation frequency. All types of plant organs investigated showed capacity to be transformed under the conditions (Fig. 5). Petiole explants performed significantly ($P < 0.001$) lower than the rest of the explants types. There was no significant difference among the remaining explants types (stem internodes, whole leaves and primary roots) in influencing transformation frequency.

Effect of duration of co-culture. The best transformation frequency was achieved with co-culture duration of 4 days, for both whole leaf and stem internode explants (Fig. 6). Similar to the experiment on concentration of *Agrobacterium*, the two types of explants, namely whole leaves and stem internode pieces did not have a significant ($P > 0.05$) difference in influencing transformation frequency. However, the duration of co-culture had a significant ($P < 0.001$) effect on transformation frequency. The best transformation results were obtained when explants were co-cultured for 4 days, although there was no significant ($P > 0.001$) reduction in transformation frequency when explants were

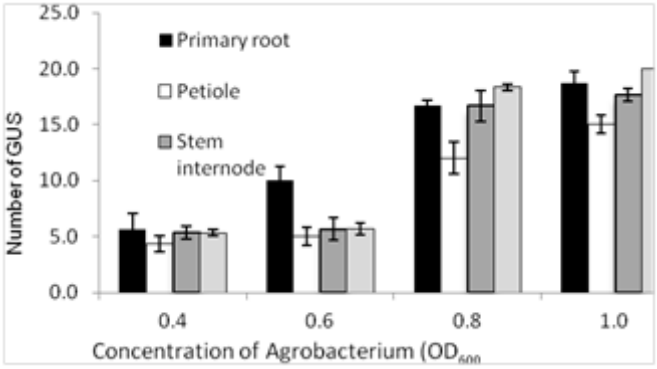

Figure 4. Effect of concentration (OD_{600} nm) of *Agrobacterium* on transient expression of the GUS gene in various explants of cv. Kyebandula.

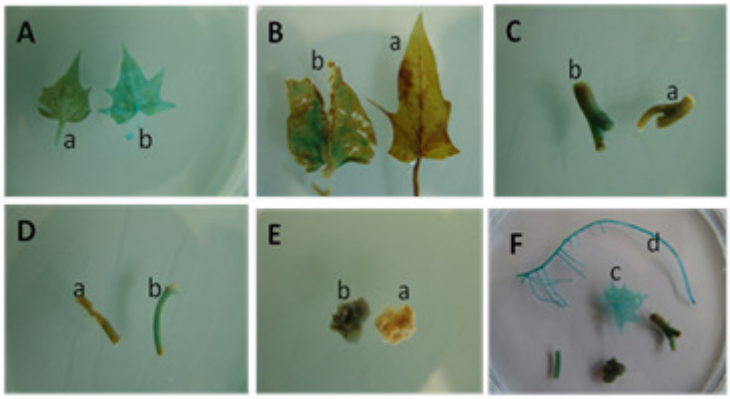

Figure 5. Different types of explants of cultivar Kyebandula showing transient GUS activity after 5 days of co-cultivation with *Agrobacterium tumefaciens* EHA105 (pCAMBIA1305.1). The transformed explants (a) showed blue colour as positive indication of transient GUS expression. Non transformed controls (b) did not show blue colour. (A) Young leaves (4 weeks old); (B) Old leaves (7 weeks old); (C) Stem nodes; (D) Petioles; (E) Callus; (F) Comparatively high Gus activity in primary roots (d) and young leaves (c).

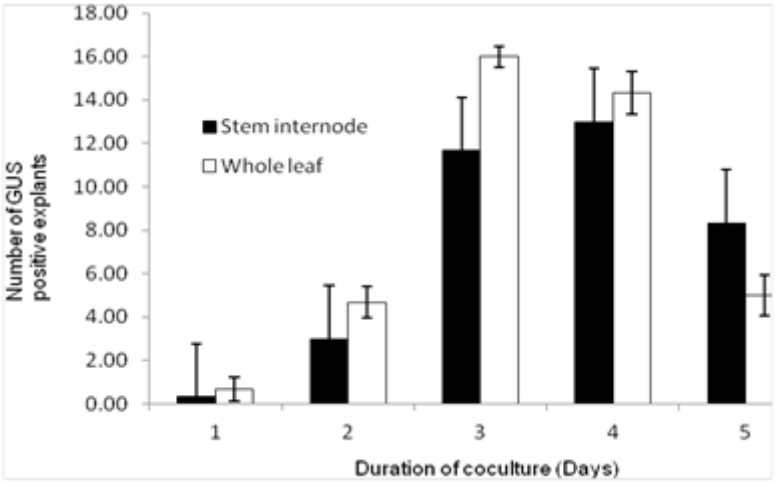

Figure 6. Effect of co-cultivation duration (days) on transient expression of GUS gene in stem and whole leaf explants of cv. Kyebandula in Uganda.

cultured for 3 days. Extending co-culture duration to more than 4 days resulted in a loss of transformation frequency, which was significant ($P < 0.001$) even when only one day was added.

Effect of placement on filter-paper. An *Agrobacterium* concentration at an OD of 1.0 at 600 nm and a co-culture duration of 4 days on co-culture medium overlaid with filter-paper significantly ($P = 0.001$) improved transformation efficiency (Fig. 7). There was little *Agrobacterium* growing around the explants and the filter-paper in contact with the explants. In the experiment without filter-paper, the explants were in direct contact with the medium and *Agrobacterium* could be seen growing around the explants and the medium for most explants. There was no significant difference ($P>0.05$) between whole leaf and stem internode pieces in influencing transformation efficiency.

Sensitivity of explants to kanamycin. Both the type of plant organ and the kanamycin concentration significantly ($P < 0.001$) influence survival of explants on CIM. Kanamycin concentration of 100 mg L^{-1} or higher completely inhibited survival and callus formation from whole leaf explants which turned brown due to necrosis within 6 weeks. There were a few stem explants that showed survival on CIM supplemented with 125 mg L^{-1} kanamycin after 8 weeks although most of them were not able to continue forming callus (Fig. 8). Therefore, a kanamycin concentration of 70 mg L^{-1} was used for whole leaves and that of 100 mg $L^{-1}$was used for stem internode pieces in subsequent experiments.

Regeneration of putatively transformed plants. After three days of co-culture on bud induction medium, explants started expanding. The swelling was more pronounced at the cut ends of the explants where callus was also formed within two weeks after co-culture. None of the induced calli regenerated shoots. In the same period, multiple adventitious shoot buds were observed along the length of most explants. The frequency of explants with adventitious buds after 4 weeks was recorded as 54%, while the mean number of buds per explants was seven.

Some buds started to elongate within the four weeks of placement on selection medium. However, most adventitious buds failed to elongate into shoots and remained green or died with the explants after 12 weeks. After 8 weeks, the shoots that had developed from the elongating buds were cut out from the explants and placed on sweetpotato propagation medium where they rooted easily by the twelfth week. There were 11, 2 and 5 plants regenerated in experiments 1, 2 and 3, respectively. In total, eighteen independent plants were regenerated. The per cent of explants regenerating shoots was 6.0%. One to two shoots were formed per explants for those explants that formed shoots.

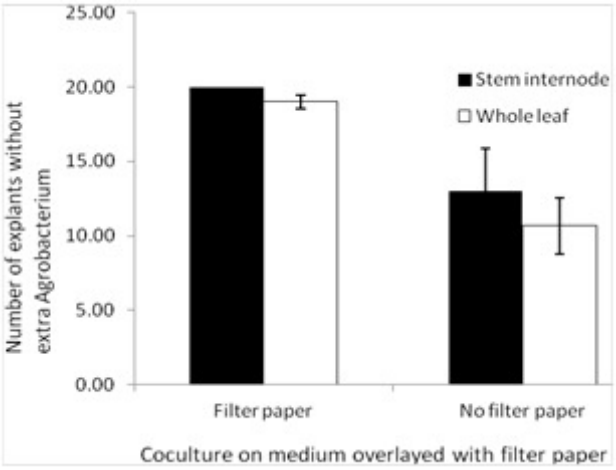

Figure 7. Effect of placing explants on filter paper during co-cultivation on elimination of excess *Agrobacterium* from stem and whole leaf explants of cv. Kyebandula in Uganda.

The regenerated plants were further multiplied by cutting into nodal segments on the sweetpotato propagation medium without antibiotics.

PCR analysis of putatively transformed plants. Among the 18 putatively transformed plants, 10 were used for initial PCR analysis and the expected fragment length for *cry7Aa1* (608 bp) was found in 8 them. It was absent in the non-transformed control. Two of the eight plants that were positive to PCR with *cry7Aa1* primers were also positive to primers for *virD2* gene producing a fragment of 338 bp length (Fig. 9).

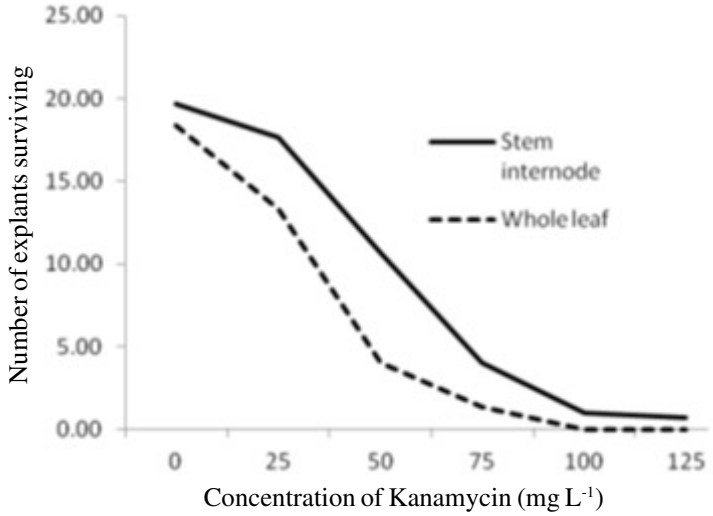

Figure 8. Effect of Kanamycin concentration (mg L^{-1}) on survival of stem and whole leaf explants of cv. Kyebandula in Uganda.

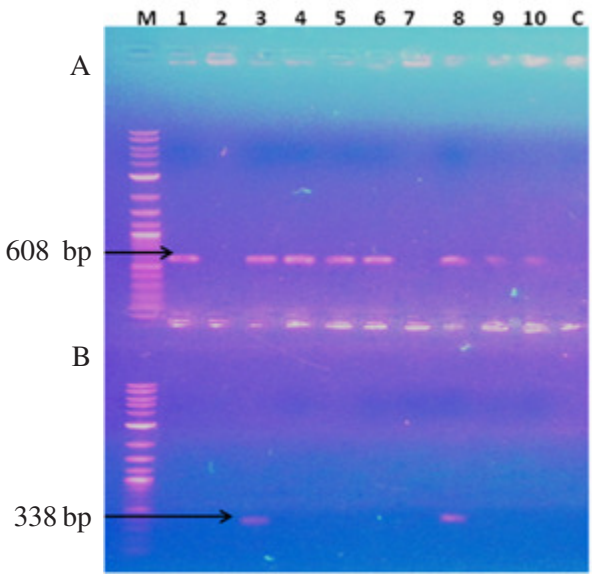

Figure 9. Molecular analysis of putatively transgenic plants. (A) PCR analysis for cry7Aa1. Lane C: Untransformed plant as negative control. Lane M: DNA marker. Lanes 1, 3-6, 8-10: Cry 7Aa1-positive plants. Lane 2 and 7: Cry 7Aa1-negative plants; (B) PCR analysis for virD2 gene. Lane 3 and 8: virD2-positve plants. Lanes 1, 2, 4-7, 9, 10: virD2-negative plants.

DISCUSSION

The transformation method combined with optimised regeneration protocol led to the first success on genetic transformation of cv. Kyebandula for weevil-resistance. Some of the important factors that determine transformation frequency are concentration of bacteria, length of co-cultivation and elimination of excess bacteria (Cheng *et al.,* 1997). There was no significant difference between whole leaves and stem internode pieces, in their response to *Agrobacterium* concentration, co-culture duration and co-culture on filter paper. However, the expression of GUS in both of these commonly used explant types was lower than that for primary roots. From the data, it is clearthat *Agrobacterium* OD concentration of 1.0 at 600 nm is the best for the transformation of these explants from cv. Kyebandula.

Xing *et al.* (2007) did not find a significant difference when various *Agrobacterium* OD concentrations (0.6, 0.8 and 1.0) at 600 nm were used for infection, although the best transformation in that work was achieved when OD concentration was 0.8. The same work demonstrated that higher OD reduced transformation efficiency due to over-infection of plant cells by the large number of *Agrobacterium* cells at high OD concentration (Xing *et al.,* 2007).

However, in the present work, the *Agrobacterium* OD concentration of 1.0 at 600 nm was significantly better than any lower concentrations investigated. For similar plant organs used as explants in this study, Song *et al.* (2004) used an *Agrobacterium* OD of 0.8 to 1.0 while Luo *et al.* (2006) and Kreuze *et al.* (2008) used an OD of 0.4 – 0.6. This could partly explain why Song *et al* (2004) obtained higher transformation efficiency than Luo *et al.* (2006). Yang *et al.* (2011) used an OD value of 1.0, while an OD of 0.5 was used in the work of Yu *et al.* (2007), who reported comparatively high transformation efficiency than any previous work (Yu *et al.,* 2007). The cultivars that have been used in previous transformation studies are Jewel (Luo *et al.,* 2006), Huachano (Kreuze *et al.,* 2008), Lizixiang (Yu *et al.,* 2007), Xu55-2 (Xing *et al.,* 2007) and Beniazuma (Song *et al.,* 2004), while Yang *et al.* (2011) used thirteen cultivars adapted to China. It is interesting to note that Yang *et al.* (2011), who used a high *Agrobacterium* OD value of 1.0, also recommended a longer co-culture duration (4 to 7 days); compared to most reports which recommend 3 days (Song *et al.,* 2004; Yu *et al.,* 2007).

Normally, 2-3 days are standard for co-cultivation in most transformation protocols (Xing *et al.,* 2007). In the current work, 4 days was the best co-cultivation time for cv. Kyebandula. Xing *et al.* (2007) also found optimal co-culture time was 4 days for cell suspensions of cv. Xu55-2. Some reports (Prakash *et al.,* 1991) recommended co-culture of more than 3 days, while others (Otani *et al.,* 1998) found 2 days sufficient.

The recurrence of *Agrobacterium* has been reported as a major problem in previous studies and Kreuze *et al.* (2008) discarded 5 to 18% of explants due to excessive bacterial overgrowth. Song *et al*. (2004) did not report any *Agrobacterium* overgrowth when they placed filter-paper, on co-culture medium before placing explants.

In the present work, the placement of explants on filter paper, followed by washing in antibiotic medium, eliminated the excess *Agrobacterium* almost completely (Fig. 7). To our knowledge, this is the first report to compare these two approaches and demonstrate that the placement of filter paper on co-culture medium has a direct and significant effect on suppression of excess *Agrobacterium*. It is possible that when the explants are placed directly on the medium, the co-culture medium supports the growth of *Agrobacterium* to an extent that has the same effect as long co-culture duration. It is evident that high *Agrobacterium* recurrence is associated with low transformation efficiency.

An efficient selection method is required for production of transgenic plants. Based on the data in the current study, it is concluded that kanamycin of 100 mg L^{-1} is sufficient for selection of transformed callus from stem internode pieces of cv. Kyebandula. The results with whole leaf explants were more conclusive than those for stem internode segments, which showed slightly more resistance to kanamycin (Fig. 8). The deployment of kanamycin for selection of transformed cells is not straight forward. Although

kanamycin has been used successfully to select transformed sweetpotato cells by some groups (Moran *et al.,* 1998), other groups demonstrate that kanamycin has a negative effect on regeneration; while others opted not to use kanamycin in the first 5 days after co-culture (Sheng-Jun *et al.*, 2004). Xing *et al.* (2008) found that a high dose of Kanamycin decreased the proliferation of embryogenic callus of cultivar Xu55-2 from China. This group found that 10 mg L^{-1} Kanamycin was sufficient for selection of transformed cell aggregates of cv. Xu55-2 and suggested that cultivar has an effect on survival in presence of Kanamycin (Xing *et al.,* 2008). This suggestion is supported by the findings of another group, which demonstrated that cultivar Yulmi has higher intrinsic resistance to kanamycin (200 mg L^{-1}) (Shin *et al.*, 2007). Generally, most researchers have demonstrated that sweetpotato cells are less sensitive to kanamycin and, therefore, a high dose (25-100 mg L^{-1}) of this antibiotic is required to kill non-transformed cells and leave out only transformed cells on medium (Prakash and Veradarajan, 1992; Song *et al.*, 2004; Luo *et al.*, 2006).

The optimised transformation conditions led to successful regeneration of putatively transformed plants. To the best of our knowledge, this is the first report on genetic transformation of a popular Ugandan landrace cultivar. Luo *et al* (2006) reported that important Chinese and African sweetpotato cultivars are difficult to transform. African cultivars that were investigated were not mentioned in their report and it was not indicated whether the experiments with African cultivars were on somatic embryogenesis or organogenesis. Later, Tovar *et al.* (2009) obtained only one regenerating transgenic shoot from African cv. Wagabolige and no regeneration from cv. Tanzania after eaxmining about 10,000 explants of each cultivar with a somatic embryogenesis protocol.

In the present study, 18 plants were regenerated through adventitious shoots from a total of 300 stem internode pieces. Importantly, there were shoots regenerated from each experiment, supporting the reproducibility of the protocol. However, the conversion of adventitious buds into shoots remains a problem, as a large number of adventitious buds failed to elongate into shoots. This phenomenon could be due to the effect of TDZ as observed in previous studies with *Vaccinium vitis-idaea* and *Paulownia tomentosa* Steud. (Debnath, 2005; Corredoira *et al.,* 2008).

PCR results with *cry7Aa1* primers showed that 2 of the 10 randomly selected plants were not transformed. Song *et al.* (2004) reported that 100 mg L^{-1} of kanamycin was effective in selection of transformed plants when they used stem internodes as explants. However, Luo *et al.* (2006) encountered non-transformed escapes under similar conditions. This latter group argued that the strong regeneration capacity of stem segments seems to compete with the regeneration from the rare transformed cells (Luo *et al.*, 2006). Later, another group proposed that the regeneration of non-transformed plants on selection medium is because non-transformed cells can sometimes survive among transformed cells within a single callus clump (Yu *et al.,* 2007). Similarly, in the present work the plants that were not transformed, but were able to survive on medium with kanamycin could have done so due to the reasons promoted by Luo *et al.* (2005) and Yu *et al.* (2007). The 8 plants are considered as putatively transformed, awaiting southern hybridisation to confirm stable integration of the *cry* genes into the sweetpotato genome and also the copy number of the genes in the events.

The two plants that showed PCR-positive to *virD2* primers revealed that the *Agrobacterium* used for genetic transformation survived on them. The implication of this is that it is likely that the PCR-positive results with *cry7Aa1* gene are due to the pCIP84 plasmid in the *Agrobacterium,* and not the transgenic nature of the plants. These two plants are currently being propagated on media with the antibiotic cefotaxime to eliminate the *Agrobacterium.* From previous studies, it looks too difficult to entirely eliminate *Agrobacterium* from plant tissue after transformation. Ogawa and Mii (2007) found that although *Agrobacterium* was not evident on media after suppression with antibiotics, the PCR with *virC* on 16-weeks-old shoots was positive. This could indicate that the *Agrobacterium* they used for transformation survived on the tissue of the regenerated plants as they developed, although the *Agrobacterium* could not survive

directly on the media on which the plants were growing.

This report is new in that it is based on readily accessible explants of an African cultivar that has not been reported in previous transformation work. It is known that *Agrobacterium* transformation has a negative effect on regeneration efficiency (Song *et al.,* 2004; Xing *et al.,* 2008). Therefore, preliminary regeneration studies to this work needed to be coupled with successful transformation to be more valuable. In regeneration experiments with TDZ, prior to transformation, the best results showed 61.0% explants formed adventitious buds, 39 buds per explant, 56.0% explants forming shoots and 9 shoots per explant for those explants that formed shoots. When the regeneration conditions were incorporated in the transformation process, the results for all the parameters dropped to 54.0% explants forming adventitious buds, 7 buds formed per explant, 6.0% explants forming shoots and one shoot per explant for those explants that formed shoots.

The current data demonstrate the ability to genetically transform cv. Kyebandula based on transient expression of the GUS reporter gene. The transformation conditions optimised through transient expression were applied in the transfer of weevil-resistance genes into cv. Kyebandula. The genetic transformation protocol reported here has potential to be extended to other sweetpotato cultivars. Southern hybridisation is the next most important step in the plants that have been regenerated in this work.

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