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A Robust *Agrobacterium*-Mediated Genetic Transformation Protocol for East African Coastal Farmer-Preferred Cassava Landraces

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Abstract: *Cassava (Manihot esculenta) is the third biggest source of carbohydrates after rice and maize, feeding almost a billion people daily in the tropics. Recently it has been proposed as one of the more ideal biomass producers for biofuel production due to its high biomass yield and low input needs. A robust reproducible genetic transformation protocol is needed for germplasm improvement or functional genomics studies in cassava. In the present study we report, for the first time the establishment of a reproducible Agrobacterium-mediated transformation protocol for cassava landraces Mkombozi and Albert preferred by farmers in the coastal region of east Africa. Friable embryogenic calli (FEC) were produced using axillary buds and infected using Agrobacterium strain LBA4404 harboring the binary vector pCAMBIA2301. About 32–40 transgenic plants per 1 ml settled cell volume (SCV) were regenerated on selective medium. Histochemical GUS assays confirmed the expression of GUS gene in transformed calli, somatic embryos and transgenic plants. The presence and expression of the gusA gene were confirmed by PCR and RT-PCR analysis of transgenic plants.*

Keywords: *Cassava, Friable embryogenic calli, genetic transformation, transgenic plants*

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I. Introduction

Cassava (Manihot Esculenta) Is The Fifth Most Important Food Crop In The World And A Major Source Of Dietary Energy To Over 800 Million People Globally (Burns Et Al., 2011; FAO, 2011). It Is The Second Most Important Source Of Starch Worldwide, After Maize (Stapleton 2012; Norton 2014), And Is The Starch Most Traded Internationally. Cassava Is A Food Security Crop And A Source Of Income That Provides Livelihood To Small-Holder Farmers And It Is One Of The Most Important Food Crops In Sub-Saharan Africa (FAO, 2011).Abuja Declaration African Union (2007) Identified Cassava As One Of The Crops With The Greatest Potential To Combat Poverty As Well As Food And Nutritional Insecurity In Africa. Due To Its High Starch Content And Its Potential For Biofuel Production, The Global Demand For Cassava Is Rapidly Growing. Despite Its Importance, The Cultivation And Production Of Cassava Is Greatly Constrained By Abiotic And Biotic Stresses. Bacteria And Viruses Are A Major Cause Of Diseases, Which Greatly Reduce Cassava Yields (Nassar, 2006), Cyanogens And Post-Harvest Physiological Deterioration Also Hamper The Consumption And Commercialization Of Cassava (Siritunga And Sayre, 2003; Zidenga, 2012).It Is Therefore Important That The Yield Of Cassava Be Improved.

Conventional breeding can be used in production of improved cassava, however difficulties in introgression of resistant traits into germplasm preferred by farmers and consumers makes breeding a challenging and a very long process (Hillocks, 2003; Yadav et al., 2011). Genetic engineering is an alternative approach in introducing a trait specific gene in a plant by using methods such as Agrobacterium mediated transformation (Mohan Jain, 2007). However, an essential pre-requisite for genetic improvement of any crop is the availability of a reproducible and robust protocol for gene transfer techniques. Somatic embryogenesis that produces friable embryogenic callus (FEC) is the preferred tissue culture method for genetic transformation of cassava (Bull et al., 2009). FEC is the most widely used explant to generate transgenic cassava plants as the high population of totipotent unicellular cells located on the surfaces of FEC reduces the likelihood of chimerism. The relatively small size of FEC cell clumps creates a large surface for exposure to the transforming agent thus more effective selection of transformed individual cells (Bull et al., 2009; Nyaboga et al., 2013; Taylor et al., 2001; Taylor et al., 2012). To date, an effective FEC-based transformation protocol has been developed for the model cultivar 60444 and few African farmer-preferred cassava cultivars Serere, Ebwanatereka, Kibandameno, TME14, TME204, TME7,(Nyaboga et al, 2013; 2015; Taylor et al., 2012;

Zainuddin et al., 2012; Chauhan et al., 2015). However, none of these cultivars is preferred by farmers in the coastal regions of east Africa.

Transformation and regeneration of farmer-preferred cassava cultivars still remain challenging due to the difficulty in FEC production and lack of an efficient transformation and regeneration protocols. The response of specific cultivars to the development of FECs remains challenging step in the production of transgenic cassava plants and FEC induction process need to be optimized for each particular cultivar because FEC production is genotype dependent (Zainuddin et al., 2012). Optimization of FEC production, regeneration and transformation protocols need to be expanded to more cultivars preferred by farmers in sub-Saharan Africa including the coastal regions where cassava is of high economic importance. Two east African farmer-preferred cassava cultivars from the coastal region namely Albert and Mkombozi were used in this study. These cultivars were selected based on their economic importance, frequency of cultivation in coastal zones of East Africa, tolerance to CMD and susceptibility to CBSD. The transformation and regeneration approaches described in this study led to development of transgenic plants from these farmer-preferred cultivars

II. Materials And Methods

1. Cassava material

Cassava cultivars 60444, Albert and Mkombozi were provided as in vitro plants by International Institute of Tropical Agriculture (IITA). Cassava cultivar 60444 was used in this study as a model cultivar. All the plants were cultured on cassava basic medium (CBM; Supplementary Table 1) for micro-propagation and incubated at 28°C under a 16/8 h photoperiod. The in vitro plantlets were sub-cultured every 3–4 weeks for multiplication and maintenance.

2. Induction and maturation of somatic embryos

In these study axillary buds (AB) of cultivars 60444 and Mkombozi were used for somatic embryogenesis and immature leaf lobe (ILL) for Albert. Somatic embryos were induced from nodal cuttings (approx. 1 to 2 cm long) obtained from young stem cuttings of in vitro cultured plantlets. The explants were placed horizontally on cassava axillary bud medium (CAM; Supplementary Table 1) for 4 days at 28°C in the dark. The enlarged axillary buds were removed from the nodal explants with a sterile hypodermic needle and transferred onto plates containing cassava embryo induction medium (CIM; Supplementary Table 1). The somatic embryos developing on CIM medium were excised with a hypodermic needle under microscope and cultured on fresh CIM medium for 14 days at 28°C in the dark. During transfer to fresh CIM medium, non-embryogenic tissues surrounding somatic embryos were removed using a hypodermic needle. For immature leaf lobe (ILL), 2 to 6 mm long piece of leaf explants were excised from in vitro mother plantlets and placed on CIM for 28 days at 28°C in in dark. The organised embryogenic structures (OES) developed from explants cultured on CIM medium and somatic embryos were excised with a hypodermic needle under microscope and transferred to Greshoff and Doy (GD) medium (GD; Supplementary Table 1) for induction of FEC.

3. Production and maintenance of friable embryogenic callus (FEC)

Production of FEC was done according to the protocols described by Nyaboga et al. (2013, 2015) with some modifications. Somatic embryos cultured on CIM medium were collected onto a wire mesh (1 to 2 mm pore size) and crushed into small pieces by passing them through the mesh with a spatula. The small pieces of somatic embryos were cultured on GD media supplemented with 12 mg/l picloram and incubated at 28 ºC in dark for 14 days. After 14 days, FEC was transferred to fresh GD media supplemented with 12 mg/l picloram and grown under 16/8 h light/dark at 28°C and further sub-cultured onto fresh media every 21 days. Homogenous FEC was recovered after the 3rd sub-culture cycle on GD medium. The effect of L-tyrosine on FEC production in cassava cultivars was evaluated by transferring the somatic clusters onto GD medium supplemented with picloram (12 mg/l) in combination with different concentrations of tyrosine (125 µM, 250 µM and 500 µM) and the frequency to form FEC observed.

4. Regeneration of FECs

To evaluate the regeneration potential of FEC obtained for the three cultivars, 0.5 ml of total settled cell volume (SCV) of FEC was used for regeneration. The FECs were spread on a mesh and cultured on embryo germination and maturation media (MSN; Supplementary Table 1) at 28°C under 16/8 h photoperiod and sub-cultured onto fresh MSN medium every fortnight. The emerging somatic embryos were counted individually, removed and placed on cassava shoot elongation medium (CEM; Supplementary Table 1) and sub-cultured every 10 days onto fresh CEM medium. The regeneration capacity of the FEC was estimated as the total number of mature somatic embryos (green cotyledons) produced and further germinated into shoots. The germinated shoots were transferred using a sterile forceps to CBM for establishment of roots. After four weeks the rooted plantlets were sub-cultured and maintained on the CBM medium. The effect of silver nitrate (AgNO3) on

somatic embryogenesis and plant regeneration from cassava was evaluated. Cotyledonary-stage embryos were cultured on CEM supplemented with BAP (0.4 mg/l) in combination with different concentrations of AgNO3 (0 mg/l, 1 mg/l, 2 mg/l, 3 mg/l, 4 mg/l, 5 mg/l) and the shoot formation frequency assessed. The cotyledons were sub-cultured after every 10 days on fresh CEM medium with the same AgNO3 concentration as the previous. The number of shoots from cotyledons was recorded at every sub-culture. Germinating shoots were transferred to CBM for establishment of plantlets.

5. Agrobacterium strain and binary vector used for transformation

Agrobacterium tumefaciens strain LBA4404 harboring binary vector pCAMBIA2301was used for cassava transformation. The plasmid pCAMBIA2301 contained neomycin phosphotransferase gene (nptII) as selectable marker, and an intron-containing β-glucuronidase (gusA) as reporter gene (Figure 1). Agrobacterium cultures were initiated from glycerol stocks stored at -80 oC. Single colonies of LBA4404 harboring pCAMBIA2301 were maintained on Luria Bertani (LB) agar (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 15 g/l agar, pH 7.5) plates containing kanamycin (50 mg/l), rifampicin (50 mg/l) and streptomycin (100 mg/l). Single colonies were used to initiate 2 ml LB medium starter cultures. After 48 h shaking at 150 rpm at 28°C, the bacterium suspension was used to inoculate a 25 ml LB medium containing the antibiotics mentioned above and grown overnight on a shaking platform at 150 rpm to reach an OD600 of 1. Bacterial culture was centrifuged at 3500 rpm for 15 min and pellet was re-suspended in liquid GD medium supplemented with 200 µM acetosyringone (Sigma Chemical Co.) and grown further for 1 h at 25°C with shaking at 50 rpm. The optical density (OD600) of culture was checked and adjusted to 0.5. The bacterium suspension was used for transformation experiments.

6. Transformation, selection and regeneration of transgenic plants

FEC at the third cycle of sub-culture on GD medium were used for transformation experiments. About 0.5 ml of total settled cell volume (SCV) were inoculated with 15 ml of the Agrobacterium tumefaciens strain LBA4404 harboring pCAMBIA2301 in sterile 50 ml falcon tubes, mixed to disaggregate the callus tissues and incubated for 20 min with gentle shaking. FEC tissues inoculated with Agrobacterium were transferred onto a nylon 100 µM mesh placed on sterilized paper towel for 5 min to remove excess bacteria suspension. The FEC were co-cultivated on GD medium for 3 days with Agrobacterium under light (16/8 h photoperiod) at 22°C. After the co-cultivation period, the Agro-infected FEC was removed from the mesh using sterile forceps, transferred to a 50 ml falcon tube and washed three times with liquid GD medium containing 500 mg/l carbenicillin. The FECs were transferred by pipetting using cut tips and spread evenly onto a fresh sterile 100 µM mesh on top of sterile paper towels for 5 min to remove excess liquid. The mesh with Agro-infected FEC was transferred onto fresh GD medium supplemented with 250 mg/l carbenicillin and incubated for 4 days of recovery at 28oC 16/8h photoperiod. After 4 days of incubation, the mesh was transferred to fresh GD medium supplemented with 250 mg/l carbenicillin and 30 mg/l paramomycine and kept under16/8 h light/dark at 28°C for 7 days. Three sub cultures were done on GD medium with the 2nd and 3rd sub-cultures containing 40 and 50 mg/l of paramomycine, respectively. The mesh was transferred to MSN medium supplemented with 250 mg/l carbenicillin and 50 mg/l paramomycine and kept under 16/8 h photoperiod at 28 ºC with sub-culturing onto fresh MSN medium after every fortnight.

Matured embryos developing cotyledons on selective MSN medium were removed and transferred to CEM supplemented with 100 mg/l carbenicillin for cotyledon maturation and shoot induction. The sub-culturing continued every 10 -14 days in the same fresh medium until fully developed elongated shoots appeared. The elongated shoots were cut off and transferred to CBM for rooting. Rooted plantlets were screened for any escapes by micro-propagating stem cuttings on CBM supplemented with carbenicilin 50 mg/l and 50 mg/l paramomycine for the rooting test.

7. Histochemical GUS analysis

Histochemical GUS assays on transformed and non-transgenic tissues was conducted according to Bull et al. (2009). Transient GUS expression was performed 3 days after co-culture. A quarter of the callus on each plate was assayed for GUS expression. The callus was incubated with histochemical GUS assay buffer {10 Mm tris(pH 7.2), 50mM NaCl, 10 mg/ml 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc, dissolved in dimethylformamide, w/v), 10% w/v triton} at 37°C for 12 h followed by a de-pigmentation step using 70% ethanol at room temperature. The number of blue foci on the callus was scored under a microscope at 10000 X. Transient transformation efficiency was determined by expressing these GUS positive calli as a proportion (%) of the total number of calli in the sample (% GUS+). Stable GUS expression was performed using the same protocol described by Bull et al. (2009) using cotyledons, leaves, stems and roots excised from paramomycine-resistant plantlets. The tissues were incubated in a GUS assay buffer at 37°C for 12 h, the tissues were washed several times with 70% v/v ethanol and observed for blue coloration.

8. Genomic DNA isolation and PCR analysis of transgenic plants

The paramomycine-resistant transgenic lines were analyzed using PCR for the presence of nptII and gusA genes. Total genomic DNA was isolated from 100 mg of in vitro leaves of transgenic and non-transgenic plants using DNAeasy plant mini kit (Qiagen, GmbH, Germany). The presence of transgenes was confirmed by PCR amplification using gene-specific primers. Plasmid DNA of pCAMBIA2301 was used as a positive control and non-transgenic plant DNA as a negative control. The presence of transgenes was confirmed by PCR amplification using gusA forward primer 5′- AAAGTGTGGGTCAATAATCAGG-3′ and reverse 5′-ATGGATTCCGGCATAGTTAAAG-3′; and the nptII gene: forward 5′-GGGTGGAGAGGCTATTCGGCTATGA-3′ and reverse 5′-ATTCGGCAAGCAGGCATCGC-3′, corresponding to a 215 and 500-bp, respectively. PCR reactions were carried out in 25 µl reaction mixtures containing 1× PCR buffer, 2.5 mM MgCl2, 0.2 mM of each dNTP, 0.5 mM of each primer, 0.5 U Hot Star Taq DNA polymerase (Qiagen, GmbH, Germany) and 150 ng of template DNA. The PCR conditions for gusA gene detection was pre-denaturation at 95°C for 5 min, then followed by 35 cycles of strand separation of 95°C for 30 sec, annealing at 58°C for 1 min and extension at 72°C for 1 min and 10 min at 72°C as final extension. The PCR conditions for nptII gene detection was pre-denaturation at 94°C for 3 min, then followed by 35 cycles of strand separation at 94°C for 45 sec, annealing at 62°C for 45 sec and extension at 72°C for 2 min and 30 sec, and 10 min at 72°C final extension. The amplified PCR products were resolved by electrophoresis on 1% v/v agarose gel stained with GelRed TM (Biotium) and visualized under UV trans illuminator.

9. RNA extraction and reverse transcriptase (RT)-PCR

Expression levels of gusA gene in cassava transgenic lines were determined by semi-quantitative RT-PCR analysis. Total RNA was isolated from leaves of in vitro plantlets as described by Chang et al. (1993) with some modifications. RNA was precipitated with ethanol 70% and the pellets resuspended in 20 µl of DEPC water. The first strand cDNA synthesis was performed using 2 µg of total RNA, oligodT18primers and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MuLV-RT; MBI Fermentas, Hanover, MD, USA) according to the manufacturer's instructions. All cDNAs were diluted 1:20 before being used for amplification. RT-PCR was performed with gusA gene specific primers as above described for PCR analysis Protein phosphatase 2A (PP2A) amplification using gene-specific primers: forward 5′-TGCAAGGCTCACACTTTCATC-3′ and reverse 5′-CTGAGCGTAAAGCAGGGAAG-3′ was used as controls to check the quality of synthesized cDNA. The specific fragments were amplified in a total volume of 25 µl containing 1× PCR buffer, 2.5 mM MgCl2, 0.2 mM of each dNTP, 0.5 mM of each primer, 0.5 U of HotStar Taq DNA polymerase (Qiagen, GmbH, Germany) and 2 µl of cDNA as template. The PCR program used for the amplification was as described above for gusA gene. The amplified PCR products were resolved by electrophoresis on 1% agarose gel stained with GelRedTM (Biotium) and visualized under UV trans illuminator.

10. Data analysis

The experiments were arranged in completely randomized design with three replicates for each treatment. Data on embryo production and FEC production such as the effect of L-tyrosine on FEC production, effect of silver nitrate on somatic embryo regeneration was analyzed by analysis of variance (ANOVA) and treatment mean comparisons by Turkey's test ($p<0.05$) using the software R (R Core team 2012). Transformation efficiency was expressed as the number of independent transgenic lines as confirmed by PCR per 0.5 ml of SCV.

III. RESULTS

1. Production of FEC

All the three cultivars Mkombozi, Albert and cv. 60444 produced FEC from crushed somatic embryos after incubation for 21days on GD medium supplemented with 12 mg/l picloram and varying concentrations of L-tyrosine. The effect of L-tyrosine was significant in the induction of FEC from all the three cultivars tested. There was also a significant difference in the number of FECs recovered from the varying concentrations of L-tyrosine. Embryos cultured on GD medium supplemented with 250 µM of L-tyrosine recorded the highest conversion rate of OES to FEC. The conversion rate was recorded highest (44%) in Mkombozi, followed by cv. 60444 (40.6%) and Albert (29.3%) on medium supplemented with 250µM L-tyrosine (Figure 1).

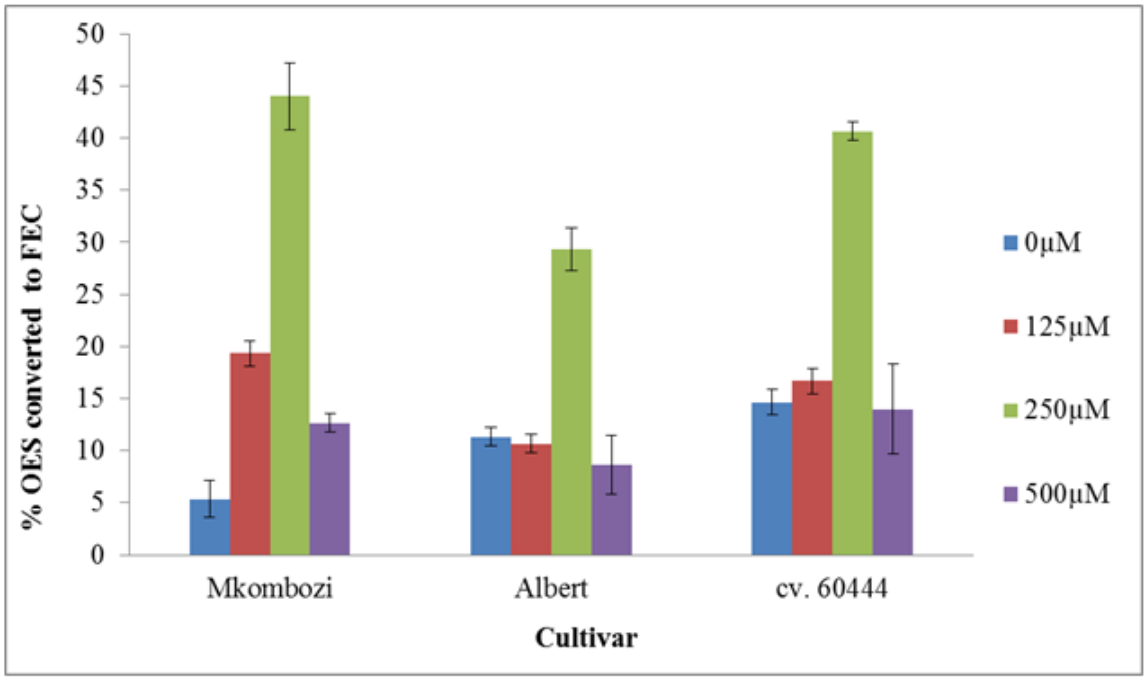


Figure 1 Effect of L-tyrosine on the production of FEC among different cassava cultivars. FEC production frequencies were recorded by calculating the ratio of FEC clusters/OES cultured. Values are means ± SE of three independent experiments.

All the cultivars tested were able to produce FEC (Figure 2). Mkombozi and cv. 60444 were the most responsive to FEC production but also FEC production in Albert was comparable to these two cultivars. The time required to generate homogenous FEC from OES differed between the tested cultivars. The earliest signs of conversion of OES to FECs occurred in Mkombozi towards the end of the first 21 days cycle on GD medium. The homogenous FECs were used for transformation and to compare transformation and regeneration efficiencies among the three cultivars.

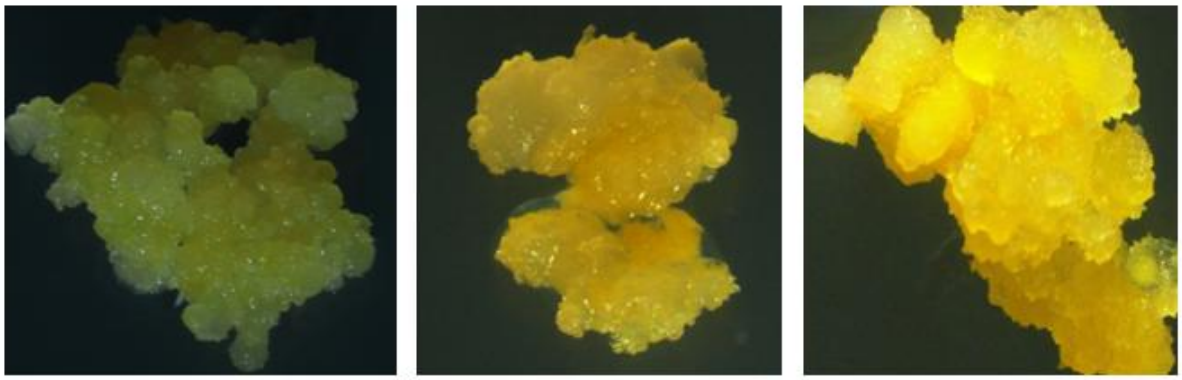


Figure 2 Generation of FEC from different cassava cultivars. (A) Homogenous FEC of Albert cultured on GD medium supplemented with 250 µM L-tyrosine; (B) Homogenous FEC of Mkombozi cultured on GD medium supplemented with 250 µM L-tyrosine; (C) Homogenous FEC of cv. 60444 cultured on GD medium supplemented with 250 µM L-tyrosine.

2. Regeneration capacity of FECs of Mkombozi and Albert

The regeneration potential of FECs from the three cultivars was assessed as indicated in (Table 1). FECs from all the cultivars were able to produce mature somatic embryos (cotyledonary-stage embryo) in 20 days after culturing on somatic embryo induction medium (MSN). All the cultivars tested were highly regenerative, producing on average more than 85 cotyledonary-stage embryos per 0.5 ml of FECs (Table 1). Cultivar 60444 and Albert produced the highest number of cotyledon-stage embryos (96 - 97 per 0.5 ml of settle cell volume of FEC) followed by Mkombozi (89 per 0.5 ml of settle cell volume of FEC). However there was a significant difference when comparing the number of cotyledons recovered to the maturing cotyledons and *in vitro* established plants (Table 1). The cotyledonary-stage embryos were highest for cv. 60444 at 48%, followed by Albert (29%) and Mkombozi

(26%). The shooting efficiency i.e. percentage of green cotyledons converting into plantlets was found to be cultivar dependent. Mkombozi and Albert had no significant difference with a conversion rate of 95.8% and 89.3% respectively whereas cultivar 60444 had a conversion rate of 43.4% (Table 1).

Table 1 Regeneration of plants from FECs of different cultivars.

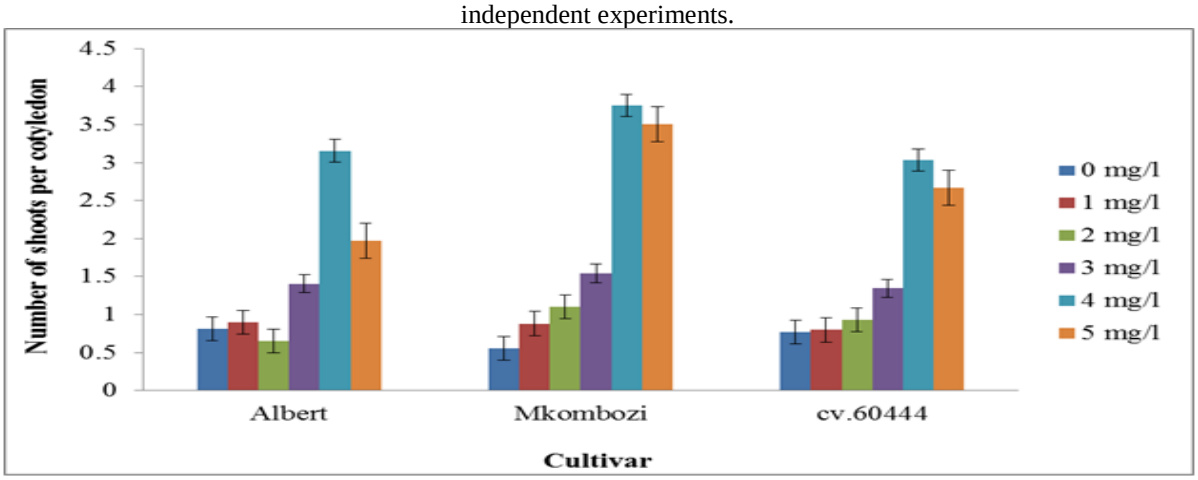
Cultivar	Amount of FEC	Average number of Cotyledonary-stage embryos recovered	Average number of maturing Cotyledonary-stage embryos (%)	Regeneration efficiency	Average number of *in vitro* plantlets established
Mkombozi	0.5ml	89 ± 10.5^{a}	26.4 ± 2.4^{b}	23/24	23 ± 4.0^{d}
Albert	0.5ml	96.3 ± 5.7^{a}	29.4 ± 3.9^{b}	25/28	25.3 ± 3.1^{d}
cv. 60444	0.5ml	97.7 ± 7.4^{a}	48.1 ± 8.0^{c}	20/46	20.3 ± 6.5^{d}

Values are means ± SE of three independent experiments. The mean values marked with the same letter are not significantly different from each other between the cultivar by Tukey-HSD test (α=0.05).

3. Effect of silver nitrate on regeneration of cotyledonary-stage embryos

The role of silver nitrate ($AgNO_3$) on shoot formation was investigated with mature somatic embryos cultured on CEM medium. Regeneration medium supplemented with $AgNO_3$ enhanced the number of shoots per cotyledonary-stage embryo (Figure 3). However, there was no significant difference ($p<0.05$) in shoot formation between the cultivars at different concentration levels apart from Albert at 5 mg/l where the mean number of shoots per cotyledon was lower at 1.8 compared to 3.6 and 3.1 for Mkombozi and cv. 60444. Cotyledonary-stage embryos cultured on CEM medium supplemented with 4 mg/l $AgNO_3$ showed a higher recovery of shoots per cotyledonary staged embryo (Figure 3). The highest mean number of shoots per cotyledonary-stage embryos was recorded for cultivar Mkombozi at 3.75 followed by Albert at 3.15 and cv. 60444 had 3.03 all on medium with 4 mg/l of $AgNO_3$ (Figure 3).

Figure 3 Effect of silver nitrate on regeneration of mature somatic embryos. Values are means ± SE of three independent experiments.



4. Generation of transgenic cassava plants

Axillary buds (Figure 4a) and immature leaf lobes were used to generate organized embryogenic structures (Figure 4b) from which homogeneous FECs on GD medium were generated(Figure 4c). The FECs were used for establishing genetic transformation procedures. *Agrobacterium* infected FECs (Figure 4d) proliferated into pale yellow colored embryogenic calli whereas non-transgenic calli did not show any growth and turned white.

Agrobacterium infected FECs from all the three cultivars were able to grow and generate transgenic somatic embryos on MSN supplemented with 50 mg/l paramomycine (Figure 4e). After 20 to 30 days on MSN medium, transformed embryogenic calli developed into cotyledonary-stage embryos. After 70 days (seven subcultures on MSN), cv. 60444 and Albert FEC gave rise to about 134 and 103 putatively transformed cotyledonary stage embryos respectively, while Mkombozi gave only 91 cotyledonary stage embryos. All the cultivars were able to form transgenic shoots (Figure 4f) 21 days (three sub cultures of 7 days each) after culturing mature somatic embryos on shoot elongation medium (CEM) (Figure 4). About 20.8% of somatic embryos were able to germinate from the total selected somatic embryos of Mkombozi, 32.3% and 39% for Albert and cv. 60444 respectively (Table 2). Albert performed better than all other cultivar at regeneration stage with 20 plantlets per 0.5 ml of SCV followed by cv. 60444 and Mkombozi with 17 and 16 plantlets per 0.5 ml of SCV (Table 2). The regenerated shoots were transferred to rooting medium (CBM) and all the shoots were able to form roots within 2 to 3 weeks (Figure 4g). All the transgenic lines grew normally in the greenhouse. No phenotypic variations were observed compared to non-transgenic plants, including leaf shape and plant stature.

Table 2 Transgenic plants regenerated from FEC of different cultivars

Cultivar	Amount of FEC	No. of somatic Embryos developed on selection medium	No. of maturing Embryos (%)	Regenerated plantlets (TE)	PCR positive plants (%)
Mkombozi	0.5ml	91.3 ± 5.7^{a}	20.8 ± 1.1^{d}	16.3 ± 1.7^{f}	100
Albert	0.5ml	103.6 ± 4.8^{b}	32.3 ± 4.3^{e}	20.6 ± 1.5^{f}	100
cv. 60444	0.5ml	134 ± 7.6^{c}	39 ± 6.4^{e}	17.6 ± 2.9^{f}	100

Values are means ± SE of three independent experiments. TE= total number of transformed plants identified to be PCR positive per 0.5 ml of SCV. The mean values marked with the same letter are not significantly different from each other between the cultivar by Tukey-HSD test (α=0.05)

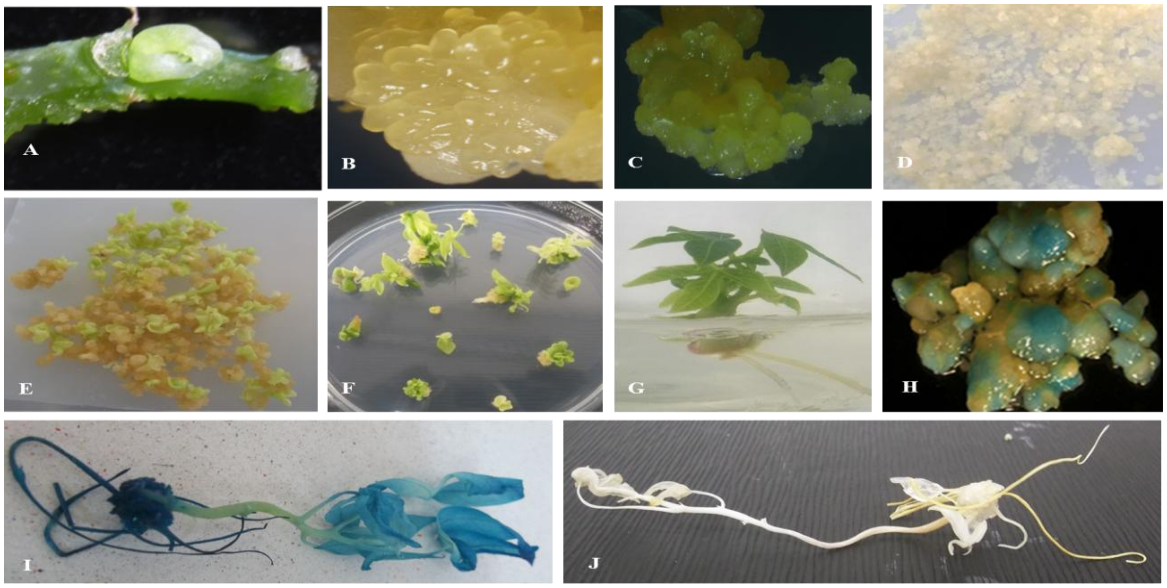


Figure 4 *Agrobacterium*-mediated genetic transformation of FEC of cassava cultivar Mkombozi and histochemical GUS assays of transformed and non-transformed tissues. (A) axillary bud cultured on CAM medium (B) organized embryogenic structure (C) friable embryogenic callus (D) *Agrobacterium*-infected FEC proliferating on selective medium, (E) developing embryos on embryo induction medium, (F) maturing cotyledonary-stage embryos on shoot induction medium, (G) transgenic plantlets regenerated on selective medium, (H) expression of *gusA* gene in mature embryos, (I) no expression of *gusA* gene in non-transgenic plant, (J) stable expression of *gusA* gene in transgenic plant

5. Histochemical GUS analysis

Transient GUS expression assay was done 3 days after co-cultivation of FEC and *Agrobacterium* containing pCAMBIA 230 (Figure 4h). Expression of GUS activity was observed as blue coloration indicating transient expression of the reporter gene in FEC. Stable GUS expression was observed in mature plants of all the three cultivars (Figure 4i). The control non-transformed plants lacked GUS staining (Figure 4j). Stable GUS expression was observed in all the tissues of regenerated transgenic plants, confirming stable expression of *gusA* gene. The GUS expression in the transgenic plant indicated that uniform transformation was achieved. No blue coloration was observed in non-transgenic plants. There was no significant difference across the cultivars and the percentage of
GUS positive calli varied among the cultivars ranging from 56.6 to 68.1% (Figure 5).

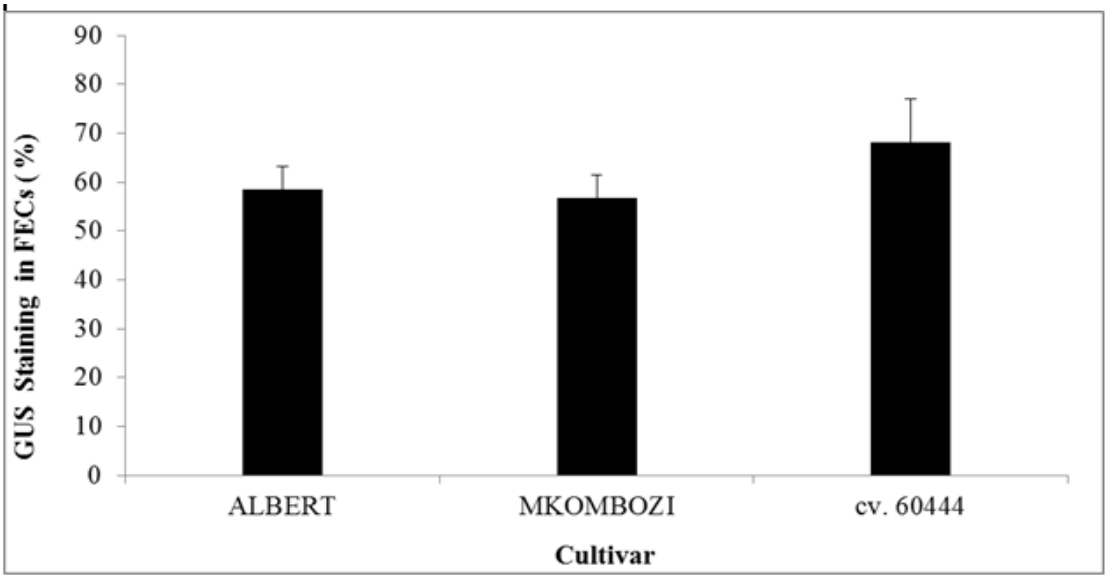


Figure 5 GUS staining in FECs of cultivars Mkombozi, Albert and cv. 60444. Values are means ± SE of three replicates of two independent experiments

6. Molecular characterization of putative transgenic plants

To confirm the presence of the transgene in the putative transgenic plants, PCR analysis was done using total genomic DNA isolated from leaves of transgenic and non-transgenic plants using specific primers designed to amplify *gusA* and *nptII* genes. The amplified product of approximately 215 bp was observed in all the analyzed transgenic plants, using *gusA*-specific primers (Figure 6a). The size of the amplified product confirmed the presence of *gus*A transgene in transgenic plants. A 500 bp fragment was amplified using *npt*II-specific primers confirming the presence of *npt*II gene (Figure 6b). No amplification was observed from the non-transgenic control plants with both *gusA* and *npt*II-specific primers. Expression of *gus*A gene was assessed by RT-PCR to confirm the expression of the gene (Figure 6c). An expected 215 bp fragment was amplified from the cDNA products of eight transgenic plantlets, but was absent in non-transgenic plants. Both transgenic and non-transgenic plants showed expression of a reference gene (endogenous PP2A) by amplification of 200 bp product (Figure 6d).

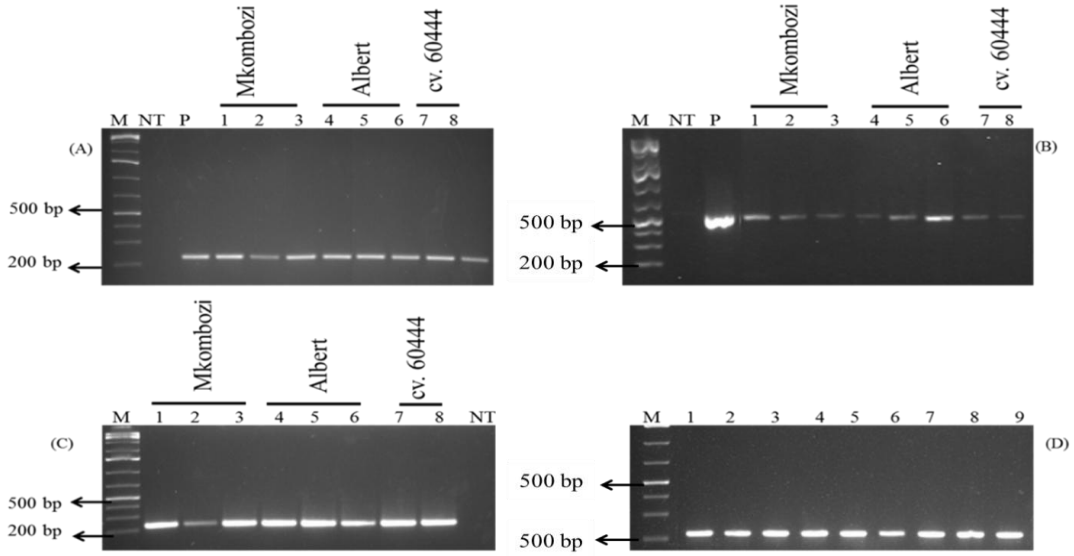


Figure 6 molecular analyses of transgenic lines of various cassava cultivars. (A) *gus*A specific primers; (B) *nptII* specific primers. Lanes: M, molecular size marker (1 kb plus DNA ladder); NT, non-transgenic control plant; P, pCAMBIA2301 plasmid DNA; 1 – 2, 3 – 5 and 6 – 8, transformed cassava lines of cultivars cv. 60444, Albert and Mkombozi, respectively;. (C) RT-PCR using *gus*A specific primers; (D) RT-PCR use housekeeping gene protein phosphatase 2A (PP2A).

IV. DISCUSSION

Regeneration and transformation of cassava has been developed based on somatic embryogenesis, shoot organogenesis from somatic embryos and friable embryogenic calli (FEC) (Li et al., 1996; Rossin, 2008; Bull et al., 2009; Taylor et al., 2012; Chetty et al., 2013; Nyaboga et al., 2013). Using FECs the explants for transformation also enables large production of independent transgenic events (Bull et al., 2009; Nyaboga et al., 2013). However, production of FEC is quite variable across different cultivars and may not even be obtained in some cultivars (Taylor et al., 2001; Bull et al.,2009; Nyaboga, 2013). Non-existence of a robust cultivar independent FEC generation protocol is considered to be a limiting step resulting in low transformation and regeneration potential of recalcitrant cassava cultivars (Raemakers et al., 2001). Therefore, difficulty in FEC production is a major constraint for the successful transformation of farmer-preferred or domesticated cassava cultivars with desired agronomic traits. In this study, an efficient FEC and transformation system for two of the farmer-preferred cassava cultivars was developed. Previous studies conducted by Nyaboga et al. (2013) to optimize transformation protocols of farmer-preferred cultivars in East Africa, two cultivars Albert and Mkombozi were not able to produce FEC. Following modifications of this protocol, in this study, a novel regeneration and transformation of FECs of these two cultivars was successfully developed.

Successful genetic transformation of cassava through FEC-based transformation systems depends initially on the production of OES, and the ability of OES to induce and proliferate into pure and homogeneous FEC (Taylor et al., 2004). In this study, OES production frequency varied among the different cultivars tested. These variations in OES production frequency was also observed in a previous study using the same cultivars (Albert, Mkombozi and the model cultivar cv. 60444) (Nyaboga et al., 2013). Production of OES was done from ABs and ILL. From a previous study by Nyaboga et al. (2013), OES from Mkombozi and cv. 60444 were induced from AB and Albert from ILL. Rapid processing of the OEC by crushing the OES through 1-2 mm size metallic mesh followed by culturing on FEC initiation medium resulted in the production of FEC for cultivars Albert and Mkombozi.

Callus (which is a mass of undifferentiated cells) forms naturally on plant organs in response to wounding, infestations, or at graft unions. Wounding of OEC proved to facilitate induction of FEC from both Albert and Mkombozi OECs that were previously not able to produce FEC. Previously, Taylor et al. (2012) reported that wounding of OES encourages FEC production in cassava. Supplementation of L-tyrosine a precursor of phenylethanoid glycosides in FEC induction medium had greatly enhanced FEC production and proliferation. This was comparable to a previous study by Nyaboga et al., (2013) that reported an increase of 1.7 to 7.2 fold in FEC production upon L-tyrosine addition in cultivar Ebwanatereka.

Though all the cultivars used were able to form FEC, there were variations in the frequency of FEC production across the different cultivars. This variations had previously been observed by Nyaboga et al, (2013) while working with other African cassava cultivars. Raemakers et al., (2001) also reported genotypic effect on the FEC production by Asian cassava cultivars.

The genotypic variations in the formation of FEC is a major impediment in improvement of cassava through genetic engineering (Nyaboga et al., 2013). The establishment of FECs for Albert and Mkombozi cultivars popularly grown in East-African is very critical in initiating their genetic improvement through transgenic approaches. This is unquestionably the case for other farmer-preferred cultivars where until now, friable embryogenic calli have never been obtained. Selection of FEC is normally done with a lot of precision because FEC coexist with non-embryogenic calli that outgrows the FEC on FEC induction medium. It is therefore important to make sure that only the embryogenic calli is picked during sub culturing to enable rapid production of FEC and to prevent depletion of the nutrients in the medium by the non-embryogenic tissues. It is also important to choose only the friable calli when setting up Agrobacterium-mediated transformation experiment in order to realize a high transformation and regeneration efficiency and to avoid regeneration of chimeric lines.

Variations in plant regeneration were observed in the different cultivars using 0.5ml of total settled cell volume. Albert had the highest plant regeneration score followed by Mkombozi then the model cultivar cv. 60444. This implies that genotypic differences have an effect on plant regeneration considering that the farmer preferred cultivars responded better than the model cultivar which has been used for many years to optimize a transformation and regeneration protocol (Nyaboga et al, 2013). In this study, we tested the effect of silver nitrate (AgNO3) on regeneration of transformed embryos. Supplementation of AgNO3 in regeneration media caused an increase in number of shoots per cotyledonary staged embryo. Silver nitrate has been shown to be effective in improving somatic embryogenesis and plant regeneration in a number of crop species; (Kumar et al., 2009). The Ag+ ions prevent a wide variety of ethylene-induced plant responses including growth inhibition and senescence, this is assumed to be mediated through the inhibition of the physiological action of ethylene which is a potential inhibitor of many plant regeneration systems (Ozudogru et al., 2005; Kumar et al., 2009). Although supplementation of AgNO3 in regeneration media caused a significant increase in number of shoots per cotyledon, the shooting efficiency was not affected implying that low regeneration efficiency of transformed embryos might not be due to ethylene production by cassava tissue cultured in vitro. In this study, the transformation frequency was also cultivar dependent. Histochemical GUS staining indicated that FECs of farmer-preferred cultivars Albert and Mkombozi and model cultivar cv. 60444 were susceptible to Agrobacterium strain LBA4404. Monitoring done by the frequency of blue foci observed during staining suggests that different cultivars respond differently to transformation.

This study has demonstrated the transformability of Albert and Mkombozi, for which transformation had not previously been, achieved (Nyaboga et al, 2013). All the tested cultivars were able to regenerate into putative transgenic plantlets. In this study, 28 out of 103, 16 out of 91 and 17 out of 134 somatic embryos of cultivars Albert, Mkombozi and cv. 60444, respectively, regenerated into plants. In a previous study, Nyaboga et al. (2013) regenerated 22 plants from 240 paromomycin resistant embryos from cassava cultivar Serere and 17 plants from 187 paromomycin resistant embryos of Ebwanatereka. The findings from this study show an increase in the embryo germination frequency that ranged between 17% to 19% for farmer-preferred cultivars while the previous studies done by Nyaboga, (2013) had a much lower embryo germination frequency for farmer-preferred cultivars ranging between 9% to 10%. The recovery of complete plants from cassava somatic embryos is cultivar dependent. Due to the low frequency of embryo germination, generation of transgenic cassava from a wider range of cultivars may be greatly challenged.

Analysis of all putative transgenic lines using PCR established the presence of the nptII and gusA genes in all regenerated lines, indicating an efficient selection system. The sequential increase of the selection pressure (30 mg/l paramomycine to 40mg/l paramomycine and finally 50 mg/l paramomycine) and the prolonged exposure to 50 mg/l paramomycine inhibited regeneration of the non-transgenic embryos. The selection pressure was only withdrawn briefly at the shoot elongation step to facilitate shoot elongation (Bull et al., 2009).

This is the first report of Agrobacterium-mediated transformation of farmer-preferred cultivars Albert and Mkombozi. These findings provide a platform for the development of transgenic farmer-preferred germplasm possessing traits of agronomic importance, such as high yield and cassava that are resistant to biotic and abiotic constrains. This protocol can be used to transform other cassava farmer-preferred cultivars and landraces.

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