

Full Length Research Paper

Production of the first transgenic cassava in Africa via direct shoot organogenesis from friable embryogenic calli and germination of maturing somatic embryos

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The impact of cassava transformation technologies for agricultural development in Africa will depend largely on how successfully these capabilities are transferred and adapted to the African environment and local needs. Here we report on the first successful establishment of cassava regeneration and transformation capacity in Africa via organogenesis, somatic embryogenesis and friable embryogenic callus (FEC). As a prerequisite for genetic engineering, we evaluated six African cassava genotypes for the ability of a) induction of FEC b) hygromycin sensitivity and c) T-DNA integration potential by different *Agrobacterium* strains. FEC was induced in genotypes TMS 60444, TME 1 and TMS 91/02327. Potential tissues for FEC formation were induced in TMS 91/02324, TME 12 and TME 13. Pure and proliferating FEC was obtained and maintained only in TMS 60444. FEC growth and shoot organogenesis were completely suppressed when hygromycin was used at a concentration of 20 mg/l in all tissue types and genotypes. With somatic cotyledons, statistically significant differences ($p \leq 0.05$) were observed between *Agrobacterium* strains and genotypes with respect to T-DNA transfer efficiency. Using somatic cotyledons, TME 8 was found to be the most amenable to transformation with maximum blue spots per GUS-positive explants, and *Agrobacterium* GV3101 proved to be superior to EHA105, LBA4404, and AGI-1 for T-DNA transfer based on transient assays with a reporter gene (GUS). With FEC, *Agrobacterium* LBA4404 was superior to other strains. This study also identified EHA105 as a new vir helper strain to recover transgenic cassava plants. PCR and Southern hybridization of genomic DNA of the hygromycin-resistant cassava plants to a *hpt* probe confirmed the integration of *hpt* with integration events varying between 1 and 2 insertions. The benefit of combining the FEC and shoot organogenesis systems for recovering transgenic cassava plants is described. The contributions of this report to enhancing the development and deployment of genetic engineering of cassava for agricultural biotechnology development in Africa are discussed.

Key words: Cassava (*Manihot esculenta* Crantz), landraces, embryogenic suspension, *Agrobacterium tumefaciens*, hygromycin, shoot organogenesis.

INTRODUCTION

Cassava is the second most important staple food in Africa (Nweke et al., 2002), where mostly low-income

Abbreviations: BAP (benzyladenine) NAA (α -Naphthalene acetic acid), SSA (sub-Saharan Africa), picloram (3, 5, 6-trichloro-4-aminopicolinic acid), IBA (indolebutyric acid), *hpt* (hygromycin phosphotransferase), OES (organized embryogenic structure), FEC (Friable embryogenic callus), NARS (national agricultural research system).

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farmers grow it in marginal lands and its swollen, starchy storage roots provide a cheap source of dietary calories to over 300 million people in the continent (Nweke et al., 2002). Although this continent produces more than half of the world's cassava, the average yield of cassava roots per hectare is significantly below the global average (FAO). Increasing cassava yields in Africa to levels comparable to the world average will contribute to increased food for million of people and economic security for low-income farmers and local industries. Succeeding in these endeavors required that commendable efforts being deployed to tackle the range of cassava production constraints in Sub-Saharan Africa (For review see Hankoua, 2003). Poor seed set and high heterozygosity constantly frustrates conventional breeding and makes genetic engineering an attractive and efficient tool to complement traditional breeding in addressing cassava production constraints in SSA (Asiedu et al., 1992; Bokanga et al., 1998; Fregene and Puonti-Kearlas, 2002; Taylor et al., 2004) and delivering enhanced germplasm to cassava consumers. Genetic transformation protocols have been developed for the crop in which transgenic plants are recovered via somatic embryogenesis and organogenic pathways. These systems are compatible with both particle bombardment and *Agrobacterium*-mediated gene-transfer and are used routinely for the production of transgenic cassava plants of Asian, African and South-American origins, expressing selectable marker genes and transgenes with potential agronomic importance (Li et al., 1996; Schopke et al., 1996; Gonzales et al., 1998; Raemakers et al., 2001; Taylor et al., 2001; Zhang et al., 2003a; Siritunga and Sayre, 2003; Zhang et al., 2005; Ihemere et al., 2006). Application of transgenic technology in cassava R&D have moved from production of cassava plants expressing agronomically useful traits to isolation of specific promoters from cassava tissues (Zhang et al., 2003b), and initiation of field testing in Africa (Taylor et al., 2004). One of a critical issue that will govern the adoption of transgenic cassava plants by farmers in Africa is the development of capacity aiming to integrate transgenes of agronomical importance into appropriate germplasm. While recognizing that only a fraction of the-se relevant germplasm will enter a specific transgenic program, there is an urgent need to develop capacities to transform the most important landraces, improved varieties and breeding lines for each major cassava growing regions (Taylor et al., 2002). Therefore, screening important African cassava genotypes for their transformation ability and integrate useful into these cultivars represent an important technical challenges for cassava biotechnologists.

To date, less than five African cassava genotypes have proved amenable to the existing plant transformation systems, a situation that must be rectified if rapid deployment of this technology is to target beneficiaries in Africa (Taylor et al., 2004). Furthermore, technological capacity for cassava genetic transformation is still concentrated in

laboratories in the USA, Europe, and the Consultative Group on International Agricultural Research (CGIAR) center, International Center for Tropical Agriculture (CIAT) based in Cali, Colombia. Long-term success of transgenic technologies for genetic improvement of cassava or any other important

African crop plants aiming at enhancing agricultural development in Africa will depend largely on the transfer and expansion of such capabilities to researchers in Africa, where these systems can be exploited for specific local needs in the relevant germplasm (Altman, 1993; Thro et al., 1998; Masona et al., 2001; Machuka, 2001; Toenniessen et al., 2003; Conway and Toenniessen, 2003; Gaillard, 2003, DaSilva et al., 1998; Hankoua et al., 2004). This report is designed to start addressing these issues by using IITA as a platform for biotechnology transfer between advanced laboratories and the NARS in Africa (Alhassan, 2003).

In this study, we tested the amenability of two existing cassava regeneration and transformation systems on a range of West-African cassava genotypes for plant regeneration, marker gene expression and recovery of first transgenic cassava plants from Africa.

MATERIALS AND METHODS

Maintenance of cassava plants, production of embryogenic tissues, and somatic cotyledons

Cassava genotypes (TME 13, TME 127, TME 8, TME 1, TMS I 91/02327 and 60444) were obtained from the *in vitro* germplasm collection maintained at the International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria. *In vitro* plantlets were maintained by monthly subcultures as shoot cuttings following the procedure described by Hankoua et al. (2005). Shoot apical meristems and immature leaf lobes (0.1-0.9 mm in length) were excised from plantlets 10-14-days after previous subculture and cultured on picloram-based embryo induction medium (P-CIM) as previously described (Li et al., 1996; Hankoua et al., 2005) for the induction of primary somatic embryos and organized embryogenic structure (OES). OES were divided into small clusters of 5-10 embryos each and transferred into cassava embryo maturation media (CMML) (Table 1) as described by Hankoua et al. (2005) to enhance their development into green cotyledon stage embryos.

Green cotyledon pieces (about 5 mm² each), obtained from 14-15 days old cotyledonary stage embryos using scapel blades, were placed on P-CIM (Table 1) for the production of secondary somatic embryos. Secondary somatic embryo clusters induced from P-CIM were transferred to CMML for maturation. Green somatic cotyledon pieces obtained from 14-15 day-old secondary stage embryos were placed on P-CIM for induction of cyclic somatic embryogenesis.

OES induced from all cassava genotypes were fragmented into small pieces of about 5 mm² each and sub cultured onto friable callus induction medium and maintenance medium (FEC-IM) for the induction of friable embryogenic callus (FEC) as described by Taylor et al. (1996). FEC-IM was modified by the inclusion of 25-mg/l-casein hydrolysate and 2 μM copper sulfate (Table 1). Calli with the potential to develop into FECs and induced FECs were sub-cultured on the same medium for the selection and proliferation of high quality friable embryogenesis callus. Embryogenic suspensions were initiated by transferring approximately 35 mg of pure FEC into 50 ml embryogenesis suspension medium (EMS) and cult-

Table 1. Composition of media used for bacteria culture, tissue culture and genetic transformation.

Media	Composition	Reference
<i>Agrobacterium</i> culture medium (YEB)	5g/l Bacto beef extract, 1g/l Bacto yeast extract, 1g/l Bacto peptone, 240 mg/l MgSO ₄ , 5g/l sucrose, 15g/l granulated agar (for semi-solid medium), pH 7.2	Lichtenstein and Draper (1985)
<i>E. coli</i> culture medium (LB Broth)	10g/l bactotryptone, 5g bacto yeast extract, 10g NaOH 15g/l granulated agar (for semi-solid medium), pH 7.2	Miller (1972)
Bacteria preinduction and inoculation	COM or FEC-IM, 200 µM acetosyringone, pH 5.2	Li et al. (1996) and this study
P-CIM	MS, 20g/l sucrose, 12mg/l picloram, and 2µM CuS ₀₄ , pH 5.7	Li et al. (1996) and this study
CMM	MS, 20g/l sucrose, 0.1mg/l BAP, pH 5.7	Li et al. (1996) and this study
FEC-MU ₁	SH, 12mg/l picloram, MS vitamins, 60g/l sucrose, 500mg/l timentin, pH 5.7	Taylor et al. (1996) and this study
FEC-MM ₁	MS, 20g/l sucrose, MS vitamins, 0.2mg/l NAA, pH 5.7	Taylor et al. (1996) and this study
ESM	SH, 12mg/l picloram, MS vitamins, 60g/l sucrose, pH 5.7	Taylor et al. (1996) and this study
FEC-GE	MS, 20g/l sucrose, MS vitamins, 0.45mg/l BAP	Taylor et al. (1996) and this study
COM	MS, 20g/l sucrose, 1mg/l BA, 0.5mg/l IBA, 2µM CuS ₀₄ , pH 5.7	Li et al. (1996) and this study
CEM	MS, 20g/l sucrose, 0.4mg/l BA, 2µM CuS ₀₄ , pH 5.7	Li et al. (1996) and this study
FEC-IM	GD, 20g/l sucrose, MS vitamins, 12mg/l picloram, 25mg/l casein hydrolysate, and 2µM CuS ₀₄ , pH 5.7	Modified from Taylor et al. (1996)
(CCM) ₁	COM, 100 µM acetosyringone, 20g/l glucose, 20g/l sucrose pH 5.2	Modified from Li et al. (1996)
(CCM) ₂	FEC-IM, 100 µM acetosyringone, 20g/l glucose, 20g/l sucrose pH 5.2	Modified from Schokpe et al. (1996)
(CRM) ₁	COM, 8mg/l AgNO ₃ , 0.8% agar, 500mg/l timentin, 7.5 mg/l hygromycin, pH 5.7	Modified from Li et al. (1996)
(CRM) ₂	-IM, 20g/l sucrose, 500mg/l timentin, 20mg/l hygromycin, pH 5.7,	Modified from Schokpe et al. (1996)
(CRM) ₃	FEC-GE, 20g/l sucrose, pH 5.7	Modified from Schokpe et al. (1996)

Murashige & Skoog basal medium (Murashige and Skoog, 1962) (MS); Gresshoff & Doy basal medium (Gresshoff & Doy, 1974) (GD); Shenk & Hildebrandt (1972) (SH); Embryogenic suspension medium (ESM); Picloram-based embryo induction medium (P-CIM); Cassava organogenesis medium (COM); Cassava elongation medium (CEM); Friable callus induction medium and maintenance (FEC-IM); Maturation medium for embryogenic callus (FEC-MM₁); Embryo germination and plant regeneration medium (FEC-GE); Cassava co-cultivation medium for somatic cotyledons (CCM)₁; Cassava co-cultivation medium for embryogenic suspensions (CCM)₂; Cassava regeneration medium for hygromycin resistant shoot; (CRM)₁; Cassava regeneration medium for hygromycin resistant embryogenic units (CRM)₂; Cassava regeneration medium for plantlet conversion of hygromycin-resistant maturing embryos (CRM)₃; Liquid medium for multiplication and maintenance of cassava embryogenic suspensions (FEC-MU₁)

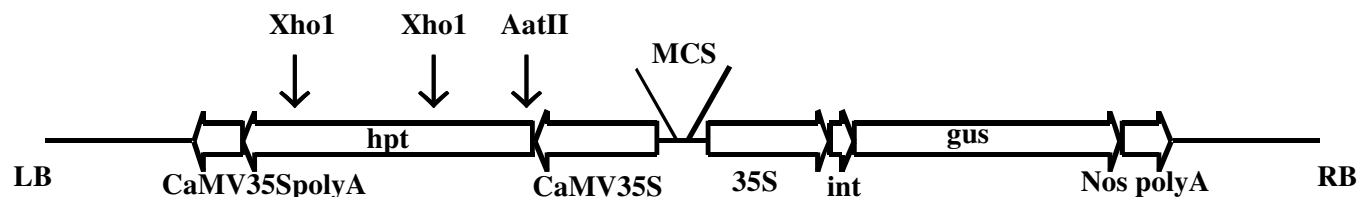


Figure 1. T-DNA region of binary vector pCambia1301, showing restriction sites used for Southern blot analysis. *Xho*I digest the plasmid and release the 1.1 kb *hpt* gene used for probing. *aat* II (enzyme with only one restriction site within and out of the T-DNA borders) digest the plasmid at one end of the *hpt* gene for determining the number of transgene insertions.

uring as described by Taylor et al. (1996). All cultures were incubated in the growth room at temperatures varying from 25°C to 28°C either under total dark (one week) for embryo induction from leaf lobe and apical meristem explants or with a photoperiod of 16 h (40-100 $\mu\text{m photon s}^{-1}\text{m}^{-2}$ PAR) for multiplication and maintenance of all other tissues. Unless otherwise stipulated, all culture media were supplemented with 0.6% agar.

Determination of phytotoxic levels of selective antibiotic

Green cotyledon pieces approximately 5 mm² in size, were excised from 10-14-day old maturing somatic embryos and FECs were sub-cultured onto cassava organogenesis (COM) and FEC-IM (Table 1) media, respectively. The media were augmented with different concentrations of the antibiotic hygromycin (0, 5, 10, 15, 20, 25, 30 mg/l). Hygromycin was filter-sterilized and added to the autoclaved media after it had cooled to 45°C. All treatments were assigned to experimental units (single petri plate) in a completely randomized manner. Ten cotyledon pieces and seven FECs clusters were used per replicate (petri plate). Four and nine replicates were used for somatic cotyledon pieces and FECs respectively. The number of somatic cotyledon piece regenerating shoot buds and FEC developing new FECs tissues were recorded after one month in culture.

Bacteria strains and vectors

Disarmed *Agrobacterium* strains EHA105, LBA4404, GV3101 and AGL1 (Hellens and Mullineaux, 2000) carrying the pCambia 1301 plasmid, obtained from the Center for Application of Molecular Biology to International Agriculture (Cambia), Australia, were used as vector systems for transformation (Figure 1). The plasmid pCambia carries the hygromycin selectable marker gene (*hpt*) and the *uidA* (*gus*) visual marker gene within its T-DNA, each under the control of the 35S cauliflower mosaic virus promoter.

Genetic transformation by *Agrobacterium tumefaciens* strains

Agrobacterium culture and pre-induction of the bacterial cells: The various media used for bacterial as well as plant tissue cultures are listed in Table 1. All tested *Agrobacterium* strains harboring pCambia1301 were kept as glycerol stocks at -80°C and streaked on the semi-solid YEB medium supplemented with appropriate antibiotics (Lichtenstein and Draper, 1985; Hellens and Mullineaux, 2000) and then grown at 28°C for 2 days. A single colony from each of the *Agrobacterium* strains was picked and inoculated to 5 ml fresh YEB liquid medium containing the appropriate antibiotics. Cultures were incubated at 28°C, with agitation at 180-200 rpm for 12 h. Subsequently; a 1 ml aliquot of the *Agrobacterium* suspensions was transferred to 50 ml of YEB liquid medium and grown overnight under the conditions described above. *Agrobac-*

terium strains were collected by centrifugation in 15 ml eppendorf tubes at 6000 rpm for 15 min at 4°C. Bacteria pellets were resuspended in pre-induction medium (PIM) (Table 1) to generate an OD₆₀₀ of 0.5-0.9, and pre-induced on a shaker set at 200 rpm for 2-5 h at 28°C prior to use for inoculation of FEC and somatic cotyledon pieces.

Plant tissue, *Agrobacterium* inoculation and co-cultivation: Green cotyledon pieces (5 mm² each) of secondary and cyclic maturing somatic embryos were immersed in the bacteria suspensions for 1 h at room temperature and excess bacterial suspension was removed by blotting explants on sterile filter paper before transferring to semi-solid co-cultivation medium (CCM₁) (Table 1), followed by incubation at 24±1°C for 5 days in the dark. The experiment was a completely randomized 3 x 4 factorial with four *Agrobacterium* strains and three cassava genotypes TME 13, TME 127 and TME 8 as treatment levels making 12 treatment combinations. Each treatment was replicated four times with 25 somatic cotyledon pieces per replicate.

For FECs, an aliquot of 0.5 ml settled cell volume (SCV) was obtained by sieving a sample of embryogenic suspension sequentially through 250 and 500 μm meshes. Embryogenic tissues (250-500 μm in diameter) were transferred to 15 ml sterile eppendorf tubes containing liquid SH medium and allow to settle for 20 min. Aliquots of FEC were transferred to petri plates containing 5-10 ml of *Agrobacterium* suspensions. After 0.5-1 h, bacterial suspension was removed using a sterile pipette and the inoculated tissues blotted dry on sterile No.1 Whatman filter papers before transfer to semi-solid co-cultivation medium (CCM₂) (Table 1) and co-cultivated for 2 days in the dark. The experiment was a single factor, laid out in a completely randomized design with three *Agrobacterium* strains EHA105, GV3101 and LBA4404 as the treatments. Each treatment was replicated four times and the number of embryogenic units making 0.5 ml SCV of embryogenic suspension was used per replicate.

Selection and regeneration of putative transgenic tissues and plants: After 4-5 days co-cultivation, cotyledon pieces from various treatments were transferred into cassava regeneration medium (CRM₁) (Table 1). CRM₁ is modified from cassava organogenesis medium (COM) by the inclusion of timentin, silver nitrate and agar for the induction of hygromycin-resistant shoot buds (Table 1). As soon as shoot buds were induced on this selection medium, they were transferred to CEM (Table 1) for plant regeneration.

In case of FEC, after 2 days co-cultivation on CCM (Table 1), embryogenic tissues were transferred to 50 ml liquid FEC-MU₁ (Table 1) in 250 ml flask and grown on a shaker at 150 rpm. Within these two weeks, suspension tissues were sub-cultured at 3-day intervals for two weeks with fresh FEC-MU₁ (Table 1). Tissues were then transferred to FEC-MU₂ (FEC-MU₁ containing 10 mg/l hygromycin), and incubated with shaking for a further 2 weeks. Subsequently, embryogenic tissues were transferred to CRM₂ (Table 1) for selecting the hygromycin-resistant FEC lines. Individual hygromycin-resistant FEC lines were first transferred to FEC-MM₂ (FEC-MM₁ containing 20 mg/l hygromycin and 500 mg/l timentin) for cotyledon emergence and COM for direct shoot bud

induction. Hygromycin-resistant maturing embryos were allowed to germinate on CRM₃ (Table 1) and the developing shoots were transferred to CEM for plant regeneration. Direct shoot buds induced from putative transgenic embryoids were also transferred to CEM for elongation.

Assay of GUS activity

The histochemical assay of GUS was carried out as described by Jefferson et al. (1987). Co-cultured FEC and somatic cotyledons, friable embryogenic units and clusters, maturing embryos, shoot apices, leaves, roots, whole plantlets from randomly selected hygromycin-resistant plantlets were incubated overnight at 37°C, except for FEC which was incubated in GUS staining solution (1% 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 50 mM K-ferricyanid (II), 50 mM K-ferrocyanid (III), 1 M potassium/sodium-buffer (pH 7.0), 100 mM NaEDTA (pH 8.0), and 1% Triton-X-100, pH 7.0) for 2-3 h. Thereafter, tissues were first fixed in 5% formaldehyde, 5% acetic acid and 20% ethanol solution and then bleached in ethanol (EtOH) gradient {10 min in 50% EtOH, 30 min in 70% EtOH, overnight in 100% EtOH}, scored and photographed. A single embryogenic unit and cotyledon piece showing one GUS blue spot or more were recorded as positive GUS-expressing explants. In addition, the number of blue spots per GUS-positive explants was recorded. All the experiments were laid out in a completely randomized manner and replicated four times with a replicate consisting of thirty embryogenic units, and 20 cotyledon pieces selected randomly among masses of stained tissues for scoring. Data were analyzed using the SAS statistical package (SAS, 1988) where standard error values were derived for each replicated set of treatments.

PCR and southern blot analysis

Total DNA was isolated from approximately 1g of *in vitro* leaves of hygromycin-resistant cassava lines (IITA-60444-1, IITA-60444-2, IITA-60444-3, IITA-60444-4, IITA-60444-10, IITA-60444-11, IITA-60444-12, IITA-60444-13, IITA-60444-SO₁, IITA-60444-SO₂ and IITA-60444-SO₃) by the modified Dellaporta procedure (Dellaporta et al., 1983). Residual RNA was removed by treating the isolated DNA with RNase. PCR analysis was conducted in a 50 µl reaction mixture containing DNA (1 µg), 200 µM of each dNTP, 1 µM of each primer, 1 unit Taq DNA polymerase, 2 mM MgCl₂ and 1x Taq buffer (Invitrogen). The standard reaction was at 95°C for 5 min followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min and 72°C for 10 min. Forward primer 5'-CGATCTTAGCCAGACGAGCGGGTTCG-3' and reverse primer 5'-CGCATAACAGCGGTCATTGACTGGAGC-3' were used to amplify an internal *hpt* sequence of 628 bp. For Southern blots hybridization analysis, 40 µg DNA isolated from *in vitro* leaves of 5 transgenic cassava lines (IITA-60444-1, IITA-60444-10, IITA-60444-12, IITA-60444-SO₃, IITA-60444-SO₁), was digested with *aat*II and separated on 1% agarose gel by electrophoresis and blotted onto a hybond-N+ (Bioscience, Amersham) membrane. The DNA was fixed to the membrane by UV-cross linking and hybridized with the 1.1 Kb fragment of *hpt* gene obtained by *Xho*I digestion of pCAMBIA1301. Probe labeling, prehybridization and hybridization were carried out as previously described (Padmanabhan et al., 2004). Blots were scanned and bands detected using phosphor images (Molecular Dynamics, Amersham).

RESULTS

Maintenance of cassava plants, production of embryogenic tissues, and somatic cotyledons

All cassava genotypes used in the present study formed OES (Figure 2a) when leaf lobe and apical meristem explants were cultured on an MS-based medium supplemented with auxin picloram. OES were successfully produced from the six West African landraces, the improved line TMS I 91/02327, and the model cultivar 60444. All the cassava genotypes produced isolated maturing somatic embryos (Figure 2b) from which maturing cotyledon pieces were obtained. Secondary and cyclic somatic embryos were produced and maintained from all genotypes and generate constant supply of high quality somatic cotyledons for subsequent transgene insertion experiments. By subculturing such OES onto Gresshoff and Doy-based medium (GD) in the manner described by Taylor et al. (2001), friable embryogenic callus could be induced from three (TME 1, TMS 91/02327, and 60444) of the six cassava genotypes tested, but proliferating FEC of high quality could only be establish in 60444. TME 13, TME 12, and TMS I 91/02324 showed early signs of conversion of OES to FEC (Figure 2c) by producing structures-like FEC tissues, but homogenous lines of friable embryogenic calli could not be recovered from these genotypes as these tissues all converted to non-morphogenic tissues upon subculture into FEC-IM.

For 60444, FEC lines were established within 12 weeks while other cassava genotypes probably required longer periods to achieve high quality FEC tissues. The differential response of these six West African cassava genotypes in induction of FEC suggested that the induction of FEC in cassava might be genotype dependent. Casein hydrolysate and copper sulfate were added to the FEC-IM in an attempt to improve FEC production but did not significantly enhance FEC production in any of the six lines tested (date not shown). Embryogenic suspension cultures were successfully established by transferring FEC of 60444 into liquid Schenk and Hildebrandt-based medium in the manner described by Taylor et al. (2001). Homogenous, high quality FEC of 60444 (Figure 2d), was maintained by repeated subculture on semi solid FEC-IM or in liquid medium for several months and used as source of plant material for subsequent regeneration and transformation experiments.

Determination of phytotoxic levels of selective antibiotic

Cotyledon fragments from six cassava genotypes, TME 13, TME 127, TME 203, TME 282, TME 1, TME 8, and FEC of 60444 were cultured on medium containing var-

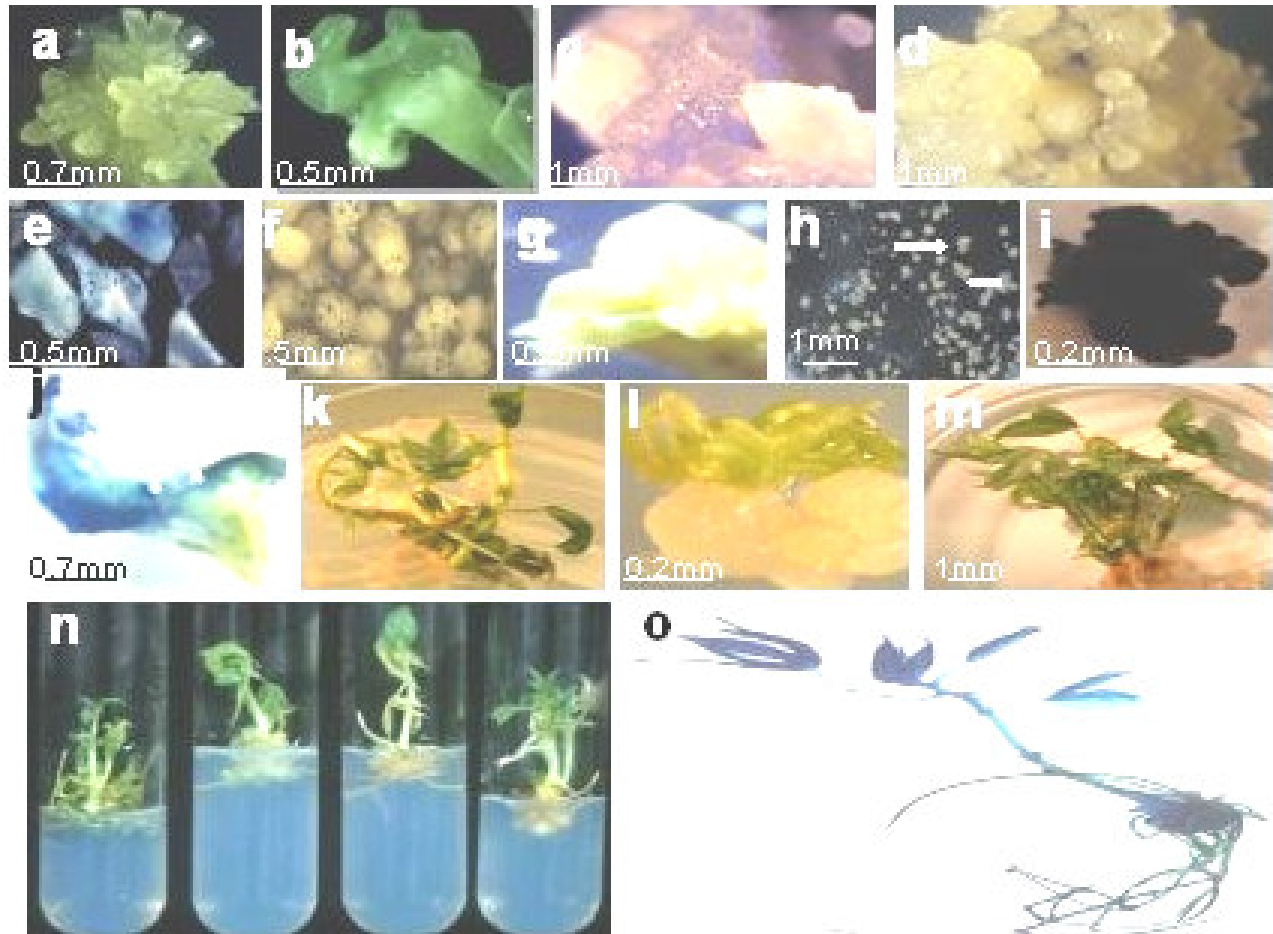


Figure 2. *In Vitro* regeneration of transgenic cassava. Organized embryogenic structures produced from leaf lobe and apical meristem explants (a); Isolated maturing somatic embryos with developed green cotyledons (b); Potential tissues for FEC formation (c); Pure and high quality FEC tissues (d); Cells expressing *uidA* visual marker gene in maturing somatic cotyledon fragment (e); Embryogenic units expressing *uidA* visual marker gene (f); Hygromycin-resistant shoot-buds from maturing somatic cotyledons cut edge (g); Hygromycin-resistant friable embryogenic callus (yellowish units) as arrow indicates (h); Embryogenic clusters (dark blue) expressing *uidA* visual marker gene (i); Maturing somatic embryos expressing *uidA* gene (j); germinated hygromycin-resistant maturing embryo (k); Direct shoot induction from hygromycin-resistant friable embryoids (l); Multiple hygromycin-resistant rooted plantlets from elongated shoots initiated from single friable embryoid (m); Hygromycin-resistant rooted plantlets from germinated maturing somatic embryos (n); Regmented rooted plantlets expressing *uidA* visual marker gene (o).

ious concentrations of hygromycin. Shoot bud regeneration frequency was assessed from cotyledons after one month of culture on cassava organogenesis medium (COM) supplemented with hygromycin up to 30 mg/l. Genotypes TME 127, TME 13, and TME 8 were found to be more sensitive to hygromycin toxicity, with shoot regeneration from cotyledon explants eliminated by antibiotic concentration as low as 5 mg/l. Tissues from these cultivars were killed outright at this level of toxic challenge. TME 282 and TME 203 were more resistant, requiring at least 15 mg/l and 20 mg/l hygromycin, respectively, to prevent shoot regeneration (Figure 3). FEC from 60444 was found to be significantly more resistant to hygromycin compared with cotyledon fragments from all cultivars tested except TME 203.

Growth of FEC was completely suppressed when the level of hygromycin in the medium reached 20 mg/l. These results suggest that hygromycin concentrations of between 4 and 15 mg/l might be adequate to select for putative transformants within the tested cassava genotypes and this concentration range was adopted for subsequent genetic transformation studies.

***Agrobacterium*-cassava compatibility screening**

Somatic cotyledon explants from five cassava genotypes TME 1, TME 13, TME 127, TME 8, TMS I 91/02327 and embryogenic units derived from FEC of 60444 were co-cultured with *Agrobacterium* strains LBA4404, GV3101,

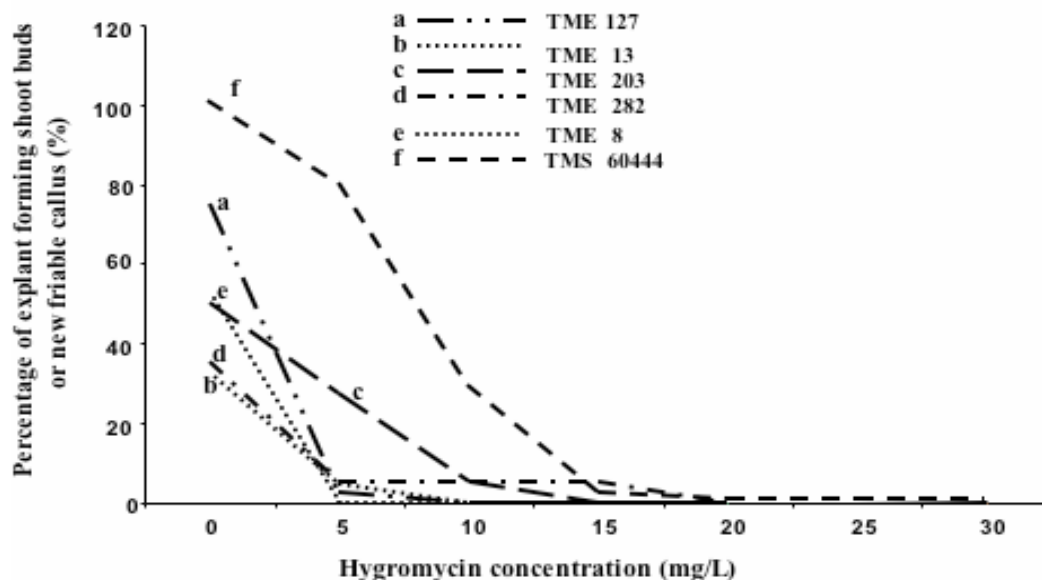


Figure 3. Hygromycin dose response curves for shoot buds, calli, and newly formed-embryogenic callus of cassava somatic cotyledons, and embryogenic callus cultured on COM and FEC-IM media containing hygromycin at various levels. Data are mean percentage of explant showing shoot buds or embryogenic cluster with new growths.

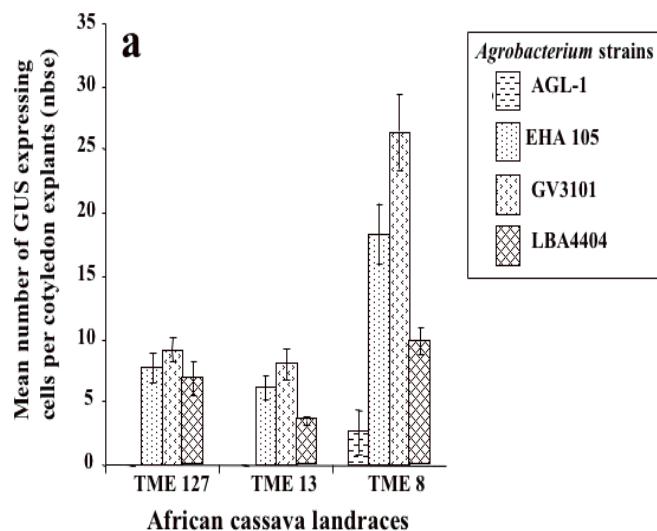


Figure 4a. Effects of four *Agrobacterium* strains and three African local cassava landraces on transient transformation efficiency in respect to mean number of GUS expressing cells per cotyledon explant (nbse) (a). EHA105, LBA4404, AGL-1, and GV3101 are *Agrobacterium* strains used for the co-cultivation. All values are mean \pm standard error.

EHA105, and AGL-1 and scored for the presence and number of GUS-expressing loci, two and four days after co-cultivation of embryogenic units and somatic cotyledon pieces, respectively. Data on GUS expression was collected for all cultivars but for TME 1 and TMS 91/

02327 data could not be statistically analyzed because of the limited number of maturing cotyledon pieces which were available in these cultivars. GUS-expressing cells were observed in all treatments indicating that both cotyledon tissues and FEC were amenable to transformation by all four *Agrobacterium* tested here. Although all cassava genotypes were susceptible to all four *Agrobacterium* strains, cotyledon pieces from TME 8 were more amenable to infection than other genotypes (Figure 2e). TME 8 also had the highest mean number of blue spots (nbse) per GUS positive explant (11.6), which was two- to four-fold higher than that recorded for TME 127 and TME 13. TME 13 and TME 127 did not significantly differ ($p \leq 0.05$) in their number of blue spots per explant. The highest number of GUS positive spots (14.52) per cotyledon explant was recorded with GV3101 and the lowest (1.42) with AGL1 in all tested cassava genotypes (Figure 4a), demonstrating that GV3101 showed a statistically significant greater efficiency of T-DNA transfer compared with EHA105, LBA4404, and AGL1. Importantly, AGL-1 could only infect TME 8 among all tested genotypes.

A fraction of the embryogenic suspension cells treated with LBA4404, GV3101, EHA105 were assayed for transient GUS expression after two days of co-cultivation. The percentage of GUS positive cell clusters was found to be *Agrobacterium* strain-dependant. All three *Agrobacterium* strains effectively infected and transformed the embryogenic cells (Figure 2f). As for the cotyledon explants, response of cassava FEC (derived from cultivar 60444) to T-DNA transfer significantly differ-

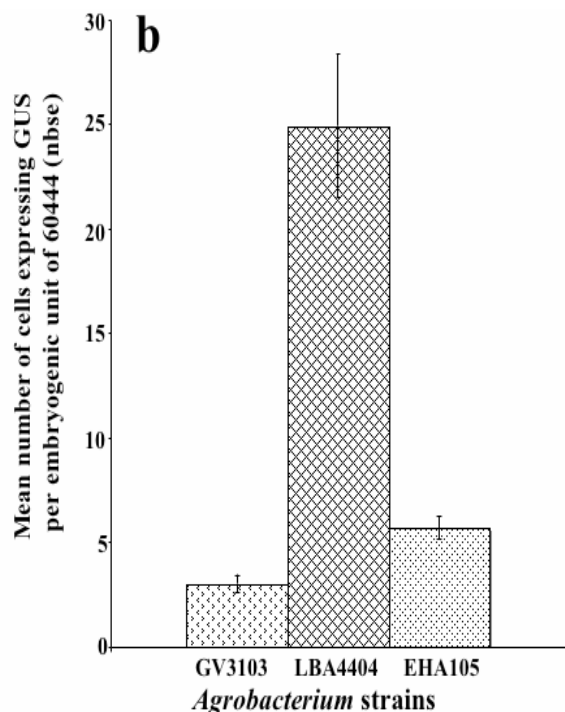


Figure 4b. Effect of three *Agrobacterium* strains on transformation frequency as assayed by number GUS expressing cells per embryogenic unit (nbse) of TMS I 60444. EHA105, LBA4404, and GV3101 are *Agrobacterium* strains used for the co-cultivation. All values are mean \pm standard error and the annotations for shading are similar as in Figure 4a.

ed between *Agrobacterium* strains. FEC co-cultured with strain LBA4404 yielded the most GUS-expressing foci. FEC derived from 60444 was observed, and evaluated based on the recorded number of blue spots per explant or embryoid (nbse). The increase in T-DNA transfer efficiency was approximately 8-fold greater in LBA4404 and 4-fold greater in EHA105, compared with GV3101 (Figure 4b). However, GUS expression was much easier to obtain from FEC than with somatic cotyledons. With somatic cotyledons, the combination of TME 8 with either GV3101 or EHA 105 appeared best for initiation of stable transformation because TME 8 had higher shoot regeneration capability than other previously tested cassava genotypes (Hankoua et al., 2005), and his response to infection was much better compare to most of the *Agrobacterium* strains. TMS I 91/02327 was also selected for stable transformation experiments since it showed a higher capability to regenerate than other improved genotypes tested (Hankoua et al., 2005) and was also found to be compatible with GV3101 and EHA105 (result not shown).

Production of stable transgenic tissues and plants

Co-cultured somatic cotyledons from maturing cyclic somatic embryos of TME 8 and TMS I 91/02327 were

transferred to CRM₁ supplemented with 4.5 mg/l hygromycin to regenerate transgenic shoots. After 4-5 weeks, regenerated putative transgenic shoot buds were transferred to CEM containing 20 mg/l hygromycin in order to induce elongation and full shoot development. However, these shoots (Figure 2g) failed to develop further in elongation medium (CEM) either at this, or higher levels of hygromycin-containing medium. A similar phenomenon was observed with shoot buds derived from cotyledon pieces of TME 1 co-cultured with *Agrobacterium* strain GV3101, indicating that these regenerated shoot buds (Figure 2g) were possibly escapes. The frequencies of such hygromycin resistant shoot buds or escapes varied from 3.4% and 6.8% (expressed per 100 co-cultured explants) with GV3101 and EHA105, respectively, for genotype TMS I 91/02327. Despite the recovery of resistant shoot buds at these frequencies, no transgenic plants were recovered using the cotyledon explants through shoot organogenesis-based cassava regeneration system.

After co-culturing embryogenic suspensions derived from FEC of 60444, they were first transferred to FEC-MU₁ for one week, to allow the co-cultured embryogenic cells to recover from the damaging effect of *Agrobacterium* during inoculation and co-cultivation. During this phase, *Agrobacterium* was eliminated from co-cultured embryogenic cells by addition of timentin at 500 mg/l. This was followed by the selection of hygromycin-resistant cell suspensions upon transfer of the cells into FEC-MU₂. When suspension cells were grown under this condition for two weeks, hygromycin-resistant suspension cells were established. These cells were selected, transfer-red to CRM₂, and cultured for 4-5 weeks by which time they had developed into hygromycin-resistant FEC lines. These FEC lines displayed yellowish tissues, similar to growth from control FEC grown under non-selective conditions (Figure 2h). Hygromycin-resistant FEC lines were obtained from the embryogenic cell suspensions co-cultured with all three *Agrobacterium* strains. Among the hygromycin-resistant FEC lines, some tested positive to GUS (Figure 2i) and some negative. Resistant-FEC lines developed into hygromycin-resistant cotyledon stage embryos four to five weeks after they were transferred to FEC-MM containing 20 mg/l hygromycin and 500 mg/l timentin. Some hygromycin-resistant maturing embryos were positive for GUS expression (Figure 2j). The mature embryos and putative transgenic embryoids were transferred to FEC-GE and COM media, respectively, without hygromycin for germination or direct shoot induction and to regenerate plants. After five weeks in FEC-GE, 50 maturing somatic embryo lines germinated into shoots (Figure 2k) and three out of ten embryoid clusters directly induced shoots (Figure 2l) after two week. These shoots were allowed to elongate on CEM. Out of these 53 total shoots regenerated, 29 grew into normal cassava plants showing vigorous root growth after four weeks in CEM (Figure 2m). The GUS pattern on

shoot apices, root and leaf explants obtained from the regenerated shoots (Figure 2n) as well as rooted plantlets (Figure 2o) indicated that they were all putative transgenics. Out of these 29 regenerants, 18 produced both blue stained and non-stained regions on the same tissues (root, leaf, petiole) when exposed to X-Gluc solution, indicating that they might have been chimeras. Out of these 11 stable transgenic lines, one originated from calli transformed with EHA105 (IITA-60444-1), three were from calli transformed with GV3101 and regenerated via direct shoot organogenesis (IITA-60444-SO₁, IITA-60444-SO₂, IITA-60444-SO₃), and seven were from calli transformed with GV3101 and regenerated via germination of hygromycin-resistant maturing embryos (IITA-60444-2, IITA-60444-3, IITA-60444-4, IITA-60444-10, IITA-60444-11, IITA-60444-12, IITA-60444-13).

Putative transgenic cassava plants obtained during this work were multiplied by *in vitro* cultures and molecularly characterized by PCR and Southern blot analysis to confirm the integration and copy number of the transgene. Using *hpt* gene primers, PCR tests amplified a 628 bp internal fragment from total genomic DNA isolated from GUS- positive plants (IITA-60444-1, IITA-60444-2, IITA-60444-3, IITA-60444-4, IITA-60444-10, IITA-60444-11, IITA-60444-12, IITA-60444-13, IITA-60444-SO₁, IITA-60444-SO₂, IITA-60444-SO₃) generated using EHA105 (pCAMBIA1301) and GV3101 (pCAMBIA1301). This indicated that the presence of the transgene in these cassava plant lines. Based on the PCR result, genomic DNA from some of the representative transgenic lines (including lines derived via direct shoot organogenesis of putatively transformed FEC and FEC co-cultivated with EHA105) were subjected to southern blot (Figure 5) to confirm transgene integration and determine the copy number of *hpt* gene. Southern blotting revealed that all transgenic lines tested had a single band except line IITA-60444-10 that showed two bands, thereby establishing that integration events were mostly single insertions.

DISCUSSION

Successful genetic transformation of cassava through FEC and somatic cotyledon-based transformation systems as described in this study depends initially on the production of organized embryogenic structures (OES), the capability of OES to convert into maturing somatic embryos, and the ability of OES to induce and proliferate into pure FEC (Taylor et al., 2004). The competence and variability of the cassava tested in this study to form OES and to convert into maturing somatic embryo was already reported (Hankoua et al., 2005). Pure FEC of 60444 was established in this study in the manner previously described by Taylor et al. (2001). The genotypic effect on the FEC production of cassava genotypes tested in this study was also observed recently in the FEC production of a range of West African (Hankoua et al., unpublished data), East-African, and Asian cassava genotypes (Taylor et al., 1997; Taylor et

al., 2001; Raemaker et al., 2001). This genotypic effect was recently recognized as one of the major limitations of the application of embryogenic suspension cultures in genetic transformation of cassava (Taylor et al., 1997; Fregene and Puonti-Kearlas, 2002; Hankoua, 2003). These observations demonstrate that there might be an underlying genetic control in the capability of a given genotype to induce, and then proliferate FEC lines. As observed by Taylor et al. (1997) on the timing of FEC establishment of various genotypes, it is probable that TME 1 and TMS 91/02327 needed more striker selection of highly competent FEC cell lines over a long period of time to produce high quality and proliferating FEC. Indeed, the establishment of pure FEC lines from these two (TME 1 and TMS 91/02327) popularly grown West-African cassava genotypes will be critical in initiating their genetic improvement through transgenic approaches. System improvement such as the supplementation of FEC-IM with tyrosine could be extremely beneficial in enhancing the FEC induction and proliferation capability of TME 13, TME 12, and TMS I 91/02324 (Hankoua and Taylor, unpublished). Antibiotics are used to select and recover transgenic tissues and whole plants after transgene integration. Optimum concentration of the selective agent should be the one that prevents regeneration of non-transformed cells/tissues without being toxic to the target explant. In order to determine effective levels of hygromycin for recovery of cassava tissues genetically transformed with the *hpt* selectable marker gene, cotyledon explants and FEC were screened to determine their sensitivity to this toxic agent. The ability of 15 mg/l hygromycin to block regeneration of adventitious shoots from somatic cotyledons is in agreement with the results of Li et al. (1996) although adventitious shoot formation were blocked at hygromycin as low as 5 mg/l for some cassava genotypes tested in this study. These observations confirmed the view that genotypes differ in their natural background resistance to antibiotics (Irwin et al., 1999). Likewise effective inhibition in regeneration of new FEC lines from embryogenic suspension of 60444 at 15 mg/l hygromycin is in agreement with the work of Zhang et al. (2000). Based on the observations that the main focus on identifying right selective antibiotic concentration in recovering transgenic events should be to balance the selection requirement against the inhibitory effects of the selective antibiotic on shoot development, our present results suggest that adequate selection of putative transformed shoots for most of the cassava genotypes tested and for FEC tissues of 60444 should be done at hygromycin concentration lying between 4.5 and 15 mg/l. In such situation, the probability that transformed cells would enter embryogenesis or shoot regeneration pathways might be higher while recovery of escapes will be limited.

Determining optimal compatibility of different strains of *Agrobacterium* with different plant species and explant types has been of great importance in successful recove-

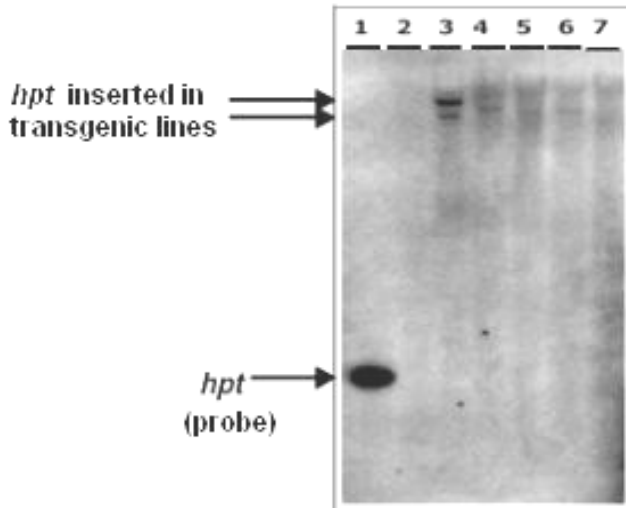


Figure 5. southern blot of genomic DNA of cassava plants transformed with GV3101 (pCambia 1301) and EHA 105 (pCambia 1301). Lane 1: pCambia 1301 plasmid DNA restricted with XhoI; Lane 2: DNA from untransformed control plant restricted with aatII; Lane 3 (IITA-60444-10, IITA-60444-12, IITA-60444-S03, IITA-60444-S01, IITA-60444-S01, IITA-60444-1): Genomic DNA from transgenic cassava was digested with aatII (cuts within the T-DNA but at one end of *hpt* gene). DNA fragments of various sizes containing the *hpt* coding sequence are released. The blot was probed with 1.1kb *hpt* gene fragment.

ry of transgenic tissues and plants (Sangwan et al., 1992; Li et al., 1992; Jin et al., 2005). We therefore evaluated the influence of four commonly used *Agrobacterium* vir helper strains on six cassava genotypes for transient expression of *uidA* (GUS) gene, establishment of hygromycin resistant tissues and regeneration of putative and stable transgenic plants.

In this study a compatibility test between four African cassava landraces, an improved variety and the model genotype 60444, and four commonly used disarmed *Agrobacterium* strains revealed genotype-dependent response on the efficiency of the T-DNA transfer as determined by the expression of *uidA* (GUS) marker gene. Previous reports describing cassava transformation have shown that the *Agrobacterium* strain LBA4404 was suitable for the transformation of the cassava genotype MCol 22 (Li et al., 1996) due to the presence of duplicated Vir D region on the co-integrated plasmid pTOK233 of LBA4404 that enhanced the virulence of such *Agrobacterium* strains in cassava (Li et al., 1996) and rice (Hiei et al., 1994). In embryogenic callus of cotton, LBA4404 was also shown to be more efficient in producing transformants than C58C3 (Jin et al., 2005). In our study, LBA4404 harbors pCambia1301 instead of p Δ gusbin19 in the work of Jin et al. (2005). This demonstrate that the superiority of LBA4404 in infecting both cassava and cotton tissues is independent of the binary plasmid type, but associated to the nature of the genome of LBA4404. Our study shows that the efficiency

of T-DNA transfer for somatic cotyledon pieces, FEC of cassava genotypes, and *Agrobacterium* strains is dependent on the i) *Agrobacterium* vir helper strains (LBA4404, GV3101, AGL-1 and EHA105), ii) cassava genotypes, and iii) cassava tissue types such as cotyledons from maturing somatic embryos or embryogenic calli from organized embryogenic tissues. This ability of different *Agrobacterium* strains to preferentially transfer their T-DNA in specific plant and tissue types is in agreement with previous findings (Jin et al., 2005). In rice transformation, the frequency of GUS expression was found to be higher in Indica than Japonica rice variety (Li et al., 1992), with leaf explants from both rice varieties tested found to be more accessible to *Agrobacterium* than roots and seed remnants.

Surprisingly, strain EHA105, a derivative of A281 previously shown not to be suitable for cassava transformation (Li et al., 1996), was highly virulent with the cassava genotypes used in this study. These observations clearly demonstrate that in cassava, the virulence of the four *Agrobacterium* strains varied based on the *Agrobacterium* strain, cassava genotype, tissue type, and the T-DNA transfer ability of the binary or co-integrated resident plasmids present in the disarmed *Agrobacterium* vectors as previously demonstrated in *Chrysanthemum morifolium* (Bush and Pueppke, 1991).

Stable transformation studies were conducted between the landrace TME 8 and *Agrobacterium* strain GV3101 and also between an improved genotype TMS I 91/02327 with both GV3101 and EHA 105 strains. TMS I 91/02327 and TME 8 were selected for stable transformation because of their high regeneration potential (shoot bud regeneration) compared with all the cassava genotypes tested (Hankoua et al., 2005). No transgenic plants could be recovered from co-cultured cotyledon explants despite the production of hygromycin-resistant shoot buds in regeneration medium. One of the constraints encountered with this genetic transformation protocol was the overgrowth of *Agrobacterium*. This was mainly due to the inability of carbenicillin to control *Agrobacterium*, especially EHA105. Replacing carbenicillin with timetin at 500 mg/l resulted in effective suppression of GV3101 and EHA 105 growth on co-cultivated explants in the selection medium. Cefotaxime was found to be the most effective antibiotic for controlling *Agrobacterium* strain LBA4404 and moxalactam the most effective against EHA101 (Cheng et al., 1998).

Several reasons could account for the difficulties in recovering transgenic tissues of TME 8 and TMS 91/02327 from co-cultivated maturing cotyledons. One of the fundamental requirements for the co-cultivated cotyledon pieces to develop hygromycin resistant shoots is that many transformable cells along the somatic cotyledon cut edges be regenerable. It was speculated that competent cells for regeneration of cassava were located on the cut edges of cotyledons pieces (Li et al., 1998), but no microscopy studies have been documented

supporting this view. Failure to regenerate a single transgenic shoot from various cassava genotypes after successful infection of cotyledon pieces with *Agrobacterium* could be explained by the fact that manifestations of regeneration and transformation does not overlap possibly in the cotyledons of co-cultivated cassava genotypes tested. Thus, cells competent for organogenesis were not necessarily those competent for transformation by *Agrobacterium* and vice-versa as has been demonstrated in *Agrobacterium*-mediated transformation of *Brassica* (Mukhopadhyay et al., 1992), *Dendrothema grandifolia* (Lowe et al., 1993), sugar beet (D'Halluin et al., 1992), *Arachis hypogaea* (Mansur et al., 1997), and *Capsicum annum* (Wolf et al., 2001). Therefore, it is likely that in cassava genotype MCol.22 successfully transformed by *Agrobacterium* via shoot organogenesis of cotyledon pieces (Li et al., 1996), a particular cell type was both competent for transformation and for shoot regeneration. Detailed anatomical studies of somatic cotyledon pieces during different phases of organogenesis of cassava are required to address these issues. Such studies had proved beneficial in identifying competent cells for genetic transformation of *Arabidopsis thaliana* (Sangwan et al., 1992). The inserted *hpt* gene may have been silenced by methylation (Van slogteren et al., 1984). Other factors include metabolites such as ethylene induced by wounding of the somatic cotyledon explants may have interfered with the transformation process, and also bacteria-host cell interaction known to block competence to *Agrobacterium* (Mukhopadhyay et al., 1992). Another important factor is the possibility that some of these cassava genotypes tested may be *rat* mutants, therefore set of plant genes (*rat*) known to be involved in *Agrobacterium*-mediated transformation might not functioning well in these genotypes (Gelvin, 2003). Presence of timentin in the regeneration medium may also have suppressed shoot development, however no negative effect on the shoot regeneration was reported when this antibiotic was used in culture systems of *Nicotiana tabacum* and *Ulmus pumila* (Cheng et al., 1998) as observed in this study. The ability to combine the hygromycin-based selection and *Agrobacterium*-mediated mode of transformation to efficiently regenerate putative transgenic cassava plants contrasts with a previous report that hygromycin was inefficient for the selection of cassava suspensions (Schopke et al., 1996). The results of this study demonstrate that hygromycin selection can be used as well for the recovery of transgenic cassava tissues and plants, and is as efficient as paramomycin and phosphinothricin selection (Gonzales et al., 1998; Munyikwa et al., 1998). Although LBA4404 was shown in the compatibility test to be more efficient than EHA105 and GV3101 for T-DNA transfer, the regeneration of putatively transformed plants from FEC was obtained mainly with EHA105 and GV3101 due to loss of LBA4404 cultures through bacteria contaminations. Modifications of the *Agrobacterium*-mediated trans-

formation of FEC, as described by Gonzales et al. (1998), and used in this study, reduced the time taken to obtain putative transformed shoots by 4 weeks. This is because of the elimination of the embryo differentiation steps, thereby reducing the time necessary to produce transformed shoots from 30 to 26 weeks when starting with embryogenic suspensions inoculated with either GV3101 or EHA105.

Combining production of transgenic FECs with plant regeneration from this tissue via an organogenic system compared to multi-step embryo maturation and germination process (Gonzales et al., 1998; Shreuder et al., 2001; Taylor et al., 2001) also contributes to shorten the time to recovery of genetically transformed plants to 22 weeks. In this study, embryoids used for direct shoot regeneration were putatively transgenic. This approach is novel and it offers the possibility to induce shoot directly from embryoids during the selection phase thereby minimizing the time required to regenerate genetically transformed cassava. The reduction of this time course for recovering transgenic cassava plants by 4 and 8 weeks via elimination of embryo differentiation step and direct shoot regeneration of transgenic embryoids, respectively, could contribute in the elimination of the risk of somaclonal variation shown to arise usually from a long FEC growth cycle during either establishment of proliferating FEC tissues or selection of transgenic FEC lines (Raemaker et al., 2001). In addition, this approach also offers the possibility to induce and select for putative transgenic shoots directly after co-cultivation of embryogenic suspension, an avenue that need to be explored in further studies. The regeneration of multiple independent transgenic shoots/events from a single transgenic embryoid is one of the most important advantages of this system over the embryo germination method. This shoots regeneration ability of single embryoid open many new avenues in commercial exploitation of cassava transgenic technology for agricultural development in Africa. Developing of a transgenic system with the ability to produce large number of independent transgenic events over a short period of time is a crucial component in achieving a commercial goal of any transgenic programs. Interestingly, putative transformed shoots and rooted plants were obtained with *Agrobacterium* strain EHA105, therefore identifying another *Agrobacterium* vir helper strains suitable for recovery of transgenic cassava plants. This vector, although known to be a suitable vector for the transformation of several plant species (Hood et al., 1986), was reported not to be a suitable vector for cassava transformation (Li et al., 1996).

This study describes the first genetically transformed cassava plants to be produced on the African continent and it is a result of collaboration process involving IITA Nigeria, ETH Switzerland, the Danforth Plant Science Center, USA, and the University of Ibadan, Nigeria. The scientific approach followed in this report will serve as model for future testing of the capability of other agrono-

mical important cassava genotypes to be transformed in Africa. Outside of South Africa (Makwarela et al., 2004), capacities for the production of transgenic crop plants remain significantly underdeveloped. We consider that the present report represents an important step towards building an endogenous capacity for transgenic technologies in Sub-Saharan Africa. This report also acts as an important beginning for the use of transgenic technologies to address cassava production constraints in Africa such as disease resistance, starch modification, nutritional enhancement and non-food genetically modified cassava for enhancement of biofuel and biodiesel production. Major technological challenges remain to transfer this technology to NARS in SSA where it will be used to tackle cassava production constrain specific to each region or country.

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