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ORIGINAL PAPER

Improvements in *Agrobacterium*-mediated transformation of cassava (*Manihot esculenta* Crantz) for large-scale production of transgenic plants

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Abstract Cassava (*Manihot esculenta* Crantz) is a major staple food crop of the humid tropics. As a heterozygous, vegetatively propagated crop, robust transformation protocols must be developed for elite cultivars that allow predictable production of large numbers of independent transgenic plant lines. A high throughput *Agrobacterium*-mediated transformation system was developed for the elite East African farmer-preferred cassava cultivar TME 204 using the GFP visual marker gene. Inclusion of the antibiotic moxalactam in culture medium used to produce embryogenic target tissues prior to inoculation with *Agrobacterium* increased recovery of independent GFP-expressing transgenic callus lines by up to 113-fold compared to the control. Enhanced transformation was also observed when TME 204 tissues were pretreated with other cephalosporins, namely cefoperazone, cefoxitin, cefmetazole and cefotaxime. Similar but less dramatic increases in transformation efficiencies were seen for the West African cultivars Oko-iyawo and 60444 when pre-treated with moxalactam. Dilution of *Agrobacterium* suspensions used for co-culture was found to increase transformation efficiencies, resulting in regeneration at an average of 33 GFP-expressing TME 204 plants per cc settled cell volume at OD_{600} 0.05, compared to 15 plants at the more commonly used OD_{600} 0.5. The optimized transformation systems were successfully utilized for the integration of genetic constructs for disease resistance and nutritional enhancement into more than 750 plants of TME 204.

Keywords Cassava · Friable embryogenic callus · Genetic transformation · Moxalactam · Oko-iyawo · TME 204

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Introduction

Cassava (*Manihot esculenta* Crantz), a perennial woody shrub, is a major staple food crop of the humid tropics. Grown for its large starchy roots, cassava is the third most important source of dietary calories for people living in the tropics after rice and maize (FAO 2008). In addition to human consumption, cassava is becoming an increasingly important industrial crop. It is extensively utilized for animal feed (Nwokoro et al. 2002); in the starch (Marcon et al. 2007; Srinivas 2007), food processing and chemical (Balagopalan 2002) industries; and for paper products (Mohd Aripin et al. 2013), textiles, cosmetics and pharmaceuticals. Efforts to utilize cassava as a source of biofuels is also increasing in countries such as Nigeria (Ademiluyi and Mepba 2013) and Uganda (Nuwamanya et al. 2012). While tolerant to drought and poor soils, and well suited to the production methods of small-holder farmers, cassava production in Africa suffers from losses to pests and pathogens, rapid post-harvest deterioration and poor nutritional content of the storage root (Sayre et al. 2011). Transgenic technologies are being applied to cassava to bring biotechnological solutions to these problems (Liu et al. 2011; Taylor et al. 2004; Yadav et al. 2011; Zhang et al. 2003, 2005). Studies on introduction of resistance to viral diseases of cassava (Ogwok et al. 2012; Yadav et al. 2011) through genetic engineering have

already been conducted. Attempts to improve the nutritional qualities are underway (Sayre et al. 2011).

The successful implementation of crop biotechnology is dependent on the capability to generate large numbers of transgenic plants from the targeted germplasm. Three *Agrobacterium*-mediated transformation systems have been reported in cassava, namely via friable embryogenic callus (FEC) (Bull et al. 2009; Schreuder et al. 2001; Taylor et al. 2012; Zhang et al. 2003), organogenesis from cotyledons of somatic embryos (Jorgensen et al. 2005; Li et al. 1996) and regeneration from organized embryogenic structures (Msikita et al. 2006). The target tissues for all methods are embryogenic tissues generated from immature leaf lobe or axillary bud explants excised from in vitro shoot cultures. The most commonly employed transformation method is the FEC system because of the relatively high transformation efficiency and low occurrence of chimeras. As for other plant species, response to tissue culture and genetic transformation systems varies significantly across different cultivars of cassava. Important progress has been reported recently for expansion of transformation capacity out of the model cv. 60444 (Bull et al. 2009; Taylor et al. 2012; Zhang et al. 2003) and into several cultivars with economic importance in East and West Africa (Nyaboga et al. 2013; Zainuddin et al. 2012). In our laboratory, focus has been on the major virus diseases impacting cassava in Africa. These include cassava mosaic disease (CMD) and cassava brown streak disease (CBSD), the two most important biotic constraints to cassava production in Sub-Saharan Africa (Legg et al. 2011). In an effort to develop resistant germplasm for delivery to farmers in East Africa, transgenic RNAi technologies are being utilized to integrate resistance to these viral diseases into farmer-preferred cultivars (Taylor et al. 2012). Most advanced is a pathogen-derived RNAi strategy against CBSD, for which proof of concept in the model cv. 60444 has been successfully demonstrated under field conditions in Uganda (Odipio et al. 2014; Ogwok et al. 2012). Because cassava is a heterozygous species with strong inbreeding depression (Ceballos et al. 2004), the challenge is now to integrate this technology directly into cassava cultivars favored by farmers in East Africa.

Within the Virus Resistant Cassava for Africa (VIRCA) project (Taylor et al. 2012), the Ugandan cultivar TME 204 has been selected for transgenic integration of resistance to CBSD. This necessitates the development of robust transformation protocols for this cultivar at a level where the predictable production of large numbers of independent plant lines can be achieved on a routine basis. We report here development of such a system for TME 204, utilizing important modifications to the method described for cv. 60444 (Taylor et al. 2012). Most notably, inclusion of cephalosporin class, β-lactam antibiotics in the medium, in combination with reduced *Agrobacterium* cell densities at co-culture, breaks recalcitrance and results in a greater than 100-fold increase in transformation efficiency. The resulting culture system has been employed to produce large-scale recovery of transgenic plant lines for the development of CBSD- resistant and nutritionally enhanced cassava.

Materials and methods

Media composition and culture conditions

All media components, antibiotics, growth regulators and additives were obtained from Sigma (St. Louis, MO, USA) unless specified. 6-benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA) were added to the media before autoclaving, while picloram, acetosyringone, tyrosine and all antibiotics were filter sterilized and added after autoclaving. The pH of picloram and tyrosine stock solutions was adjusted to 5.8 prior to addition to the media. Compositions of media used in this study followed those of Taylor et al. (2012) for 60444 cultivar and with some modification for TME 204 and Oko-iyawo (TME 7). Generation of organized embryogenic structures (OES) took place on media consisting of DKW/Juglans basal salts (Driver and Kuniyuki 1984) (*Phyto*Technology Laboratories, Kansas, USA) plus Murashige and Skoog (MS) (Murashige and Skoog 1962) vitamins supplemented with 2 % w/v sucrose and 50 μM picloram (DKW 50P). Tyrosine at 250 μM and 500 μM was added to Gresshoff and Doy (Gresshoff and Doy 1974) basal medium supplemented with 2 % w/v sucrose and 50 μM picloram (GD2 50P) for generation of FEC in TME 204 and Oko-iyawo (TME 7), respectively. After co-culture, *Agrobacterium* was controlled by addition of 125 mg/l cefotaxime or 150 mg/l carbenicillin to GD2 50P based medium containing 27.5 μM paromomycin for selection of transgenic FEC colonies, followed by 30 mg/l timentin added to Stage 1 regeneration medium (MS supplemented with 2 % w/v sucrose (MS2), 5 μM NAA and 45 μM paromomycin). All cultures were incubated at 28 ± 1 °C with 16 h illumination provided by fluorescent lamps at 75 μmol m^{-2} s^{-1} unless specified.

Plant material and gene constructs

Stem cuttings of cassava cultivar TME 204 from the National Crops Resources Research Institute (NaCRRI), Uganda; and 60444 and Oko-iyawo (TME 7) from the International Institute for Tropical Agriculture (IITA), Nigeria, were imported to the Donald Danforth Plant Science Center (DDPSC), St. Louis. Stakes were established in pots containing Fafard 51 growing mixture in a

quarantine growth chamber at 28 ± 1 °C day/25 ± 1 °C night temperature and a 14 h light/10 h dark photoperiod (400 µmol m^{-2} s^{-1} irradiance). Axillary buds produced from new stem growth were excised and surface sterilized with 15 % v/v sodium hypochlorite for 30 min, washed three times with sterile water and cultured on MS2 medium solidified with 2.2 g/l phytagel. Approximately 3 weeks later, apical shoots were excised and subcultured onto MS2 medium supplemented with 8 g/l of Noble agar (MS2 Agar) and maintained as described by Taylor et al. (2012).

Agrobacterium tumefaciens strain LBA4404 harboring pCAMBIA2300-based binary vectors p5001, p8001 and pCAMBIA2300-*EGFP* (Fig. 1) were used for transformation. The enhanced green fluorescent protein gene (*eGFP)* in pCAMBIA2300-*eGFP* construct (Taylor et al. 2012) was under control of the Cauliflower mosaic virus (CaMV) 35S promoter. The p5001 construct consisted of an inverted repeat of coat protein gene fusion derived from *Ugandan cassava brown streak virus* (UCBSV) and *Cassava brown streak virus* (CBSV) driven by the *Cassava vein mosaic virus* (CsVMV) promoter. p8001 carried the same inverted repeat cassette plus expression cassettes of phytoene synthase (*crtB*) and 1-deoxy-D-xylulose 5-phosphate synthase (*DXS*), from *Pantoea ananas* (formerly *Erwinia uredovora*) and *Arabidopsis thaliana* (Fregene et al. 2010), both under the control of the patatin promoter (Fig. 1) (Sayre et al. 2011).

Production of target tissues for transformation

Embryogenic callus used as target tissues for genetic transformation of cv. 60444 was generated following the methods described by Taylor et al. (2012), with some modifications for production of these tissues from cvs. TME 204 and Oko-iyawo (TME 7). For the latter, immature leaf lobes were excised from 4 to 6 week-old micropropagated shoot cultures, placed on DKW 50P and incubated in the dark at 28 °C for 4 weeks for induction of OES. OES obtained was excised from the explants using fine forceps and forced through a 1 mm wire mesh. Seven to ten resulting tissue fragments were placed together to form a group. Five to six such groups were cultured in each Petri dish containing GD2 50P media supplemented with tyrosine and incubated in the dark for 5 days. After 5 days, tissues were then transferred to fresh medium of the same type and cultured in the dark for a further three to 4 weeks. Small groups of FEC formed after this time were carefully separated from the non-embryogenic tissue using fine forceps and subcultured onto fresh GD2 50P of the same type. After a further 21 days, FEC was transferred to same media with or without various types and concentrations of cephalosporin antibiotics prior to co-culture with *Agrobacterium*.

Transformation and plant regeneration

Agrobacterium suspensions were prepared as described by Taylor et al. (2012). To study the effect of dilution of *Agrobacterium* cell density on transformation efficiency, suspensions were grown to OD_{600} 0.5 and then diluted by adding GD2 50P liquid media containing 200 µM acetosyringone to attain ODs of 0.05 and 0.025.

FEC obtained after 18–20 days culture on a third 21-day cycle on GD2 50P media supplemented with tyrosine and moxalactam or the antibiotics cefotaxime, cefoperazone, cefoxitin or cefmetazole was used as target tissue for transformation. FECs were placed at 0.5–0.8 cc settled cell volume (SCV) in 12-well plates, and each sample inoculated with 2 ml of *Agrobacterium* suspension for 45 min. After inoculation, the FEC was transferred to

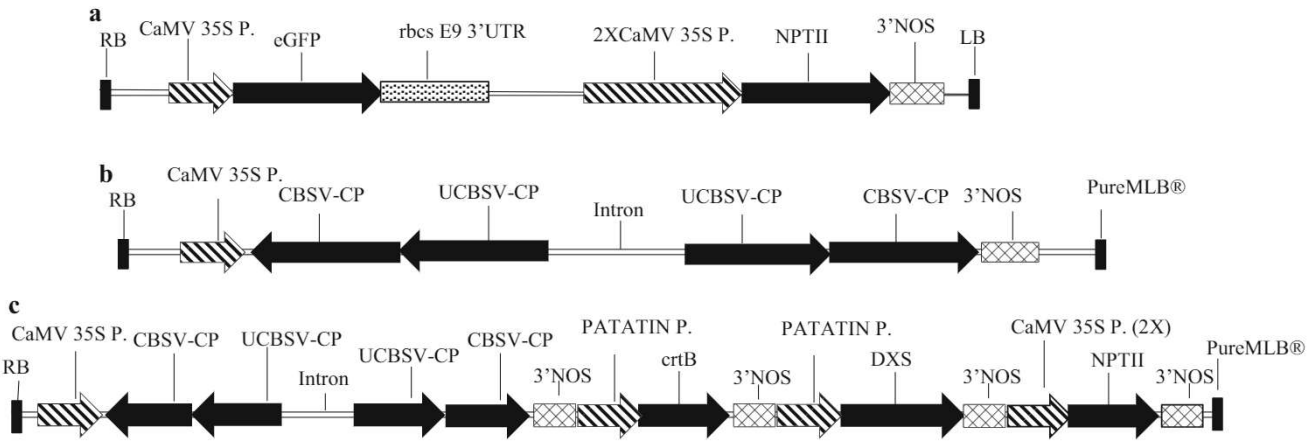

Fig. 1 Diagrammatic representation of the T-DNA of *eGFP* (**a**); p5001 (**b**) and p8001 (**c**)

sterile nylon mesh (110 μm pore size), placed on co-culture media containing acetosyringone and incubated at 22 °C under 76 μmol m^{-2} s^{-1} irradiance for 3–4 days. After co-culture FEC samples were transferred to graduated tubes and washed with liquid GD2 50P containing 125 mg/l cefotaxime for TME 204 and Oko-iyawo or 150 mg/l carbenicillin for 60444. Settled cell volume (SCV) was determined before transferring the FEC onto new sterile nylon mesh and placing on GD2 50P supplemented with 125 mg/l cefotaxime or 150 mg/l carbenicillin, depending on the cultivar. Ten days later, tissues were re-suspended by vortexing with liquid GD2 50P containing 125 mg/l cefotaxime or 150 mg/l carbenicillin and spread evenly across the surface of Petri dishes containing GD2 50P supplemented with 125 mg/l cefotaxime or 150 mg/l carbenicillin and 27.5 μM paromomycin, and cultured in low light (20–25 μmol m^{-2} s^{-1} irradiance). Twenty-one days later, actively growing units of FEC were subcultured sequentially to embryo regeneration (Stage 1 medium, MS2 supplemented with 5 μM NAA and 30 mg/l timentin for TME 204 and Oko-iyawo or 150 mg/l carbenicillin for 60444 and 45 μM paromomycin) for 21 days followed by embryo maturation media (Stage 2, MS2 supplemented with 0.5 μM NAA and 45 μM paromomycin). Shoots were recovered on MS2 containing 2 μM BAP and maintained on MS2 Agar. Micropropagated plants were planted in Fafard 51 mixture and established in the greenhouse (Taylor et al. 2012).

Assessment of *Agrobacterium*-mediated transformation efficiency

Tissues were scored for GFP expression at multiple stages after co-culture with *Agrobacterium* using a Nikon C15304 dissecting microscope equipped with an excitation filter of 460–500 nm and barrier filter 510 LP. Transient transformation was assessed by scoring each FEC sample at the end of the co-culture period using a scale of 0–5 where the number of visible GFP-expressing cells was: 0 = no visible signal; 1 = 1–10 GFP-expressing cells; 2 = 11–50 cells; 3 = 51–100 cells; 4 = 101–500 cells; and 5 = greater than 500 GFP-expressing cells visible. GFP expression was scored again 10 days after subculture onto selection-free medium (GD2 50P supplemented with 125 mg/l cefotaxime or 150 mg/l carbenicillin paromomycin). Each sample was assessed for the number of visible GFP-expressing cells on the 0–5 scale as described above. Lastly, efficiency of stable transformation was determined after 21 days culture on selection medium containing 27.5 μM paromomycin by counting the number of individual dividing FEC units seen to be expressing GFP. The numbers of GFP-positive cotyledon stage and rooted plantlet stage lines obtained were recorded at the end of embryo maturation (Stage 2) and rooting (MS2 Agar) stages, respectively.

T-DNA copy number determination

Genomic DNA was isolated from young in vitro leaves using DNeasy plant mini kit (Qiagen, Hilden, Germany), and copy numbers were estimated by dot blot analysis (Sambrook and Russell 2001), as described by Taylor et al. (2012). For Southern blot analysis, high molecular weight genomic DNA was extracted from 1 to 2 g of young leaves obtained from 4 to 6 week-old greenhouse-grown plants using the CTAB protocol (Doyle and Doyle 1990). Extracted DNA was isopropanol precipitated, washed in 70 % ethanol and RNase treated using 5 μg/μl unit of DNase free RNAseA according to the manufacturer's instructions (Roche, Indianapolis, USA). Genomic DNA (20 μg) from each sample was digested with 80 units of NcoI (New England Biolabs) overnight and subjected to electrophoresis on a 1 % agarose gel. The fractionated DNA was transferred to a positively charged nylon membrane using downward capillary transfer in 0.4 M NaOH buffer (Sambrook and Russell 2001).

Membranes for dot blots were hybridized to a DIG-labeled (Roche, Indianapolis, USA) probe generated by PCR amplification of a 430 bp fragment or *eGFP* for analysis of GFP transgenic events or a 323 bp fragment of the CaMV 35S promoter for plants transgenic for p5001 and p8001. For Southern blot analysis of p5001 transgenic plant lines, a 406 bp fragment of UCBSV CP gene was used (Table 1). Hybridization of membrane-bound DNA to DIG-labeled probes followed by washing and detection using CDP-Star (Roche, Indianapolis, USA) was performed per manufacturer instructions.

Experimental design and statistical analysis

Each experiment was performed three times and data generated subjected to ANOVA to determine significant differences between the treatments and, where appropriate, mean separation was done with Dunnett or Turkey test. Both ANOVA and mean separation were done using Minitab 17 statistical software package (Minitab Inc. State College, PA).

Results

Agrobacterium-mediated transformation was evaluated using the green fluorescent (GFP) visual reporter to assess transformation efficiencies at various stages from transient expression a few days after co-culture to recovery of transgenic FEC and whole plants.

Table 1 Primers used to amplify and label the probes used for dot blot and Southern blot analysis

Primer	Primer sequence (5′–3′)	Amplicon size (bp)	Purpose	Hybridization temp. (°C)
eGFP F	AACTCAGTAGGATTCTGGTGTGTGCG	430	Dot blot	40
eGFP R	CGGCCACAAGTTGGAATACAAC			
35S F	CACATCAATCCACTTGCTTTGAAG	323	Dot blot	40
35S R	CATGGTGGAGCACGACACT			
5001 F	CAAACGGACAAAGGAAAAGC	406	Southern blot	55
5001 R	CTTGAATGGTGACTCCATAAGC			

Effect of cephalosporin antibiotics on transformation

Observations taken during experiments to test the efficacy of various antibiotics for controlling growth of *Agrobacterium* indicated that FEC tissues cultured on medium supplemented with moxalactam at 25–100 mg/l showed enhanced vigor while not having the ability to suppress growth of the bacterium (data not shown). In order to study the effect of moxalactam on transformation efficiency of TME 204, FEC tissues were cultured on GD2 50P medium supplemented with 250 µM tyrosine plus 25, 50 or 100 mg/l moxalactam for 18-20 days prior to co-culture with *Agrobacterium* at an OD_{600} of 0.05. Tissues were scored for the number of GFP-expressing cells seen after three to 4 days of co-culture and after 21 days on selection media (27.5 µM paramomycin). Culture of cells on medium containing moxalactam was correlated with a significant ($p < 0.001$) increase in the number of cells transiently expressing visible GFP at the end of the co-culture period. In the control, to which no antibiotic was added prior to co-culture with *Agrobacterium*, an average score of 0.4 GFP (scale 0–5) was recorded after 4 days of co-culture, but increased significantly to reach levels of 1.4, 3.4, and 3.9 upon addition of 25, 50 and 100 mg/l moxalactam, respectively (Figs. 2a, 3, 4a). The average number of GFP-expressing callus lines observed after 21 days culture on selection medium was also significantly enhanced when moxalactam was included in the medium prior to *Agrobacterium* co-culture, increasing from an average of 2 in control to 45, 129 and 230 in FEC pre-treated with 25, 50 and 200 mg/l of the antibiotic, respectively (Fig. 2b). The addition of moxalactam to the final subculture cycle therefore increased recovery of stably transformed, GFP-expressing FEC lines by up to 113-fold compared to the control tissues which saw no such pre-treatment (Fig. 2b). As evident from Tables 2 and 3, the beneficial effects of moxalactam were maintained through all subsequent steps of selection and recovery of transgenic tissues and plants, such that between 22 and 30 % of the GFP-expressing callus lines subcultured onto Stage 1 regenerated into robust rooted plantlets (Table 3; Fig. 4). While more than 65 % of the callus lines that moved to Stage 1 matured to form cotyledon stage embryos, only 40 % of later germinated to form plantlets. The germination rate increased upon transferring the embryos to fresh media 2-times every 14 days after removing the cotyledons. No adverse effect of the antibiotic pretreatment was noticed at any stage of the regeneration (Fig. 4).

In order to determine the impact of moxalactam on other cassava cultivars, FEC tissues from cvs. 60444 and Oko-iyawo were cultured on GD based media supplemented with this antibiotic prior to transformation with *Agrobacterium*. An increase in recovery of GFP-expressing FEC lines of 60444 and Oko-iyawo was observed but was less dramatic than seen for TME 204 (Fig. 2). In the case of Oko-iyawo, GFP dividing units were higher (177 fold at 100 mg/l moxalactam as compared to untreated control), but the GFP dividing units per cc SCV were only 46 for Oko-iyawo FEC as compared to 230 for TME 204 FEC treated with same antibiotic concentration (Fig. 2b, d). The cultivar 60444 is amenable to transformation without addition of moxalactam (Fig. 2c) with an average of 67.8 GFP dividing units obtained per cc SCV for this treatment compared to 2.0 and 0.3 for TME 204 and Oko-iyawo, respectively. Inclusion of a moxalactam treatment prior to *Agrobacterium* co-culture was least effective in cv. 60444 where only the highest level of moxalactam (100 mg/l) significantly ($p < 0.05$) enhanced recovery of transgenic FEC events at a two-fold increase compared to the control treatment (Fig. 2c).

Effect of additional cephalosporin antibiotics on transformation efficiency of TME 204

Other cephalosporin antibiotics were tested to determine if they have similar stimulatory effects on transformation of cassava FEC. TME 204 FECs were cultured on GD2 50P medium supplemented with cefoperazone, cefoxitin, cefmetazole, cefotaxime or moxalactam at 50 mg/l for 18–20 days prior to co-culture with *Agrobacterium*. Fifty milligrams was the level of antibiotic chosen due to the stimulatory effects seen at this level (Figs. 2, 3) and associated costs and availability of the chemicals. All antibiotics promoted production of healthy FEC and

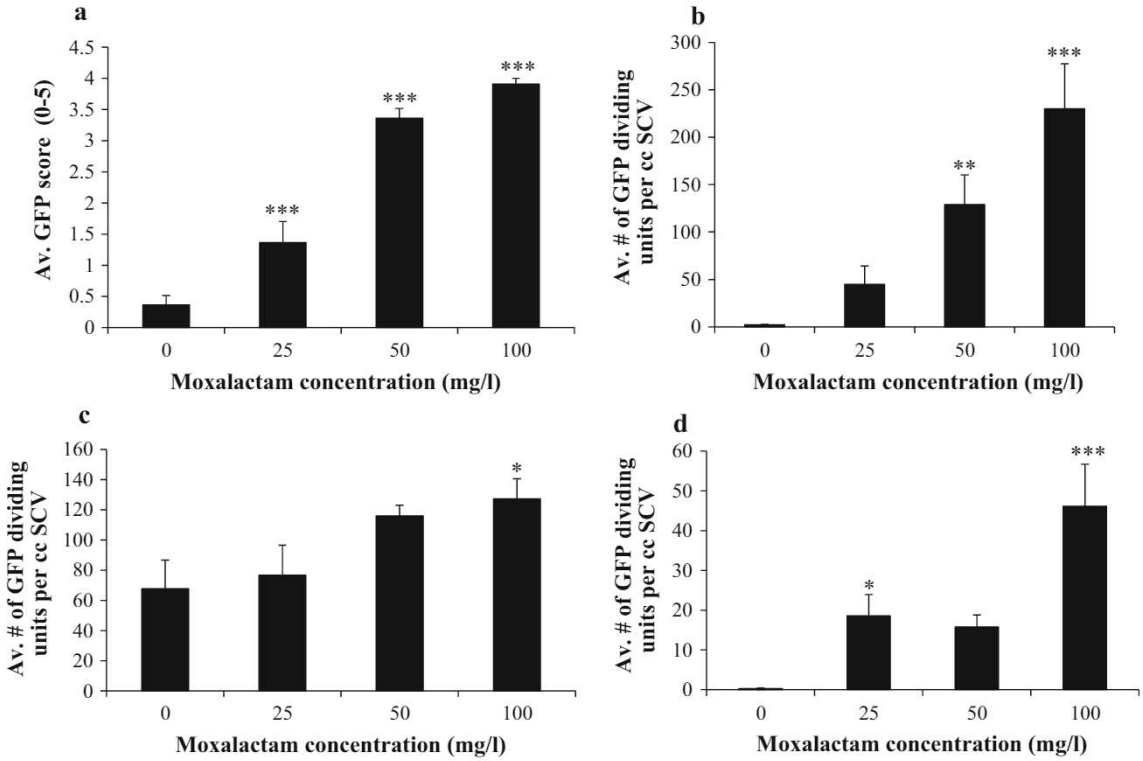

Fig. 2 Effect of moxalactam on transient and stable GFP expression. Transient GFP score recorded after 4 days of co-culture using TME 204 FEC (**a**); Average number of GFP-expressing, dividing units (stable transformants) recorded after 5 weeks of co-culture for TME 204 (**b**); 60444 (**c**) and Oko-iyawo (**d**). Values represent the average of three experiments each replicated 3–4 times ± SE where * stands for $p < 0.05$ ** stands for $p < 0.01$ and *** $p < 0.001$

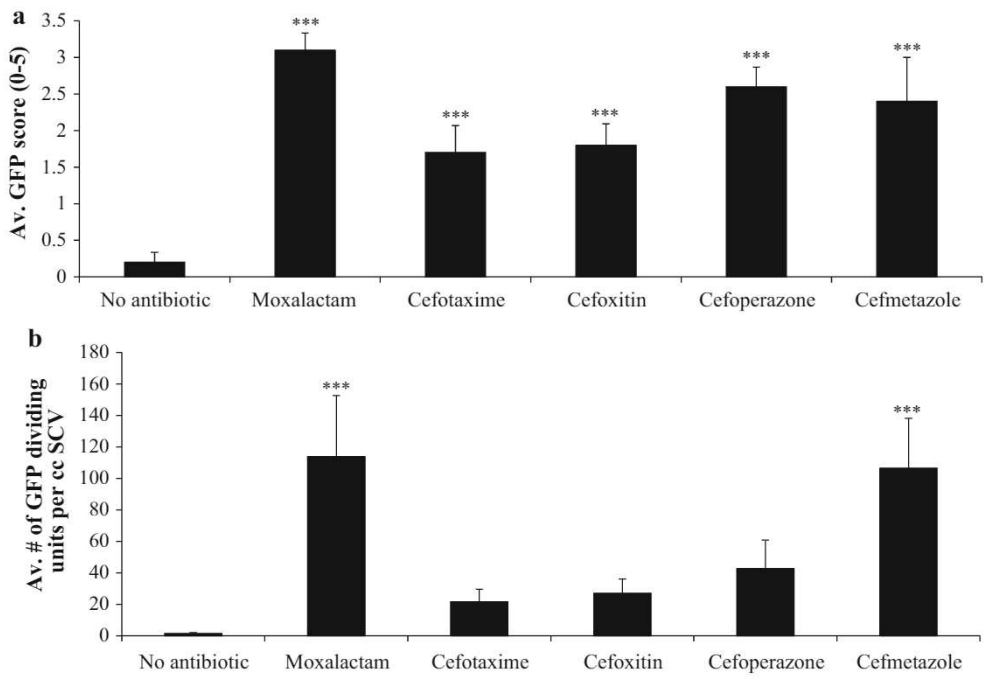

Fig. 3 Effect of different cephalosporins (50 mg/l each) on transient and stable GFP expression in TME 204. Transient GFP score recorded after 4 days of co-culture (**a**); Average number of GFP-expressing, dividing units (stable transformants) recorded after 5 weeks of co-culture (**b**). Values represent the average of three experiments each replicated three times ± SE where *** means $p < 0.001$

enhanced transient and stable transformation with GFP (Fig. 3). However, differing levels of transformation efficiency were observed across the five cephalosporins tested. Transient GFP expression increased from an average of 0.2 in control (no antibiotic treatment) to 1.7, 1.8, 2.4, 2.6 in FEC pre-treated with cefotaxime, cefoxitin, cefmetazole,

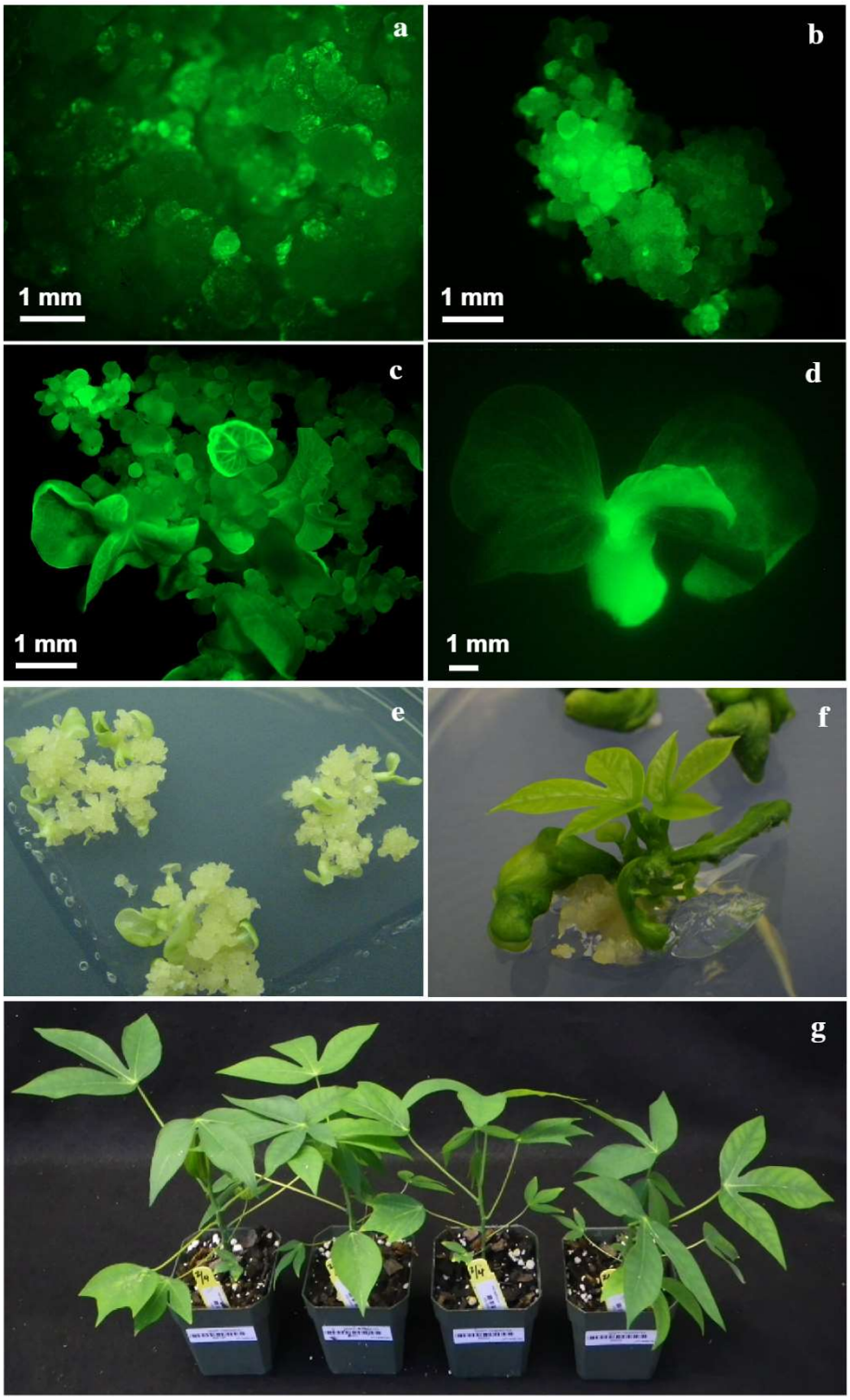

Fig. 4 Stages in the transformation of cassava cultivar TME 204 transformed with *A. tumefaciens* LBA4404 carrying eGFP. Transient GFP expression in FEC after 4 days of co-cultivation (**a**); Stable GFP expression in embryogenic *callus line* (**b**); Maturing embryos showing GFP expression (**c**); GFP-expressing cotyledon stage embryo on germination medium (**d**); Transgenic callus on Stage 2 medium (**e**); Germinating embryo on MS2 2BAP (**f**); One-month-old transgenic TME 204 plants in greenhouse (**g**)

cefoperazone, respectively. Moxalactam was the most effective antibiotic tested, generating average transient scores of 3.3 (Fig. 3a). Recovery of stable GFP-expressing transgenic callus lines was elevated compared to the control by presence of all tested antibiotics in the GD-based medium prior to *Agrobacterium* co-culture such that media containing no antibiotics produced an average of two dividing GFP-positive callus lines per cc SCV, while that supplemented with cefmetazole allowed recovery of an average of 107 callus lines (a 69-fold increase), cefoperazone 43 (a 28-fold increase), cefoxitin 27 (an 18-fold increase) and cefotaxime 22 (a 14-fold increase) (Fig. 3b).

Table 2 Effect of antibiotics on recovery of TME 204 GFP-expressing tissues and plants

	Number of FEC samples treated with *Agrobacterium*[a]	Number of callus lines moved to stage 1 (average ± SD)[b]	Number of callus lines surviving on Stage 2 (average ± SD)[c]	Number of lines that developed to cotyledon stage embryos (average ± SD)[d]	Number of lines rooted plantlets (average ± SD)[e]	Percentage germination (average ± SD)[f]
Moxalactam	3	50.0 ± 0.0 x	46.7 ± 3.5 x	32.7 ± 3.8 x	13.0 ± 6.6 x	26.0 ± 13.1 x
Cefotaxime	3	23.7 ± 0.0 x	13.7 ± 3.2	10.3 ± 2.9	6.0 ± 1.0	27.9 ± 9.3 x
Cefoxitin	3	16.3 ± 7.6	12.0 ± 5.2	8.0 ± 4.6	3.3 ± 3.5	20.4 ± 5.2
Cefoperazone	3	22.7 ± 12.7 x	21.0 ± 3.5 x	19.0 ± 1.1 x	8.3 ± 5.0	36.8 ± 6.7 x
Cefmetazole	3	50.0 ± 0.0 x	37.7 ± 1.2 x	32.3 ± 1.2 x	8.0 ± 1.0	16.0 ± 2.0
No antibiotic	3	1.0 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Means labeled with a letter "x" are significantly different from the control (no antibiotic treatment) within each column at $\alpha = 0.05$ according to Dunnett test

[a] *A. tumefaciens* strain LBA4404 harboring pCAMBIA2300 carrying *eGFP* at an $OD_{600} = 0.05$ was used for transformation of FEC samples

[b] Putatively transformed callus lines transferred to MS2 medium containing 5 μM NAA and 45 μM paramomycin (Stage 1) for 21 days

[c] Surviving callus lines transferred to MS2 medium containing 0.5 μM NAA and 45 μM paramomycin (Stage 2) for 21 days

[d] Cotyledon stage embryos transferred to selection free MS2 medium containing 2 μM BAP for germination

[e] Rooted plantlets on MS2 Agar medium

[f] The percentage germination was calculated as percentage of callus lines from which rooted plantlets were recovered

Of the chemicals tested, moxalactam proved to be the most effective, generating an average of 144 transgenic callus lines, equivalent to a 74-fold increase compared to control tissues. An average of 13 plants out of 50 callus lines were recovered from moxalactam treated plants as compared to no plant recovery from control untreated FECs, whereas only eight plants were recovered from 50 callus lines from cefmetazole treated FEC. Fully regenerated plants were recovered from the tissues treated with different antibiotics at equivalent frequencies (Table 2), indicating that there is no adverse impact of these antibiotics on plant regeneration. Rooted plants of five independent GFP-expressing transgenic events obtained from each of the above antibiotic treatments were transferred to 3-inch pots containing soilless compost and grown in the greenhouse (Fig. 4g). No phenotypic abnormalities were observed in these plants over a 3-month growing period.

Having established that addition of moxalactam to the final subculture cycle significantly enhanced recovery of transgenic tissues and plants, it was employed as a standard supplement at this stage for subsequent genetic transformations of cv. TME 204, and due to cost and availability, it was included at a level of 50 mg/l in the final cycle of GD-based medium prior to *Agrobacterium* co-culture.

Effect of *Agrobacterium* cell density at co-culture on transformation efficiency

The effect of density of *Agrobacterium* suspensions on transformation of TME 204 was assessed by inoculation and co-culture of FEC tissues with OD_{600} of 0.5, 0.05 and 0.025. Transformation efficiencies were assessed by scoring for GFP expression at the end of the 4-day co-culture period and as stable callus formation on selection medium and all phases through selection and regeneration. Transient GFP expression recorded after 4 days of co-culture indicated no significant difference between the FEC samples treated with different *Agrobacterium* cell densities (Fig. 5a). However, recovery of stable GFP-expressing callus lines was significantly ($p < 0.001$) greater from FEC inoculated OD_{600} 0.05 as compared to other treatments (Fig. 5b) and most notably compared to OD_{600} 0.5. Reducing the density of *Agrobacterium* suspension used for inoculation and co-culture of FEC by a factor of ten was seen, therefore, to increase the number of stable GFP-expressing callus lines recovered by a factor of greater than 2.25, from an average of 74–177 per cc SCV. The regeneration potential of the transformed FEC was not significantly different among the three *Agrobacterium* cell densities compared in this experiment (Table 3).

Application of optimized transformation systems for large-scale production of transgenic TME 204

Improved culture systems utilizing both moxalactam prior to *Agrobacterium* co-culture and an OD_{600} of 0.05 were utilized to enable large-scale production of transgenic plants of cassava cv. TME 204 within the VIRCA (Taylor et al. 2012) and BioCassava Plus (Sayre et al. 2011) projects.

Table 4 summarizes data on efficiency of callus and plant recovery from the three constructs *eGFP*, p5001 and p8001. Transformed callus lines were determined by

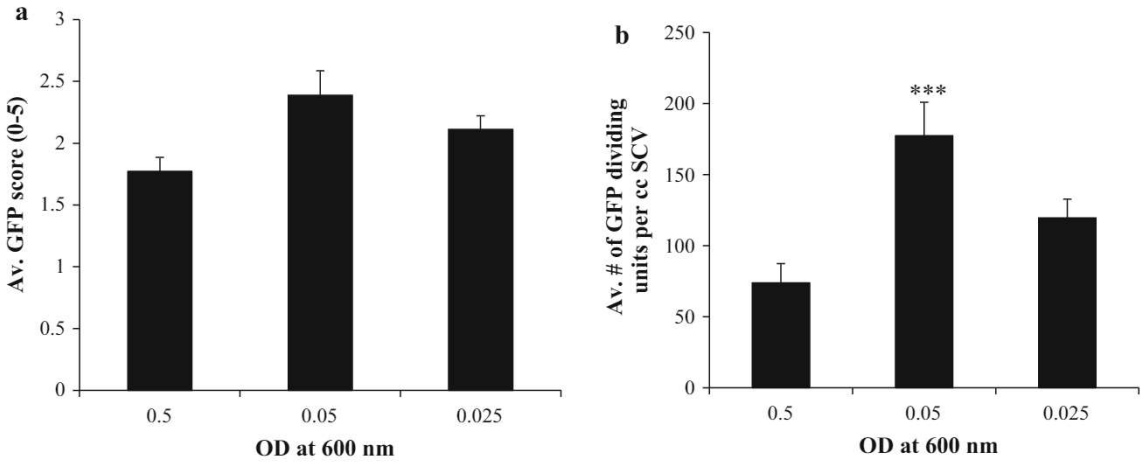

Fig. 5 Effect of lowering *Agrobacterium* cell densities on transient and stable GFP expression in TME 204. Transient GFP score recorded after 4 days of co-culture (**a**); Average number of GFP-expressing, dividing units (stable transformants) recorded after 5 weeks of co-culture (**b**); Values represent the average of three experiments each replicated 3–4 times ± SE where *** means $p < 0.001$

Table 3 Effect of *Agrobacterium* cell densities on recovery of TME 204 GFP-expressing tissues and plants

OD's at 600 nm	SCV (cc)	Number of FEC samples treated with *Agrobacterium*[a]	Total number of callus lines recovered (Stage 2)[b]	Total number of lines forming cotyledon stage embryos (MS2 2BAP)[c]	Total number of independent transgenic plants recovered (MS2 Agar)[d]	Number of callus lines recovered per cc SCV (average ± SE)[b]	Number of cotyledon stage per cc SCV (average ± SE)[c]	Number of rooted plantlets per cc SCV (average ± SE)[d]
0.5	3.1	10	138	73	44	50.5 ± 15.0 y	25.4 ± 6.0 y	15.3 ± 4.0 y
0.05	3.2	10	273	173	90	105.3 ± 33.0 x	66.4 ± 22.0 x	33.1 ± 8.0 x
0.025	3.65	10	230	104	57	77.3 ± 27.0 xy	34.6 ± 13.0 y	16.9 ± 3.0 y

Means followed by common letter(s) are not significantly different within each column at $\alpha = 0.05$ according to Tukey method of pairwise comparison

[a] *A. tumefaciens* strain LBA4404 harboring pCAMBIA2300 carrying e*GFP* was used for transformation of the FEC samples

[b] Surviving callus lines transferred to MS2 medium containing 0.5 µM NAA and containing 45 µM paramomycin (Stage 2) for 21 days

[c] Cotyledon stage embryos transferred to selection free MS2 medium containing 2 µM BAP for germination

[d] Rooted plantlets on MS2 Agar medium

Table 4 Tissue and plant production from cultivar TME 204 transgenic for pCAMBIA2300-*eGFP*, p5001 and p8001

Construct	SCV (cc)	Number of FEC samples treated with *Agrobacterium*[a]	Number of callus lines recovered[b]	Number of lines forming cotyledon stage embryos[c]	Number of independent transgenic plants recovered[d]	Number of callus lines recovered per cc SCV (average ± SE)[b]	Number of cotyledon stage per cc SCV (average ± SE)[c]	Number of rooted plantlets per cc SCV (average ± SE)[d]
eGFP	3.2	10	273	173	90	105.3 ± 33.0	66.4 ± 22.0	33.1 ± 8.0
p5001	14.3	25	1,652	401	230	116.8 ± 32.0	28.8 ± 4.0	16.3 ± 1.0
p8001	10.5	19	626	362	203	61.9 ± 13.2	35.5 ± 8.3	19.6 ± 5.9

[a] *A. tumefaciens* strain LBA4404 harboring pCAMBIA2300 carrying e*GFP,* p5001 and p8001 was used for transformation of the FEC samples

[b] Surviving callus lines transferred to MS2 medium containing 0.5 µM NAA and containing 45 µM paramomycin (Stage 2) for 21 days

[c] Cotyledon stage embryos transferred to selection free MS2 medium containing 2 µM BAP for germination

[d] Rooted plantlets on MS2 Agar medium

visualization of GFP after culture on Stages 1 and 2 regeneration media. For the *eGFP* construct, a total of 273 callus lines were recovered, from which 90 transgenic plants lines were regenerated across the three experiments shown. When corrected for the amount of starting target tissues used in each sample, efficiency of transgenic plant production averaged 33 ± 8.0 transgenic plants per cc SCV FEC. This compared to an average of 16 ± 1.0 and 19.6 ± 5.9 transgenic events per cc SCV FEC recovered from p5001 to p8001 constructs across 5 and 4 experiments each, respectively. Transgenic plants for p5001 and p8001 were established in the greenhouse and displayed normal morphology under greenhouse and confined field trial conditions (results not shown).

Analysis of transgenic plants for T-DNA copy number

T-DNA copy number was estimated by dot blot analysis for plants transgenic for *eGFP*, p5001 and p8001. A total of 74, 91 and 79 independent transgenic events recovered from *eGFP* transformation using OD_{600} 0.5, 0.05 and 0.025, respectively, were analyzed. Single or double copies of the *eGFP* were found in 57, 56 and 51 % of the events recovered, respectively, from tissues treated by co-culture with *Agrobacterium* at 0.5, 0.05 and 0.025 ODs. This indicated that while reduction of the *Agrobacterium* density at inoculation and co-culture increased recovery of transgenic plants, it did not have an effect on copy number of T-DNAs integrated into the cassava genome (Table 5). The events recovered for p5001 using 0.05 and 0.025 OD_{600} revealed that 71 and 76 % of the events had 1–2 copies of the transgene (Table 5). In case of p8001 transgenic events, 86, 82 and 87 % of analyzed events obtained from FEC samples inoculated with *Agrobacterium* at OD_{600} = 0.5, 0.05 and 0.025, respectively, had 1–2 copies of the transgene. This data indicates that while the *Agrobacterium* OD_{600} did not affect copy number, the number of 1–2 copy number events recovered positively correlated with size of the T-DNA (Fig. 1; Table 5).

Southern blot analysis was performed on 61 independent plant lines transgenic for p5001 showing 1–2 copy T-DNA integration by dot blot (Fig. 6), with 44 of these events determined as single copy events. Data from the Southern blot analysis confirmed that 80 % of the dot blot single copy events did carry single insertion of the T-DNA. Out of 17 events showing two copies according to dot blot analysis, five events were confirmed as double copies of the transgene. Southern blotting therefore indicated that overall, 96.7 % of these events had 1–2 copies as determined by dot blot analysis, whereas 3.3 % of the events showed more than two copies of the transgene. Therefore, the dot blot results are in accordance with the Southern blot analysis.

Discussion

Agrobacterium-mediated transformation of cassava cultivars 60444 (Bull et al. 2009; Schreuder et al. 2001; Taylor et al. 2012), TME 7 (Zainuddin et al. 2012), Serrere and Ebwanatereka (Nyaboga et al. 2013) have been previously reported but the transformation efficiencies vary with the cultivar. TME 204 and Oko-iyawo were chosen as the target cultivars for the generation of transgenic events for VIRCA and BioCassava Plus projects because of their popularity among East and West African farmers and innate resistance to CMD. However, the already existing protocol was not applicable for high-throughput transformation of these cultivars and most especially of TME 204. Modifications were therefore made to the methods described by Taylor et al. (Taylor et al. 2012). Addition of the cephalosporin antibiotic moxalactam to the media prior to transformation, and lowering of the *Agrobacterium* cell density at co-culture were found to significantly enhance recovery of transgenic tissues. The resulting greater than 100-fold enhancement in transformation efficiency has allowed the cultivar TME 204, which was previously recalcitrant in our hands, to enter high-throughput production with the generation of more than 1,360 independent transgenic events for VIRCA and BioCassava Plus projects in a period of less than 2 years. A total of approximately 1,500 independent transgenic events in three cultivar backgrounds, namely 60444, Oko-iyawo and TME 204, have been recovered since 2012 for BioCassava Plus project alone.

Exposing FEC target tissues to the five different cephalosporin antibiotics assessed in the present study has

Table 5 Effect of T-DNA size on frequency of low copy transgenic events in cultivar TME 204

Construct	T-DNA size (bp)	Total # of events analyzed	Percentage of 1–2 copy number events		
			OD_{600} = 0.5	OD_{600} = 0.05	OD_{600} = 0.025
eGFP	4,515	244	57	56	51
5001	7,500	373	ND	71	76
8001	13,354	117	86	82	87

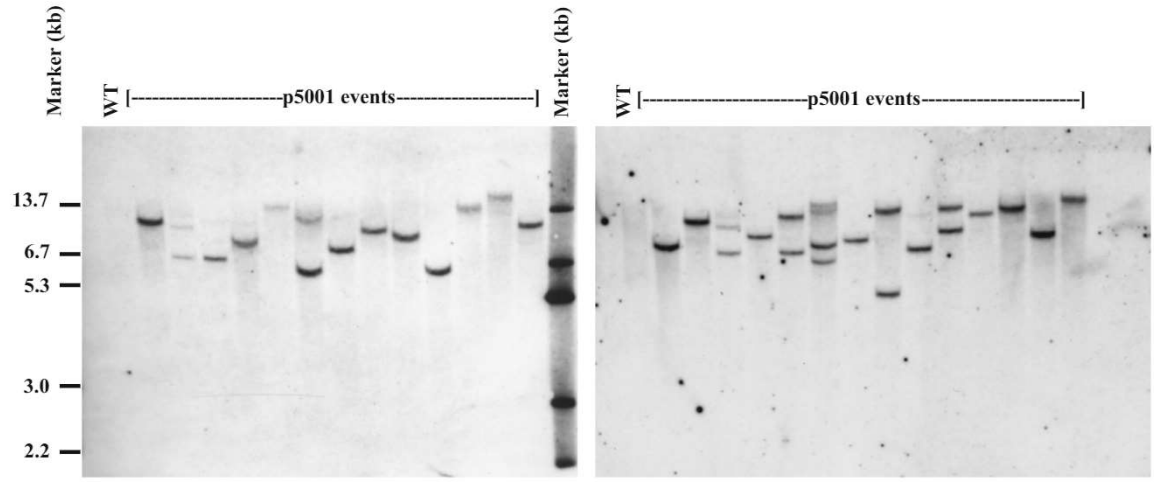

Fig. 6 Southern Blot analysis of 27 selected independent transgenic events recovered from p5001 construct. *Lane* WT-wild type TME 204; *Lane* marker-restricted plasmid DNA as positive control

shown positive effects on the transformability of these tissues. The transformation efficiency varied with the antibiotic type (Fig. 3; Table 2). Maximum transformation rates were achieved by addition of moxalactam followed by cefmetazole at concentrations of 50 mg/l. The causal mechanism behind the beneficial effects described here for cassava is not known. Cephalosporins have been successfully employed to eliminate *Agrobacterium* from putatively transformed tissues post-inoculation (Mayolo et al. 2003; Ogawa and Mii 2007). Addition of moxalactam does not appear to kill the *Agrobacterium,* therefore cefotaxime and timentin were used to eliminate bacteria in this study. Cefotaxime has been reported to be effective against *A. tumefaciens* LBA4404 and moxalactam against EHA101 (Shackelford and Chlan 1996).

Increased regeneration from cacao was reported by Mayolo et al. (2003) after exposing somatic embryos to media containing 200 mg/l moxalactam for 4 weeks. A possible mechanism of action proposed by the authors was production of phenylacetic acid, a naturally occurring auxin, resulting from metabolic breakdown of moxalactam. Efficacy of moxalactam on cassava transformation was found to be cultivar dependent with recovery of GFP-expressing callus greatest in TME 204 and having little effect in 60444. More studies are required to understand the mode of action of moxalactam in cassava. Identification of genes and gene products that are up- or down-regulated with exposure to this compound could lead to a greater understanding of the mechanisms underlying interaction between totipotent plant cells and *Agrobacterium*, and how this varies between cultivars, resulting in recalcitrance in some cultivars and not others.

The ability to recover transgenic events of TME 204 FEC exposed to moxalactam varied with the amount of *Agrobacterium* applied to the tissues. An OD_{600} of 0.5 is the standard cell density used for co-culture of cassava FEC (Nyaboga et al. 2013; Taylor et al. 2012; Zainuddin et al. 2012). Reducing this inoculum ten times had no effect on efficiency of transient transformation but significantly enhanced the recovery of stable transformation events (Fig. 2a, b; Table 3). Successful transformation was also efficient even at a 20 times reduction of *Agrobacterium* density. A correlation between *Agrobacterium* OD_{600} and transformation efficiency has been studied in other woody transformation systems such as almond (Archilletti et al. 1995) and chestnut (Corredoira et al. 2005). However, no significant effect of the *Agrobacterium* cell density was noticed when the bacterial cultures at different stages of growth curve were used for chestnut transformation. The maximum transformation was achieved when the *Agrobacterium* cultures were at exponential phase ($OD_{600} = 0.6$) as compared to early exponential phase ($OD_{600} = 0.3$) and late exponential phase ($OD_{600} = 0.9$) (Corredoira et al. 2005). On the contrary, increasing the *Agrobacterium* cell density to 1.0–1.5 substantially increased the transient GUS expression in wheat inflorescence tissues (Amoah et al. 2001). Similarly, a higher OD_{600} (0.8) gave a better transformation response in *Vigna radiata* (Yadav et al. 2012). In cassava, increasing the cell density appears to compromise cell viability leading to lower stable transformation rate.

Transgenic plants were analyzed to determine if reduced levels of *Agrobacterium* inoculation density had an effect on copy number of T-DNA integrations. A simple high-throughput dot blot method, subsequently confirmed by Southern blotting, indicated that inoculum density had no impact on the generation of low (1–2) copy events (Table 5). What was observed was a correlation between copy number and size of T-DNA. Analysis of GFP transgene copy number in 244 independently transformed lines

revealed that between 51 and 57 % of the lines contained 1–2 copies of the transgene. In a previous study of 372 putatively transgenic lines in cv. 60444, 59.5 % were found to have 1–2 copies of the transgene (Taylor et al. 2012), whereas out of 373 lines of p5001 and 117 lines of p8001 tested for presence of the 2X CaMV 35S promoter, 71–76 % and 82–87 % lines had 1–2 copies of the transgene, respectively (Table 5). Dot blot analysis for copy number is a simple and rapid method for estimation of T-DNA copy number (Taylor et al. 2012). Additional analysis of sixty-one p5001 transgenic lines by Southern blotting confirmed the accuracy of dot blotting for identifying low (1–2) copy events, with a greater than 95 % agreement between the two methods. Although the ability of dot blotting to distinguish between one and two copies was less accurate at 67 %, this method can be employed effectively for screening and removal of high copy events from a population of transgenic plants. For transgenic trait product development, it is essential to obtain transgenic lines with single transgene integration that are free from vector backbone. The single copy number p5001 events obtained in our study were screened for expression and vector backbone followed by transfer to the greenhouse. The selected events are being evaluated at multiple-location confined field trials in Africa.

The protocol demonstrates significant improvements in both efficiency and recovery of low copy events over existing methods. This opens up opportunities for using TME 204 as a new model cultivar for testing desirable traits instead of cv. 60444. It is likely that with further optimization, the protocol presented in this paper will be applicable to other elite cultivars of cassava.

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Conflict of interest The authors declare that they do not have any conflict of interest.

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