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ORIGINAL PAPER

Agrobacterium-mediated genetic transformation of selected tropical inbred and hybrid maize (*Zea mays* L.) lines

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Abstract The study was carried out to evaluate the amenability of tropical inbred and hybrid maize lines, using *Agrobacterium* mediated transformation technique. *Agrobacterium tumefaciens* strains EHA101 harbouring a pTF102 binary vector, EHA101, AGL1, and LBA4404 harbouring pBECK2000.4 plasmid, LBA4404, GV and EHA105 harbouring pCAMBIA2301 plasmid, and AGL1 harbouring the pSB223 plasmid were used. Delivery of transgenes into plant tissues was assessed using transient *β*-glucuronidase (*gus*) activity on the 3rd and 4th day of co-cultivation of the infected Immature Zygotic Embryos (IZEs) and embryogenic callus. Transient *gus* expression was influenced by the co-cultivation period, maize genotype and *Agrobacterium* strain. The expression was highest after the 3rd day of co-culture compared to the 4th day with intense blue staining was detected for IZEs which were infected with *Agrobacterium* strains EHA105 harbouring pCAMBIA2301 and EHA101 harbouring pTF102 vector. Putative transformants (T_o) were regenerated from bialaphos resistant callus. Differences were detected on the number of putative transformants regenerated among the maize lines. Polymerase chain reaction (PCR) amplification of Phosphinothricin acetyltransferase (*bar*) and *gus* gene confirmed the transfer of the transgenes into the maize cells. Southern blot hybridization confirmed stable integration of *gus* into PTL02 maize genome and segregation analysis confirmed the inheritance of the *gus*. A transformation efficiency of 1.4 % was achieved. This transformation system can be used to introduce genes of interest into tropical maize lines for genetic improvement.

Keywords *Agrobacterium tumefaciens* · Genetic transformation · *gus* expression · PCR · Southern blot hybridization · *Zea mays*

Abbreviations

CaMV	Cauliflower mosaic virus
CTAB	Cetyltrimethylammonium bromide
2,4-D	2,4-Dichlorophenoxyacetic acid
bar	Phosphinothricin acetyltransferase gene
gus	*β*-Glucuronidase
IZEs	Immature zygotic embryos
MS	Murashige and Skoog
nptII	Neomycin phosphotransferase II gene
T_o	Primary transformants
YEP	Yeast peptones extract

Introduction

Maize is one of the most important staple human food crops to Kenya's rural and urban population and it is also used as animal feed. Globally 21 % of maize grain is consumed as food and it is Africa's staple food crop which

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makes up more than 50 % of total caloric intake in the diets (OCED 2007; Sinha 2007). Due to its importance, improvement of this crop is of particular value.

Despite great effort made to increase maize production by use of conventional breeding (Campos et al. 2004; Kumar 2002), the demand has occasionally outstripped the supply due to various production constraints such as drought, low soil fertility (Mwangi and Ely 2001; Ngugi 2002), pests (stem borers), diseases (maize streak and leaf blight) and *Striga* (Conway and Toenniessen 2003; Kiiya et al. 2002; Mugo and Hoisington 2001; Mwangi and Ely 2001). Although conventional breeding methods have been used to develop some of the cultivars with improved agronomic performance and resistance to both biotic and abiotic stresses (Diallo et al. 2001), it is laborious, space consuming and the recovery of important agronomic traits is difficult due to incompatibility of heterotic groups. Thus there is an urgent need to complement conventional breeding with current biotechnological techniques such as genetic transformation. Transgenic plants are rapidly becoming a common feature of modern agriculture in many parts of the world. In 1996 1.7 million hectares (M ha) of land was under genetically modified crops worldwide, by 2004 this figure increased to 81.0 M ha (Chapman and Burke 2006) and by 2005 it was 90 M ha (Darbani et al. 2008a). Genetic transformation holds a great potential for obtaining improved genotypes in a shortened period of time (5–6 years) compared to conventional breeding technique (7–10 years) (Sharma et al. 2002). *Agrobacterium tumefaciens* is a preferred transgene delivery vehicle in maize transformation in many research laboratories. This preference is largely due to the advantages that *Agrobacterium*-mediated T-DNA transfer process has over direct gene delivery systems, such as a greater proportion of stable transgenic events (Frame et al. 2002; Shrawat and Lörz 2006; Travella et al. 2005; Zhang et al. 2005), it is highly efficient (Ishida et al. 2003), it has the capacity of transferring relatively larger DNA fragments with defined ends into recipient cells (Darbani et al. 2008b), it is a simple technology with lower cost and it inserts lower copy number of transgenes which help to minimize gene silencing. However transformation efficiency of the monocots, particularly maize, is relatively low of some genotypes because of their extreme recalcitrance to manipulation in vitro, hence this remains a major problem that requires appropriate methods and approaches (Birch 1997; Zhang et al. 2002). The overall efficiency of transformation of genetically modifying plants depends upon the efficiency of the transformation technique(s) used to stably incorporate the desired genetic material into plant cells and subsequent regeneration of the whole plants from transformed cells (Bommineni and Jauhar 1997; Darbani et al. 2008a). Establishment of a genetic transformation system serves as an important tool in the development of transgenic germplasm and will also help to address fundamental biological questions (Armstrong 1999).

Successful *Agrobacterium*-mediated transformation of maize lines has been reported using various explants including silks (Chumakov et al. 2006), seedling derived maize callus (Sidorov et al. 2006), IZEs (Frame et al. 2002; Frame et al. 2006; Hiei et al. 2006; Horn et al. 2006; Huang and Wei 2005; Huang et al. 2004; Ishida et al. 1996; Ishida et al. 2003; Luppotto et al. 1999; Negrotto et al. 2000; Taniguchi et al. 2000; Valdez-Ortiz et al. 2007; Vega et al. 2008; Zhao et al. 1998, 2001; Zhang et al. 2003), embryogenic callus (Darbani et al. 2008a; Yang et al. 2006), leaf derived callus (Ahmadabadi et al. 2007) and shoot apical meristem (O'Connor-Sánchez et al. 2002; Sairam et al. 2003; Sticklen and Oraby 2005) confined to a few lines. Reports on successful transformation of *Zea mays*, has focused on genotypes adapted to temperate zones while limited attention has been given to those adapted to tropical regions particularly sub-Saharan Africa where crop productivity is often low mainly due to abiotic and biotic stresses. Freshly isolated immature zygotic embryo explants have been reported to be highly competent for *Agrobacterium* infection in maize for the production of transgenic plants (Frame et al. 2002; Frame et al. 2006; Ishida et al. 1996; Negrotto et al. 2000; Zhao et al. 2001; Zhang et al. 2003). Genetic transformation of maize is still a challenge in a number of lines due to the genetic variability, differences in their response in vitro culture, genotype compatibility with *Agrobacterium* strains and differences in transformation procedures have resulted to low transformation frequency.

Gene expression studies constitute a critical component of molecular biological research in plants. As an alternative, assessment of the transfer of transgenes into plant cells or tissues is often performed by use of transient expression assays that are rapid and results are obtained in days. Transient *gus* expression systems are valuable tools for understanding the functions of genes in specific organs of plants. Transient gene expression and/or stable gene expression in cereals has been reported after the delivery of the DNA into cells via *Agrobaterium*-mediated transformation (Cheng et al. 2004; Frame et al. 2002; Rachmawati and Anzai 2006; Rubio et al. 2005) and direct gene transfer (O'Connor-Sánchez et al. 2002; Rafiq et al. 2006; Russell and Fromm 1997; Shen, et al. 1999).

To date there is no available published report on the assessment of the delivery and integration of transgenes into tropical maize genotypes available in Kenya using *Agrobacterium*-mediated transformation or direct gene transfer. This study was carried out with the objective of assessing the efficacy of *A. tumefaciens* strains in the transformation of selected tropical maize inbred and hybrids genotypes.

Materials and methods

Plant material

IZEs of CML78, CML216, CML331, TL18, TL27, MU25, A188 maize inbred, H627 and PTL02 hybrid lines and IZEs derived embryogenic callus of H627 hybrid were used. IZEs (1–2 mm in length) were obtained from maize grown in the greenhouse in Kenyatta University, Kenya. Plant transformation research work was carried out in level II Plant Transformation Biosafety Laboratory, Kenyatta University.

Agrobacterium tumefaciens strains, vectors and bacteria culture

Disarmed *A. tumefaciens* strains (AGL1 (Lazo et al. 1991), EHA101 (Hood et al. 1986) and LBA4404 (Hoekema et al. 1983) habouring pBECK2000.4 vector; EHA105 (Hood et al. 1986), GV and LBA4404 harbouring pCAMBIA2301 (http://www.cambia.org) were used to infect IZEs and embryogenic calli for the assessment of *gus* expression while EHA101 harbouring pTF102 vector (Frame et al. 2002), and AGL1 harbouring pSB223 were used to infect IZEs and embryogenic calli for the assessment for both *gus* expression and regeneration of putative transformants. AGL1, EHA101, LBA4404, EHA105, GV and EHA101 harbouring the binary vector were kindly provided by Prof. K. Wang, Iowa state University, USA while AGL1 harbouring pSB223 from Dr. J. Kumlehn, IPK Gatersleben, Germany. The T-DNA region of pBECK2000.4, pTF102 and pSB223 vectors contained the right and left border fragments, *bar* (Phosphinothricin acetyltransfarase gene) a selectable marker gene and *gus* reporter gene. The T-DNA region of the pCAMBIA2301 vector contains neomycin phosphotransferase gene (*nptII)* as a plant selectable marker for selection on kanamycin and the β-glucuronidase (*gus*) as the reporter gene under the control of cauliflower mosaic virus (CaMV 35S) promoter (Sujatha et al. 2012). The *gus* reporter gene had an intron in its codon region to prevent expression in *A. tumefaciens* cells.

One loop of *A. tumefaciens* strains harbouring the vector from the glycerol stocks kept at −80 °C were streaked onto a solidified Yeast peptone Extract (YEP) medium. The YEP media was supplemented with different concentrations of antibiotics 200 mg l^{-1} Spectomycin (Spec) for AGL1, EHA101 and LBA4404 with pBECK2000.4 vector, 50 mg l^{-1} Rifampicin (Rif) and 50 mg l^{-1} Kanamycin (Kan) for EHA105 and GV with pCAMBIA2301 vector, 100 mg l^{-1} Streptomycin (Strep) and 50 mg l^{-1} Kan for EHA101(pTF102), 150 mg l^{-1} Spec and 50 mg l^{-1} Kan for AGL1(pSB223) and 10 mg l^{-1} Rif, 30 mg l^{-1} Strep and 50 mg l^{-1} Kan for LBA4404(pCAMBIA2301). The cultures were incubated overnight at a temperature of 28 °C in the dark for colonies to appear after which one loop of bacteria was scrapped and suspended in 5 ml of liquid YEP broth medium supplemented with relevant antibiotics. The cultures were grown at a temperature of 28 °C overnight with shaking at 5 Xg. The *Agrobacterium* cell suspensions were centrifuged at 706 Xg for 5 min using a table centrifuge at room temperature. A loop full of *Agrobacterium* strain was then re-suspending in 2.5 ml of liquid infection medium supplemented with 200 µM acetosyringone (AS) in a 50 ml falcon tube with shaking for 4 h at a temperature of 28 °C. Bacteria cell densities were then adjusted to give an OD_{660} of 0.8 before callus or embryos infection.

Infection, co-cultivation, resting, selection, regeneration of putative T_o transformants and their progeny

Disinfection, isolation of IZEs (1–2 mm) and callus initiation were carried out according to the procedure described previously (Omwoyo et al. 2008). Media preparation and procedure used in the infection, co-cultivation, resting, selection and regeneration were as previously described by Frame et al. (2002) with a slight modification where 200 µM AS was used during infection and co-cultivation instead of 100 µM. Disinfected IZEs (1–2 mm) and embryogenic callus (2 mm in diameter after 4 days of subculture) were immersed separately in the infection medium in 1.5 ml eppendorf tubes for 5 and 60 min, respectively in *A. tumefaciens* suspension supplemented with 200 µM AS in the dark. The infected callus or IZEs were transferred onto the solidified co-cultivation medium. The embryos were placed with embryo axis side in contact with the co-cultivation medium. The cultures were then incubated in the dark at a temperature of 20 °C for 3–4 days. After co-cultivation, the callus and embryos were rinsed 4 times with sterilize distilled water followed by 2 times (5 min) with liquid N6 medium containing 500 mg l^{-1} carbanicillin (Duchefa, The Netherlands) and were blotted dry on a sterile filter paper (Whatman No. 1). They were then transferred onto the resting medium and incubated at a temperature of 28 °C for 7 days in the dark. Immature zygotic embryos/callus were then transferred onto selection medium I for two week followed by two subcultures at two week intervals on selection medium II in the dark at a temperature of 28 °C. Transformation frequency (%) based on the resistant callus was expressed as the number of resistant callus recovered at the end of culturing on selection medium II relative to the total number of callus or IZEs inoculated.

Surviving resistant embryogenic callus from selection medium II was transferred onto embryo maturation

medium consisting of Murashige and Skoog (MS) basal salts (Murashige and Skoog 1962) supplemented with 6 % sucrose, 3 mg l^{-1} bialaphos, 250 mg l^{-1} carbanicillin, devoid of growth regulators and solidified with 0.3 % gerlite (w/v). The cultures were incubated in the dark at a temperature of 28 °C for 2 weeks. Embryogenic callus or somatic embryos were transferred onto MS medium supplemented with 3 % sucrose and 0.3 % gerlite and incubated under 16/8 h (light/dark) for shoot and root formation. The number of successfully regenerated putative transgenic plants was recorded.

Plantlets (T_o), 7–10 cm high, with healthy roots from culture bottles were rinsed with distilled water to remove the gelling agent and then transferred onto pots containing peat moss for 3–5 days before being transplanted into plastic pots containing sterilized soil mixed with humus and sand (2:2:1). The seeds from T_o putative transgenic plants were grown in pots in the greenhouse to produce T_1 plants.

Histochemical analysis of transient and stable *gus* expression

Embryos or small pieces of callus on the 3rd and 4th day of co-cultivation were assessed for transient *gus* activity using procedure described previously (Jefferson et al. 1987). The *gus* expression was also examined in the root and leaf tissue of the plants in the T_o and T_1 generations. Leaf explants from putatively transformed plantlets were immersed in 70 % (v/v) ethanol to remove the chlorophyll for ease of visualization. Blue staining of the IEZs, callus and leaves was visualized using a Leica 2000 microscope, and scored for transient *gus* expression. The frequency of the IZEs or callus showing *gus* activity was calculated as the ratio between the number of callus or IZEs showing blue staining due to *gus* activity relative to total number inoculated.

DNA extraction and PCR analysis

Total genomic DNA was extracted as described by Pallota et al. (2000). The concentration of DNA was determined using a spectrophotometer technique at A260/A280. The plant genomic DNA was resolved in 0.8 % agarose gel containing ethidium bromide (0.5 μg ml^{-1}) at 200 V and 120 mA for 40 min and viewed using Image Master VDS (Biopharmacia, Germany) and gel photographed.

Putative transformants were screened using PCR for *gus* and *bar* genes. PCR amplification was performed in a 20 μl reaction, comprising of 100 ng of maize genomic DNA or 1 ng plasmid DNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM (10 pM) of each primer (forward and reverse), 0.2 mM dNTPs and 0.5 U of Taq polymerase (Bioline, Germany). The primers used to amplify a 730 bp fragment of the *gus* gene were: forward 5′CCGGTTCGTTGGCAA TACTC-3′ and reverse 5′CGCAGCGTAATGCTCTACA C-3′. The primers used to amplify a 457-bp fragment of the *bar* gene were: forward 5′-GGTCTGCACCATCGTCAA CC-3′ and reverse 5′TACCGGCAGGCTGAAGTCCA-3′. PCR reactions were performed using a thermal cycler (Biometra, Germany). PCR conditions were: initial DNA denaturation for 5 min at temperature of 95 °C, followed by 30 amplification cycles (denaturation at 95 °C for 30 s, annealing at 60 °C for 45 s and extension at 72 °C for 1 min 15 s) and final extension at 72 °C for 7 min. PCR products were separated in a 0.8 % agarose gel with ethidium bromide staining ran at 200 V, 120 mA for 40 min, viewed using Image Master VDS (Biopharmacia, Germany) and gel photographed.

Southern blot hybridization analysis

Genomic DNA (25 μg) from the control (non-transformed), putative transformant, 1 ng of pTF102 plasmid (positive control) was digested with Hin*dIII* enzyme in a 40 μl reaction overnight. The digestion products were separated by electrophoresis in a 0.8 % (w/v) agarose Tris borate ethylenediaminetetraacetic acid (TBE) gel at 25 V, 15 mA overnight. The gel was depurinated using 0.25 M HCl for 10 min followed by washing in deionized water on a slow rotating shaker for 5 min, twice (15 min each) in denaturation buffer solution (0.5 M NaOH, 1.5 M NaCl) and then neutralized two times in a neutralization buffer (1 M Tris, 1.5 M NaCl, pH 7) for 30 min each. The gel was soaked in 20× Standard sodium citrate (SSC) for 5 min. The DNA was transferred onto a positively charged nylon membrane by capillary blotting under 20× SSC conditions (pH 7) (Sambrook et al. 1989).

The blot was washed in 2× SSC for 5 min and DNA was fixed onto the blot by UV cross-link for 45 s with a UV transilluminator machine. The blot was wet on both sides using 6× SSC and pre-hybridized for one hour using pre-hybridization buffer (20× SSC, 50× Denhardts reagent, 20 % Sodium dodecyl sulphate (SDS), 100 μl salmon sperm DNA). The blot was then hybridized at a temperature of 68 °C overnight using the same solution as pre-hybridization buffer in which 1 μl of the denatured biotin labelled *gus* probe generated using PCR biotin probe synthesis kit was included. The blot was washed twice in stringent low washing buffer solution (2× SSC, 0.1 % (w/v) SDS at room temperature for 5 min, followed by washing using 0.5× SSC plus 0.1 % (w/v) SDS and then 0.1 × SSC plus 0.1 % (w/v) SDS buffer solution at a temperature of 68 °C each lasting 10 min. Chemiluminescent detection was performed as described in the users‘ instruction

manual (New England Biolabs inc., USA). The signals were visualized using X-ray detection film.

Segregation analysis

The seeds of the self-pollinated progeny (T_o) were germinated in the in the potted soil and grown for seven days and then the youngest leaves were assessed for *gus* activity according to the procedure described by Jefferson et al. (1987). To determine the segregation ratios of the transgene, the number of T_1 plants showing the *gus* activity and those which did not were recorded.

Experimental design and statistical analysis

The experiments were organized according to completely randomized design. The experiments were repeated three times. The data on transformation were analyzed for significance ($p \leq 0.05$) using analysis of variance (ANOVA) with MINITAB computer software version 23.22. Means were separated using Tukey's Honest Significant Difference (HSD) at 5 % level. The Chi square was used to determine if the segregation observed in the T_1 plants corresponds to the expected 3:1 ratio according to mendelian fashion.

Results

Co-cultivation and selection of putative transformed embryo/callus

Browning and apoptosis of non-infected IZEs was observed when they were co-cultivated with *A. tumefaciens.* All the non-infected embryos (controls) later turned brown, necrotic and died when they were grown on a medium containing bialaphos as a selective agent (Fig. 1a). Subculture of the callus formed from infected IZEs onto selection medium II twice each lasting 2 weeks resulted in the survival of sectors of resistant embryogenic callus which proliferated to form somatic embryos globular in shape (Fig. 1b). Embryogenic callus continued to proliferate when they were isolated from non-proliferating callus and subcultured onto fresh media with the same concentrations of selection medium II. Differences were detected on the frequency of bialaphos resistant callus among the maize lines. Transformation frequency of the bialaphos resistant callus on the medium containing bialaphos ranged between 0.1–5.4 % and 0.1–8.7 % for the callus obtained from IZEs which were infected with AGL1(pSB223) and EHA101(pTF102) *Agrobacterium* respectively (Table 1). A188 had the highest frequency of bialaphos resistant callus (5.4 %) and CML216 (8.7 %) from IZEs infected with AGL1(pSB223) and EHA101(pTF102) respectively. Bialaphos resistant callus were not obtained in CML78 and CML331 maize lines.

Regeneration of putative transformants (T_o) and growth of the progeny (T_1)

Regeneration of T_o plantlets was successfully achieved from bialaphos resistant embryogenic callus initiated from IZEs which were infected with EHA101(pTF102) and AGL1(pSB223) (Fig. 1c) after 3 weeks of culture on regeneration medium. Putative T_o transformants were obtained 11–12 weeks after infection of IZEs of TL18, CML216, A188, PTL02 and H627 lines when infected with AGL1(pSB223) and all genotypes tested except CML78 and CML331 when infected with EHA101(pTF102) (Table 1).

Rooted putative transformants (T_o) were hardened in pots containing peat moss and transplanted into the soil in the containment greenhouse conditions (Fig. 1d). Some of the T_o plants grew to maturity to set seeds (Fig. 1e). Fertile and infertile putative T_o plants were produced 6–7 months after the infection of IZEs. The seed set of the putative transformants varied from a few seeds per cob per plant to nearly full cob (Fig. 1f). Seeds of T_o regenerants which were planted in potted soil were found to be viable. The T_1 plants in this study were normal in morphology and grew to maturity to set seeds.

Histochemical *GUS* assays

Presence of *gus* activity was detected on the 3rd and 4th day of co-cultivation of IZEs and callus infected with *Agrobacterium* strains. *Gus* activity was also observed in the leaves and roots of T_o and T_1 plants. Contrary, *gus* activity was not detected when IZEs of CML331 maize genotype was infected with any of the *Agrobacterium* strains or in non-transformed (control) IZEs. On the 3rd and 4th day of co-cultivation of IZEs with *A. tumefaciens* carrying pBECK2000.4, pSB233, pCAMBIA2301 and pTF102 vectors *gus* activity was observed mostly at the edges of the embryos. The frequency of IZEs expressing *gus* activity when infected with different *Agrobacterium* strains ranged from 3 to 50 % and 0 to 32.6 % on the 3rd and 4th day of co-cultivation respectively. The number of embryos expressing the *gus* activity on the 4th day of co-cultivation was significantly ($p < 0.05$) lower compared to the 3rd day for all maize lines except CML331 in which *gus* activity was not detected. The frequency of IZEs expressing *gus* activity among the genotypes ranged from 0 to 50 % and 0 to 32.6 % on the 3rd and 4th day of co-cultivation respectively. Significant differences ($p < 0.05$) in the percentage area (%) of blue staining due to transient *gus* expression were noted when IZEs of the same maize genotypes were infected with different *Agrobacterium* strains except CML78 and CML331.

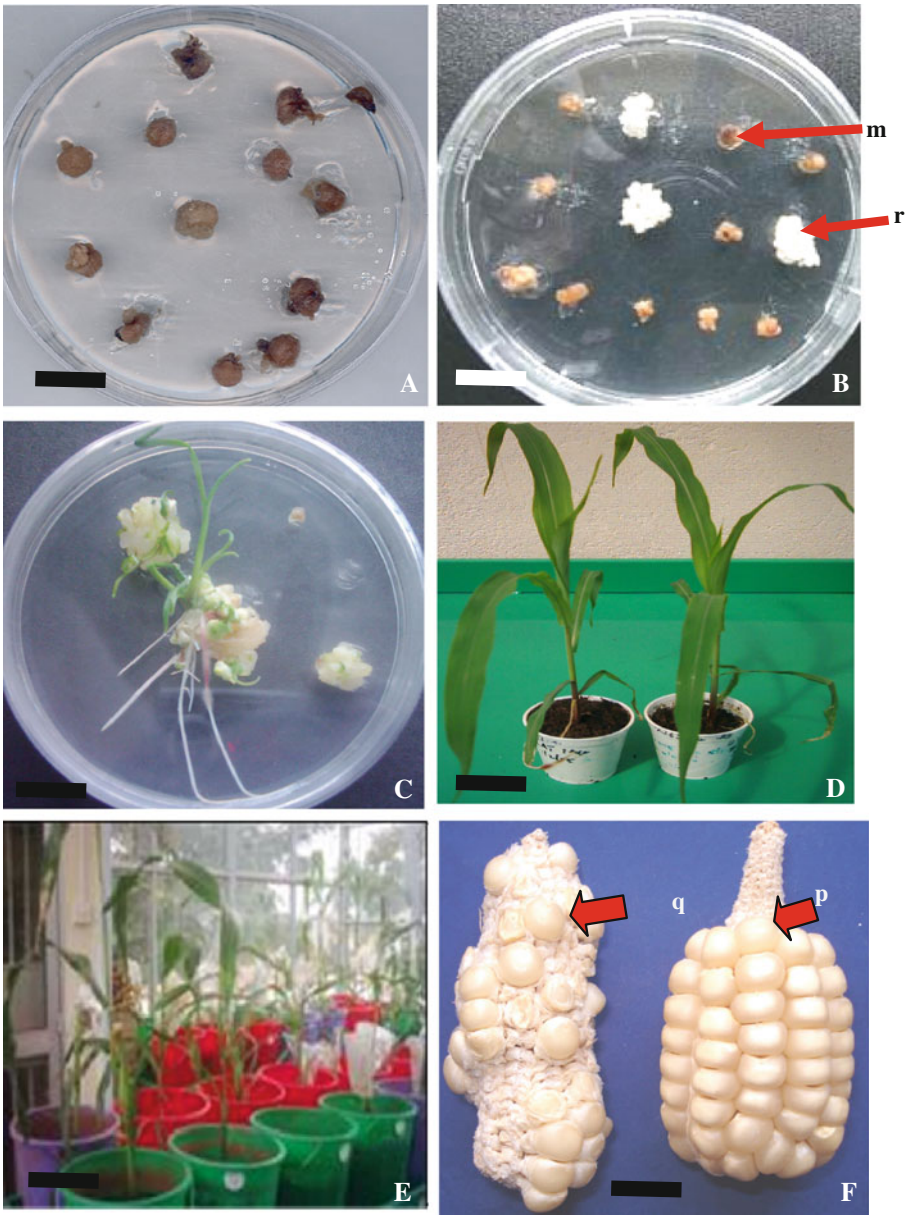

Fig. 1 *Agrobacterium*-mediated transformation of IZEs, regeneration of putative transformants and growth of the progeny of PTL02 maize line. **a** Non-transformed (control) IZEs on selection medium containing 3 mg l^{-1} bialaphos; *Bar* 0.5 cm. **b** Development of resistant putatively transformed callus (*r*), no-resistant callus (*m*) on selection medium containing 3 mg l^{-1} bialaphos; *Bar* 0.5 cm. **c** Regeneration of putatively transformed plantlets on MS medium supplemented with 3 % sucrose and 0.3 % gerlite; *Bar* 0.5 cm. **d** Putative transgenic plants (T_o) in pots; *Bar* 5 cm. **e** Fertile putative transgenic plants (T_o) with ears and tassels growing in the greenhouse; *Bar* 10 cm. **f** A cob with a few seeds (*q*) and a full cob (*p*) in T_o plants; *Bar* 1 cm

No *gus* activity was detected in non-infected H627 callus. Blue patches were observed in the putatively transformed embryogenic callus of H627 on the 3rd and 4th day of co-cultivation. The intensity of blue staining detected in the callus was generally low compared to that of IZEs. The percentage number of callus with blue staining on the 3rd day of co-cultivation was significantly ($p < 0.05$) higher compared to that on the 4th day except the callus infected with GV(pCAMBIA2301) in which transient *gus* activity was not detected (Fig. 2). The frequency of H627 embryogenic callus with *gus* activity when infected with different *Agrobacterium* strains ranged from 0 to 66.67 % and 0 to 26.60 % on the 3rd and 4th day of co-cultivation respectively.

The leaves and roots of some of the T_o maize plants exhibited blue colouration with root staining observed in the tips, elongation zones and cut edges. The *gus* activity was mostly observed in the young leaves and roots of the T_o plants, but faded as they matured. In most T_o plants of TL18, CML216, A188, PTL02 and H627 from IZEs infected with AGL1(pSB223) (Table 1) *gus* activity was observed at the cut edges and veins of the leaves. When the leaves of T_o plants obtained from IZEs which were infected with EHA101(pTF102) *gus* activity was observed in TL18, MU25, CML216, A188, H627 and PTL02 lines but not detected in the leaves of TL27 and CML331 and the non-transformed plants. Transformation frequency of T_o plants based on *gus* activity when leaves were assessed among the

Table 1 The *gus* expression in the leaf and PCR detection of putative transgenic maize T_o plants obtained from immature zygotic embryo explants mediated by two *A. tumefaciens* strains

Agrobacterium strain	Genotype	Total number of IZEs inoculated	Bialaphos resistant callus	Callus TF (%)[a]	Number of callus forming plants	Total number of plants regenerated	GUS positive T_o plants	TF (%) based on GUS positive T_o plants[b]	PCR positive T_o plants	TF (%) based on PCR positive T_o plants[c]
AGL1(pSB223)	TL18	1,509	15	1.0	2	5	3	0.2	2	0.1
	TL27	1,334	1	0.1	0	0	0	0	0	0
	MU25	192	3	1.6	0	0	0	0	0	0
	CML78	1,250	0	0	0	0	0	0	0	0
	CML216	717	10	1.4	3	4	2	0.3	2	0.3
	CML331	1,220	0	0	0	0	0	0	0	0
	A188	1,664	90	5.4	14	40	2	0.1	7	0.4
	PTL02	295	6	2.0	2	7	5	1.7	3	1.0
	H627	2,426	104	4.2	21	38	4	0.2	2	0.1
EHA101(pTF102)	TL18	2,525	8	0.3	3	3	2	0.1	1	0.0
	TL27	257	1	0.4	1	1	0	0	0	0
	MU25	883	1	0.1	1	2	1	0.1	0	0
	CML78	1,250	0	0	0	0	0	0	0	0
	CML216	228	20	8.7	2	4	3	1.3	2	0.9
	CML331	1,300	0	0	0	0	0	0	0	0
	A188	471	40	8.5	42	31	4	0.9	10	2.1
	PTL02	370	32	8.7	6	29	3	0.8	7	1.9
	H627	920	4	0.4	3	5	2	0.2	2	0.2

[a] Number of bialaphos resistant callus out of the total number of IZEs inoculated × 100

[b] GUS positive T_o plants out of the total number of IZEs inoculated × 100 based on *gus* expression

[c] Number of PCR positive T_o plants out of the total number of IZEs inoculated × 100

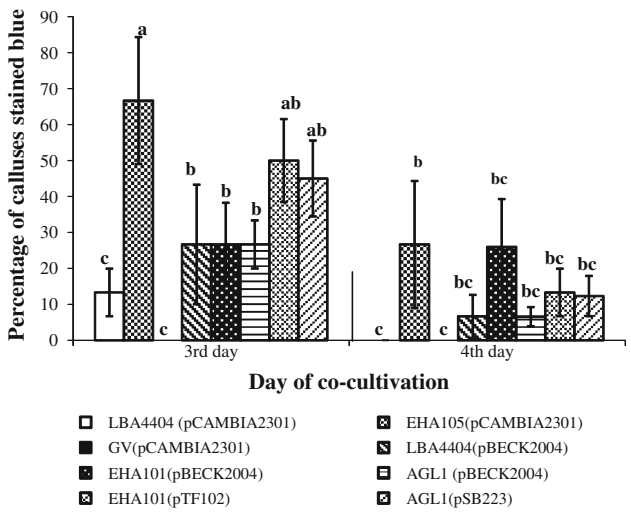

Fig. 2 Effect of co-cultivation period and *A. tumefaciens* strains on the percentage of embryogenic callus of H627 showing blue staining. Mean percentage followed by the same *letters* indicated above the *bars* are not significantly different according to Tukey's Honest Significant difference at 5 % level for each co-cultivation day. *Error bars* in the figure indicate standard errors

genotypes studied ranged from 0 to 1.3 % and from 0 to 1.7 % for those recovered from IZEs infected with EHA101(pTF102) and AGL1(pSB223) respectively (Table 1). Stable *gus* expression was observed in the roots and leaves of PTL02 T_1 plants.

Molecular analysis

PCR analysis of the genomic DNA revealed the presence of the transgenes in the putative transformants of T_o and T_1 maize plants. Expected fragment of 730 bp size for *gus* and 457 bp size for *bar* were detected in the T_o plants. A730 bp size *gus* fragment was also amplified in T_1 plants (Fig. 3a) while *gus* and *bar* gene fragments were not detected in the non-transformed plants.

Transformation frequencies based on the PCR positive T_o plants ranged from 0 to 1.0 % and 0 to 2.1 % on plants recovered from IZEs infected with AGL1(pSB223) and EHA101(pTF102) respectively (Table 1). PTL02 had the highest transformation frequency (1.0 %) for plants recovered from IZEs which were infected with AGL1(pSB223).

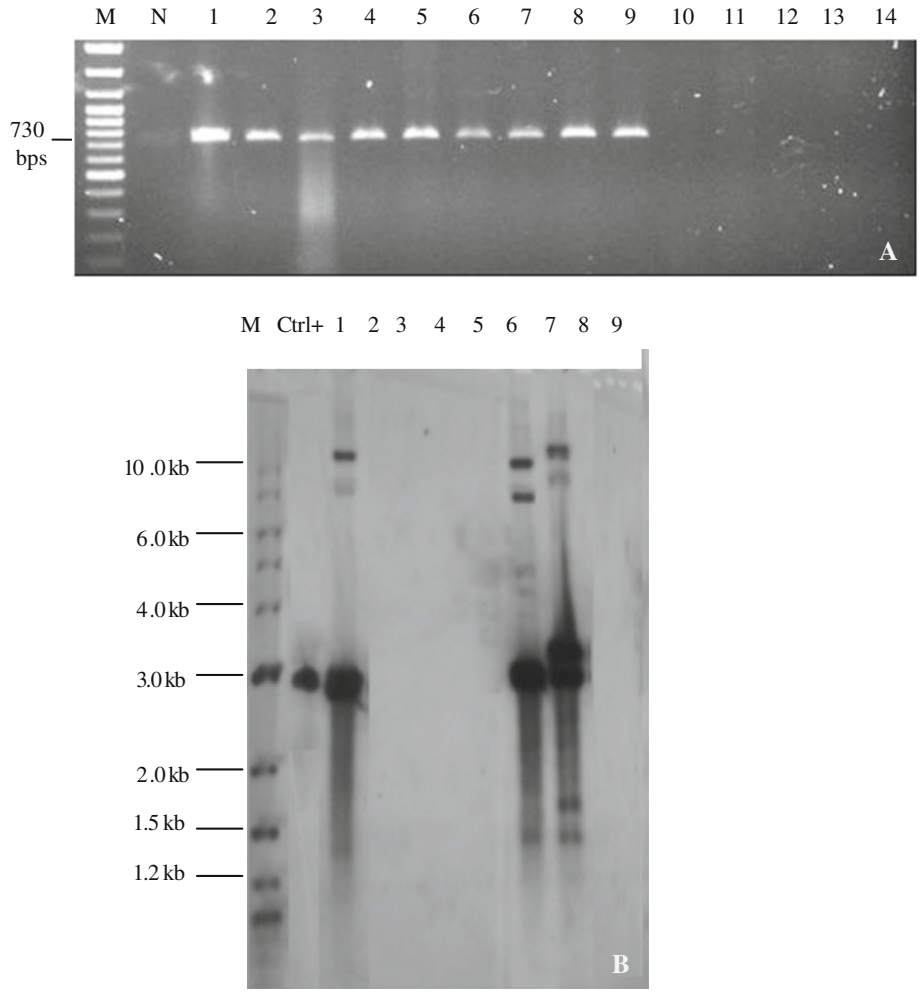

Fig. 3 **a** PCR amplification of 730 bp size *gus* fragment of the leaf genomic DNA extracted from T_1 maize plants resolved by agarose (0.8 %) gel. *M*, GeneRuler^{TM} DNA ladder (Fermentas) 100 bp. *N*. Negative control. Lane *1* Positive control (pSB223 plasmid DNA). Transformation carried out using AGL1(pSB233); lane *2* (CML216), lane *3* (TL18), lanes *4–5* (PTL02), lane *6* (H627). Transformation carried out using EHA101(pTF102); lanes *7–9* (PTL02), lanes *10–13* (A188). Lane *14*, Non-transformed plant. PCR bands shows expected 730 bp fragment of the *gus* gene. **b** Southern blot hybridization of genomic DNA from T_1 transformed maize plants; DNA was digested with *Hin*dIII enzyme and hybridized with biotin labelled PCR *gus* probe. *M*, 1 Kb mass ladder (New England Biolabs Inc., USA). Ctrl+, Positive control (pTF102). Lanes *1–3*, *5–7*, plants obtained from IZEs infected with EHA101(pTF102). Lane *4*, Non-transformed PTL02 maize plants. Lanes *8–9*, PTL02 T_1 plants from IZEs infected with AGL1(pSB223)

A188 had the highest transformation frequency (2.1 %) followed by PTL02 (1.9 %) in transformants recovered from IZEs infected with EHA101(pTF102).

Southern blot hybridization using biotin labelled PCR *gus* probe confirmed the integration of *gus* gene into the genome of three PTL02 maize transformants (T_1) recovered from IZEs which were infected with EHA101(pTF102) (Fig. 3b). One of PTL02 transformant had three copies of the *gus* gene and two had 6 copies. Each hybridization band observed was estimated as one transgene insertion copy into PTL02 maize genome. Hence the number of hybridizing bands reflected the number of copies of *gus* gene fragment integrated into the plant genome. Hybridization signal was absent in the non-transformed plant (Fig. 3b, Lane 4). A stable transformation efficiency of 1.4 % was achieved in PTL02 maize line.

Segregation analysis

Two of the transgenic events showed the expected 3:1 mendelian segregation ratio while one exhibited non-mendelian segregation ratio of 15:1 (Table 2).

Discussion

Scutellar cells of maize embryos are the most commonly used as target tissues for recovering transgenic plants, due to their ability to induce and maintain high embryogenic callus induction frequency (Bommineni and Jauhar 1997; Ishida et al. 2003; Lupotto et al. 2004). A prerequisite for successful transfer of the transgenes into plant cells is the

Table 2 Segregation analysis of PTL02 in T_1 transgenic plants

Transgenic line	Number of T_1 plants					
	Total number of plants	*Gus* positive	*Gus* negative	Observed segregation ratio	Expected segregation ratio	χ^2
PTL102-1	12	9	3	3:1	3:1	0
PTL102-2	31	23	8	3:1	3:1	0.01
PTL102-3	32	29	3	15:1	3:1	1.07

availability of target tissues which are actively dividing, amenable to gene transfer and which have a large number of regenerable cells (Birch 1997). Due to lack of efficient transformation and regeneration procedures, the application of *Agrobacterium* mediated transformation has not been utilized for tropical maize genotypes available in Kenya. The present study provides a platform for the genetic transformation and regeneration of maize genotypes in Kenya and describes for the first time the successful transformation of tropical maize genotypes.

In this study, necrosis and death of some of the embryos and pieces of callus infected with *Agrobacterium* strains resulted in low transformation frequencies. Maize tissues co-cultivated with *Agrobacterium* have been reported to result to rapid tissue necrosis and cell death (Hansen 2000; Karthikeyan et al. 2012). Tissue browning, necrosis, cell death after *Agrobacterium* infection have been reported to be some of the major factors which reduce the efficiency of *Agrobacterium* transformation in many crops. This has been attributed to hypersensitivity defence mechanisms of plants to *Agrobacterium* infection (Kuta and Tripathi 2005; Shrawat and Lörz 2006).

The frequency of bialaphos resistant callus maize lines were found to be genotype dependent and varied with the *Agrobacterium* strain used for the infection. Similar results have been reported in maize inbred lines (Frame et al. 2006). Several factors that influence *Agrobacterium*-mediated transformation and recovery of stable monocotyledonous plants, including cereals have been investigated and elucidated (Ali et al. 2007; Carvalho et al. 2004; Cheng et al. 2004; El-Itriby et al. 2003; Frame et al. 2006; Hiei et al. 1997; Jones 2005; Huang and Wei 2005; Opabode 2006; Shrawat and Lörz 2006; Kumar et al. 2011; Sharma et al. 2011). These factors include; genotype, type and developmental stage of the infected explant, type and concentration of *Agrobacterium* strains, binary vector, selectable marker genes and promoters, inoculation and co-culture conditions and tissue culture and regeneration media. However a major drawback in utilizing *Agrobacterium* for routine introduction of genes of interest in major cereals is the competence of the *Agrobacterium* to infect specific tissue, genotype or species and this posses a challenge in the future of developing transgenic plants (Shrawat and Lörz 2006). There is therefore need to assess a wide range of genotypes to determine those with higher transformation frequency.

Putative transgenic maize T_o plants were recovered following selection of IZEs on bialaphos after they were infected with EHA101(pTF102) and AGL1(pSB223). However there were significant differences on the number of putative plants regenerated among the maize lines.

High number of embryos with blue staining was observed on the 3rd day of co-cultivation compared to the 4th day showing that transient *gus* expression is dependent on the number of days of co-cultivation. Co-cultivation period has been reported to influence *gus* expression in blueberry (Pandey et al. 2010), maize (Huang and Wei 2005), lettuce, tomato and *Arabidopsis* (Wroblewski et al. 2005) and sweet potatoes (Xing et al. 2007). This could be attributed to environmental conditions under which the plants from which the explants are obtained are grown.

The expression of the *gus* in some IZEs was observed mostly at the edges. Similar observations were made when roots and leaf explants of T_o plants were assessed for *gus* activity. Staining of the cut edges of the leaf segments due to *gus* activity has also been reported in AT-3 maize genotype (Chumakov et al. 2006). This may be attributed to uneven or poor penetration of xGluc into the tissues (Wroblewski et al. 2005). Intensity of blue staining as a result of *gus* activity observed in the younger tissues was higher compared to that in mature and older ones. Similar results have been reported in other plants (Sudan et al. 2006). The *gus* expression has been reported to fade with age after the transformed tobacco plants were transferred into the greenhouse conditions (Kuvshinov et al. 2004).

The number of embryos which showed transient *gus* activity was higher compared to those which survived during selection, to form callus and plants. This is could be due to transient expression where the transgene is transferred into the cytoplasm of the plant cell but stable integration into the maize genome does not occur. The number of putatively transgenic plants formed was relatively low. This could be due to the conditions during integration of the transgenes and also in the recovery of the plants from the cells with the integrated transgenes.

Differential *gus* expression was noted among different plant tissues with higher blue staining intensity detected in IZEs compared to embryogenic callus of the same line.

This may be due to low penetration of the *Agrobacterium* suspensions into the callus tissues compared to IZEs which are small and thinner. Freshly isolated IZEs have been reported to be the best explant for successful *Agrobacterium*-mediated genetic transformation in cereals due to their competency (Bommineni and Jauhar 1997; Shrawat and Lörz 2006). Differences in expression of *gus* has been reported in sorghum (Carvalho et al. 2004) and maize genotypes (Songstad et al. 1996). Strong *gus* expression has been reported in the roots, leaves and stem but poorly expressed in the pollen (Songstad et al. 1996). Differences in the intensity and percentage area with blue staining due to *gus* activity in IZEs of the same or various maize line(s) when infected with the same *Agrobacterium* strain was detected. For example immature embryo explants which were infected with *Agrobacterium* strains harbouring pCAMBIA2301 vector exhibited high intensity of blue staining compared to other *Agrobacterium* strains. The pCAMBIA2301 vector has many inserts of the *gus* gene which could have contributed to high intensity of blue staining. In addition, these differences could have been influenced by compatibility between the genotype and *Agrobacterium* strain. The sensitivity of *Arabidopsis* cells to bacterial strain has been attributed to differences in their attachment or differences in bacterial or plant encoded T-DNA transfer machinery (Nam et al. 1997). Differences in the ability of the *Agrobacterium* strains to transfer transgenes and subsequent transient *gus* activity has also been reported in other maize genotypes (Huang and Wei 2005), pigeonpea (Surekha and Arundhati 2007), rice (Al-Forkan et al. 2004) and switch grass (Song et al. 2012).

Susceptibility of IZEs to *Agrobacterium* infection was genotype dependent. Differences in susceptibility of the genotypes to *Agrobacterium* infection may also be due to the presence of inhibitors of *Agrobacterium* sensory machinery and their competence. The presence of inhibitors such as DIMBOA, a major organic exudate released in varying amounts in different genotypes of maize specifically inhibits the induction of the *vir* gene expression (Zhang et al. 2000). The level of *gus* activity after co-cultivation of callus with *Agrobacterium* strain in two rice cultivars has been reported to be genotype dependent (Saharan et al. 2004). The difference in the competence of *Agrobacterium* to infect particular tissues and genotype has been a major drawback in the genetic transformation of elite cultivars of cereals. The *gus* expression was not observed in the roots of T_o plants of A188 in the present study. This is contrary to the results reported previously in which A188 maize genotype had a higher efficiency of expression of *gus* activity in all tissues tested following inoculation with *A. tumefaciens* compared to other genotypes (Ritchie et al. 1993).

PCR amplification of the transgene is often taken as an indication of the transfer of transgene into the regenerants. However, southern blot hybridization analysis is essential to prove the integration of transgene into the host genome and can also be used to assess the number of copies of the transgenes inserted (Ishida et al. 1996). Single digestion of pTF102 vector was carried out which released the full T-DNA cassette. The transfer of the transgenes (*bar* and *gus*) into the maize cell of T_o and T_1 plants was established by the use of PCR (Fig. 3a).

Stable integration of transgene was achieved only in three of T_1 transformants of PTL02 maize line with a transformation efficiency of 1.4 %. This shows that the formation of stable transgenic plants is not always related to the transient *gus* expression frequencies (Wang et al. 2012). Under many conditions increased T-DNA delivery do not result in increased stable transformation despite the fact that efficient T-DNA delivery is a requirement for efficient transformation in most cases (Cheng et al. 2004). Transformation efficiency ranging from 40.2 to 48.9 % has been reported when IZEs of inbred line A188 were inoculated with *Agrobacterium* strain LBA4404 harboring pSB13 vector using an improved protocol (Ishida et al. 2003). A transformation efficiency of 5.5 % (Frame et al. 2002) compared to 33–51 % (Zhao et al. 1998) based on the surviving events of HiII maize hybrid line has been reported. Transgenic plants with a transformation frequency of 30 % have been recovered from IZEs inoculated with *A. tumefaciens* (Negrotto et al. 2000). The lower transformation efficiency obtained in the present study may be due to low efficiency of T-DNA integration into the plant genome (Tie et al. 2012).

Inspite of having PCR positive plants for *gus* and *bar* in T_o plants of inbred (TL18, CML216 and A188) and H627 hybrid lines stable integration was not detected in T_1 plants. This could be due to the fact that some of the putative transformants in T_o generation did not grow to maturity to synchronize in silk and maturation of pollen grains for fertilization to take place. Thus some of the transformants did not produce seeds for the inheritance of the transgene to be assessed in T_1 generation. Failure to obtain transgenic plants from IZEs of other tropical maize inbred lines infected with *A. tumefaciens* has been reported previously (Horn et al. 2006). Forty-seven percent of the primary transgenics failed to yield progeny carrying the transgene (Joersbo et al. 1999). The success of maize genetic manipulation requires not only the ability to deliver transgenes into the cell, but also to produce many transgenic plants which stably inherit, express the transgene in a predictable and stable way over generations to be useful in plant breeding programs.

The number of copies of transgenes detected using southern blot hybridization ranged between 2 and 6 in PTL02. An estimated transgene copy number in a range of 1–5 has been reported when Hi II maize line was transformed with *Agrobacterium* strain (Frame et al. 2002; Zhao et al. 1998). Low transgene copies could help reduce the possibility of gene silencing and increase the stability of the transgenes (Diallo et al. 2001).

In conclusion, a critical step in *Agrobacterium*-mediated transformation is the establishment of optimum conditions of T-DNA delivery into the plant tissues from which plants can be regenerated. Maize genotype and day of co-cultivation were found to have a significant effect on transient *gus* expression and subsequent transformation. Results on southern blot hybridization analysis provided proof of successful transgene integration into genome of PTL02 maize line via *Agrobacterium*-mediated transformation while segregation experiment confirmed inheritance of the inserted gene (*gus*).

To the best of our knowledge this is the first report on successful *Agrobacterium* mediated transformation of tropical maize lines in Kenya.

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