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ORIGINAL PAPER

Somatic embryogenesis and plant regeneration of tropical maize genotypes

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Abstract In Latin America and sub-Saharan Africa, tropical maize (*Zea mays* L.) is a major crop for human consumption. To cope with the increasing population and changing environment, there is a need for improving tropical maize germplasm. As part of a biotechnological approach, efficient in vitro regeneration of two tropical maize inbred lines (CML216 and CML244) was established. A number of parameters were optimized, such as age of the immature embryos, plant media and growth regulator concentration. After 6 weeks of culture, somatic embryos that had already reached the coleoptilar stage produced shoots after light induction and developed into fertile plants after acclimation in the soil. The callus induction frequencies and somatic embryo-derived plantlet formation were higher when cultured with the Linsmaier and Skoog medium than those with the Chu's N6 basal medium. Regeneration of tropical maize shoots depended on the 2,4-dichlorophenoxyacetic acid (2,4-D) concentration at the callus initiation stage from immature embryos. The recalcitrance of the tropical maize inbred line TL26 to in vitro regeneration was overcome in a single-cross hybrid with the CML216 and CML244 genotypes. Remarkably, tropical maize somatic embryos were formed at the abaxial side of the scutellum facing the medium, probably from the axis of the immature embryos, as shown by histological sections. Upon co-cultivation, agrobacteria transiently expressed their intronless β-glucuronidase-encoding gene at the embryogenic tissue, but not with an intron-containing gene, suggesting that virulence genes are induced in *Agrobacterium*, but that subsequent steps in the T-DNA transfer are inhibited.

Keywords Tropical maize · Callus induction · Somatic embryogenesis

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
bar	Phosphinothricin acetyltransferase-encoding gene
CIM	Callus induction medium or resting medium
CMM	Callus maturation medium
ESM/Inf	Embryo suspension medium/infection medium
GUS	β-glucuronidase
LS	Linsmaier and Skoog
MES	2-(*N*-morpholino)ethanesulphonic acid
MS	Murashige and Skoog
N6	Chu's N6 basal medium
P35	Cauliflower mosaic virus 35S promoter
PIPES	Piperazine-*N,N*-bis(2-ethanesulphonic acid)
R0	Regenerated shoot from somatic embryo
R1	Progeny of R0 shoot
SIM	Shoot induction medium or regeneration medium
T-DNA	Transfer DNA

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T35S	Cauliflower mosaic virus 35S terminator
Tnos	Nopaline synthase terminator
UBIL	Maize long ubiquitin promoter
uidA	β-D-glucuronidase gene from *Escherichia coli*
X-Gluc	5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid
YEP	Yeast Extract Peptone

Introduction

Maize (*Zea mays* L.) is a C4 crop with a remarkable ability to maintain high rates of photosynthetic activity with an important grain and biomass yield potential. It is mainly a cross-pollinating species, a feature that has contributed to its broad morphological and genetic variability and geographical adaptability. Maize is classified in tropical and temperate varieties that grow at different latitudes and under different climatic conditions. Tropical maize is indigenous to tropical regions, including Latin America and sub-Saharan Africa (Anami et al. 2009). Economically, maize is an important crop, boasting a multibillion dollar annual revenue (Schnable et al. 2009). Large collections of maize germplasm exist, such as the International Maize and Wheat Improvement Center (CIMMYT), which possesses inbreds, hybrids, synthetic cultivars, open-pollinating varieties, and improved landraces, and represent resources for genetic diversity to be used in breeding programmes and agriculture.

In addition to its agronomic importance, maize is a model organism for basic and applied research (Strable and Scanlon 2009). Its genome is diploid, with a size of 2.3 gigabase, and contains more than 32,000 genes as determined in the temperate B73 line (Schnable et al. 2009). The genome of the ancient Mexican tropical landrace, Palomero Toluqueno, has been sequenced as well and is 22% smaller than that of B73, but a high number of identical sequence regions across all chromosomes are present in both genotypes. The study of specific loci between the Palomero landrace and Teosinte wild relatives showed the impact of the local environment on domestication (Vielle-Calzada et al. 2009).

Thus far, tropical maize improvement relied on conventional breeding and clonal selections for elite maize types that were multiplied for distribution to growers, especially in developing countries. Nevertheless, the success of this strategy is limited because of low genetic variations in the gene pools as a result of domestication, high costs and long generation times to produce a single improved selection (Xu et al. 2009). Future demands require new and ameliorated high-yield cultivars. A biotechnological approach combining tissue culture and genetic transformation with genes of interest can generate genetically transformed germplasm (Anami et al. 2009). Although tropical maize genotypes have been regenerated through immature embryo culture (Prioli and da Silva 1989; Bohorova et al. 1995; Carvalho et al. 1997; Oduor et al. 2006), neither a routine regeneration system has been established yet, nor has its response to *Agrobacterium tumefaciens* infection through transient β-glucuronidase (GUS) assay been reported.

Here, the tropical maize inbred lines CML216 and CML244 were chosen because they grow well under field conditions with excellent synchronization of both pollen and silk. Both genotypes are small in size (150 cm in height), allowing an easily controlled pollination. These lines have a short generation time (~3–4 months) and, in addition, they tolerate drought stress to a certain extent. The inbred line TL26 was selected for its prevalence in breeding programmes in Kenya to develop highland maize that grows in a high rain-fed environment.

In vitro culture conditions were optimized for efficient embryogenic callus induction from immature embryos of tropical maize genotypes and resulted in highly efficient shoot regeneration. Remarkably, callus was induced from the embryo axis side. *Agrobacterium* strains armed with a T-DNA binary vector compatible with Gateway recombinational cloning and that carry a *GUS* reporter gene were used to assess the competence of the tissue for infection. The results will be discussed in the context of the future establishment of genetic transformation in tropical maize genotypes.

Materials and methods

Maize inbred lines

Seeds of the tropical maize (*Zea mays* L.) inbred lines CML216 and CML244 were obtained from CIMMYT (Kenya). A local Kenyan tropical inbred line TL26 was supplied by the Kenya Agricultural Research Institute (Nairobi, Kenya). Seeds from the temperate maize genotype B104 were obtained from the Maize Genetics and Genomics Database (Iowa State University, Ames, IA, USA). The tropical genotypes were grown under field conditions at Kenyatta University (Nairobi, Kenya), while the temperate genotype (B104) was grown under controlled greenhouse conditions at Ghent University (Gent, Belgium). The first ears that appeared on the tropical maize plants were covered with bags before silk emergence and self-fertilized when the optimum length for silk receptivity had been reached to obtain immature embryos. Crosses were made between CML216 and CML244 as maternal

donor and TL26 as pollen donor (CML216 × TL26 and CML244 × TL26).

In vitro plant growth conditions

Ears of the three tropical maize inbred lines and of the temperate genotype (B104) were harvested from the field and from the greenhouse 14–16 and 12 days after pollination, respectively, and dehusked. The kernels were sterilized in 70% ethanol for 3 min and for 20 min in a 2.5% (v/v) sodium hypochlorite solution containing a drop of polyoxyethylene (20) sorbitan monolaurate in an airflow cabinet and rinsed four times with double-distilled water. The kernel crowns (1–2 mm) were removed with a sterile scalpel blade and immature embryos (1–1.5 mm in size) were excised by inserting a spatula between the endosperm and the pericarp, releasing the endosperm from the seed coat and exposing the embryo.

Immature embryos were transferred to sterile Petri dishes containing 20 ml of embryo suspension medium (ESM) containing Linsmaier and Skoog (LS) (Linsmaier and Skoog 1965) or N6 salts and vitamins (Chu et al. 1975; Negrotto et al. 2000) (Table 1). Immature embryos were plated on callus induction medium (CIM) (Table 1), oriented with the embryo axis facing the medium (scutellum side up). Plates were wrapped with Urgopore gas-diffusible tape (Chenôve, France) and incubated in the dark at 28°C. After 2 weeks on CIM, calli were transferred to callus maturation medium (CMM) with the same composition as CIM, but without silver nitrate and a sucrose concentration of 60 mg l^{-1} (Table 1). Thereafter, individual clones of calli were transferred to shoot induction medium (SIM) containing Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog 1962) (Table 1) without growth regulators and cultivated at 28°C with 80 µM m^{-2} s^{-1} light intensity and a 16-h light/8-h dark cycle.

Agrobacterium strain and vector

For the co-cultivation of immature embryos, *Agrobacterium tumefaciens* strain EHA101 (Hood et al. 1986) was used that harbored either vector pXBb7-SI-UBIL or vector pBbm42GW7_GUS that both contained the *uidA* gene (*gus*) under the control of the ubiquitin promoter (UBIL) (Fig. 1). Both vectors had a selectable marker gene cassette consisting of the phosphinothricin acetyltransferase gene (*bar*) driven by the cauliflower mosaic virus 35S promoter (P35S) and a nopaline synthase terminator (T*nos*) (Kay et al. 1987). The pXBb7-SI-UBIL vector contained a reporter gene cassette in which the *GUS* reporter coding sequence is interrupted with a potato invertase (PIV2) intron (UBIL-gus-int) (Karimi et al. 2007), whereas the pBbm42GW7_GUS vector carries a *UBIL-gus* reporter gene without intron. The detailed structure of the plasmids will be presented elsewhere.

Agrobacterium infection of immature embryos of tropical and temperate maize

Bacterial inoculation and co-cultivation with immature maize embryos was done according to Negrotto et al. (2000) and Frame et al. (2002, 2006) with modifications. Immature zygotic embryos, 1–1.5 mm in length, were placed in LS or MS infection (Inf) medium supplemented with 200 µM of acetosyringone in sterile Petri dishes (90 × 15 cm) and washed twice with the medium (Table 1). *Agrobacterium* cultures were grown for 3 days at 28°C on Yeast Extract Peptone (YEP) medium supplemented with 100 mg l^{-1} spectinomycin and 50 mg l^{-1} kanamycin. One full loop

Table 1 Media composition for maize tissue culture

Organic/inorganic supplements + vitamins	ESM/Inf			CCM			CIM			CMM		SIM	
	LS	N6	MS	LS	N6	MS	LS	N6	MS	LS	N6	MS	MS
Casein hydrolysate (mg l^{-1})	100	100	100	–	–	–	–	100	–	–	–	–	–
2,4-D (mg l^{-1})	2	1.5	1.5	2	1.5	1.5	2	1.5	1.5	–	2	–	–
Sucrose (g l^{-1})	68.4	68.4	68.4	30	30	30	30	20	30	60	60	30	20
Glucose (g l^{-1})	36	36	36	36	36	–	–	–	–	–	–	–	–
L-Proline (g l^{-1})	–	–	0.7	–	–	–	0.7	2.9	0.7	0.7	2.9	–	–
MES (g l^{-1})	–	–	–	0.5	–	–	0.5	0.01	0.5	0.5	0.01	0.5	–
$AgNO_3$ (mg l^{-1})	–	–	–	1.6	0.85	0.85	1.6	0.85	0.85	–	–	–	–
Acetosyrigone (µM)[a]	200	100	100	200	100	100	–	–	–	–	–	–	–
Glycine (mg l^{-1})	–	–	–	–	–	–	–	2	–	–	2	–	2
pH	5.8	5.8	5.2	5.8	5.8	5.2	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Cefotaximum (mg l^{-1})	–	–	–	–	–	–	100	100	100	100	100	–	–
Gelrite (g l^{-1})	–	–	–	3	3	3	3	3	3	3	3	3	3

[a] Acetosyringone was only used during embryo infection and co-cultivation with *Agrobacterium*

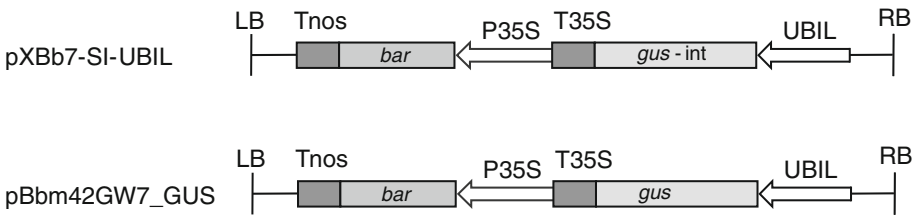

Fig. 1 T-DNA regions of pXBb7-SI-UBIL and pBbm42GW7_GUS vectors. The pXBb7-SI-UBIL contains a PIV2 intron in the *uidA* gene, the pBbm42GW7_GUS vector does not contain an intron. *LB* left border, *RB* right border, *bar* phosphinothricin acetyltransferase gene, *Tnos* nopaline synthase terminator, *P35* cauliflower mosaic virus 35S promoter, *T35S* cauliflower mosaic virus 35S terminator, *UBIL* maize long ubiquitin promoter, *gus uidA* gene, *gus-int uidA* gene interrupted by a PIV2 intron

(3 mm) of bacterial culture was scraped from the 3-day-old plate and suspended in 5 ml of the liquid infection medium supplemented with 200 µM acetosyringone in a 50-ml Falcon tube. The tube was fixed horizontally on a bench-top shaker and shaken at low speed, approximately 100 revolution per min (rpm) for 5 h at 28°C. Upon removal of the final wash of the immature embryos, 5 ml of the *Agrobacterium* suspension diluted with 40 ml of sterile LS or MS infection medium were added, the Petri dish gently swirled 10 times, and incubated for 5 min. After infection, embryos were transferred to LS or MS co-cultivation medium (CCM) (Negrotto et al. 2000; Frame et al. 2006; Table 1) at pH 5.8 or 5.2 for tropical and temperate maize, respectively and excess *Agrobacterium* suspension was pipetted off the medium surface. Embryos were oriented with the embryo axis in contact with the medium (scutellum side up). Plates were wrapped with parafilm and incubated in the dark for 3–5 days at 28°C. Subsequently, immature embryos were transferred on CIM for 5 days and assayed histochemically for GUS activity.

Histochemical GUS assay

GUS activity was assayed (Jefferson 1987) on infected immature embryos incubated for 5 days on CIM. The embryos were fixed in 3% formaldehyde, 0.3 M mannitol and 10 mM 2-(*N*-morpholino)ethanesulphonic acid (MES) for 1 h at room temperature in 1.5-ml Eppendorf tubes, washed three times in 50 mM sodium dihydrogen phosphate, and incubated for 12 h at 37°C in a buffer containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) and 50 mM NaH_2PO_4 at pH 7.0. Transient GUS activity was scored per embryo.

Histological study of somatic embryogenesis in tropical maize

Tissues of tropical maize immature embryos cultured on CIM for 1 week were fixed overnight in 4% paraformaldehyde in 50 mM piperazine-*N*,*N*-bis(2-ethanesulphonic acid) (PIPES buffer, pH 8.0) followed by two 15-min washes in 50 mM PIPES buffer and serial washes (20 min each) in a graded ethanol series followed by a xylene series (Bancroft and Stevens 1990). Tissue samples were embedded in paraffin wax and 10-µm blocks were sectioned with a rotary microtome (Reichert Histostat, Germany). Sections were dewaxed with xylene and stained with toluidine blue and examined under a light microscope. Images were taken at various stages of somatic embryo development, from nascent pre-embryo mass on.

Results

Callus induction from tropical maize immature embryos

The ability to induce embryogenic callus was assessed in immature embryos 14–16 days after fertilization, ranging from 1 to 1.5 mm in size. The immature embryos of the lines CML216, CML244, and TL26 were incubated for 2 weeks on CIM with the scutellum side up and the embryo axis facing the medium. Generally, callus initiation was observed from the fourth day of culture. Tissues swelled, increased remarkably in volume, and maintained their healthy light yellow appearance, except for inbred line TL26 that turned brown and became organogenic. Callus was induced only on 2,4-dichlorophenoxyacetic acid (2,4-D)-supplemented CIM, because lack of 2,4-D resulted in precocious germination of immature embryos. The number of immature embryos producing callus was higher on LS than on N6 basal medium with a callus induction frequency of 96 and 73% and 96 and 80% in CML216 and CML244, respectively (Table 2).

After 2 weeks of culture on CIM, the nascent calli were transferred to CMM and 2 weeks later a lot of somatic embryos were observed (Fig. 2a). Embryogenic callus was induced exclusively from cells at the embryo axis (Fig. 2b), in contrast to the temperate maize B104 that formed embryogenic callus from the upper scutellum side (Fig. 2c); it was visualized in histological sections as meristematic foci at the surface of the explants that formed recognizable somatic pro-embryos held by suspensor cells (Fig. 2d) after

Table 2 Influence of LS and N6 basal media on callus induction from 14- to 16-day-old immature embryos in selected tropical maize inbred lines supplemented with 2 mg l^{-1} 2,4-D

Genotype	Embryos	Medium	Callus-forming embryos	Callus induction frequency (%)
CML216	303	LS	292[a]	96
CML216	85	N6	58[a]	70
CML244	495	LS	477[a]	96
CML244	460	N6	370[a]	80
TL26	290	LS	213[b]	73
TL26	190	N6	126[b]	66

The results were obtained based on six independent experiments for each of the genotypes. Callus induction was observed after 4 days of culture on CIM

[a] Embryos forming embryogenic callus

[b] Embryos forming nonembryogenic, watery callus that turned brown and became organogenic

2 weeks on CMM. The pro-embryos developed into coleoptile-shaped single somatic embryos that were transferred to SIM after 4 weeks of culture on CMM.

Competence of tropical and temperate maize immature embryos to *Agrobacterium* infection

The different position of the callus induction of the tropical CML216 and temperate B104 lines prompted us to test their susceptibility to *Agrobacterium* infection by means of two novel Gateway vectors pBbm42GW7_GUS and pXBb7-SI-UBIL, with a p35S-*bar* selectable marker and the pUBIL promoter driving an intronless and intron-containing *GUS* gene, respectively. MS and LS media at pH 5.2 and 5.8 were used during infection and co-cultivation according to D'Halluin et al. (2007) and Frame et al. (2002, 2006), respectively (Table 3). The pH is a parameter that influences the induction of the *Agrobacterium* virulence functions (Mondal et al. 2001; Yu et al. 2004). Immature zygotic embryos were co-cultivated with *Agrobacterium* strain EHA101 harboring pXBb7-SI-UBIL and pBbm42GW7_GUS (Fig. 1) for 3 days, subsequently cultured on CIM medium for 5 days, and assayed for GUS activity. Control embryos were not exposed to *Agrobacterium* but cultured similarly. When the intronless GUS vector, pBbm42GW7_GUS was used (Fig. 2e, g), blue spots were observed beneath and on top of the scutellum region in the tropical and temperate genotypes, respectively. With the pXBb7-SI-UBIL vector with an intron-containing *GUS* gene, no blue spots were visible in the tropical maize immature embryos in contrast to the temperate maize embryos (Fig. 2f; Table 3).

Regeneration improvement of the recalcitrant inbred TL26 by hybridization

The addition of 2 mg l^{-1} 2,4-D in CIM was optimal for embryogenic callus initiation from immature embryos of the tropical maize lines CML216 and CML244, but non-embryogenic and watery callus was initiated from the recalcitrant TL26 line at this concentration. Therefore, CML216 and CML244 were crossed with TL26 (pollen donor) and the callus induction from immature embryos was investigated on CIM with 2,4-D concentrations ranging from 0, 2, 4, 6, 8 to 10 mg l^{-1}. At 2 mg l^{-1} 2,4-D, embryogenic callus was induced in both hybrids. The CML216 × TL26 hybrid was the most efficient because 100% of the immature embryos formed embryogenic callus at all 2,4-D concentrations, whereas the efficiency of the CML244 × TL26 hybrid was the highest at 2 mg l^{-1} 2,4-D and decreased with increasing concentrations (Table 4), indicating that the embryogenic callus induction capacity was inherited maternally. High concentrations of 2,4-D (10 mg l^{-1}) induced callus formation in the hybrids, but the subsequent maintenance of the callus on the same medium resulted in nonembryogenic and rhizogenic calli. In the inbred line TL26, no callus was induced at 2,4-D concentrations as high as 6–10 mg l^{-1}. In conclusion, optimal embryogenic type-II callus induction and plant regeneration from the F1 hybrids was achieved when the immature embryos were incubated on a medium containing 2.0 mg l^{-1} 2,4-D.

Regeneration of somatic embryos and acclimatization into plantlets

Shoots of embryogenic callus were regenerated on growth regulator-free SIM media supplemented with 30% sucrose (Table 1). Mature embryos turned green after 4–5 days on SIM under continuous illumination and shoots were formed within 1 week (Fig. 2h). CML216 produced 167 regenerated plants, followed by CML244 with 33 shoots (Table 5), whereas the TL26 inbred line formed calli that were difficult to maintain on CMM, were nonembryogenic, and failed to regenerate. The single-cross hybrids CML216 × TL26 and CML244 × TL26 formed embryogenic callus that resulted

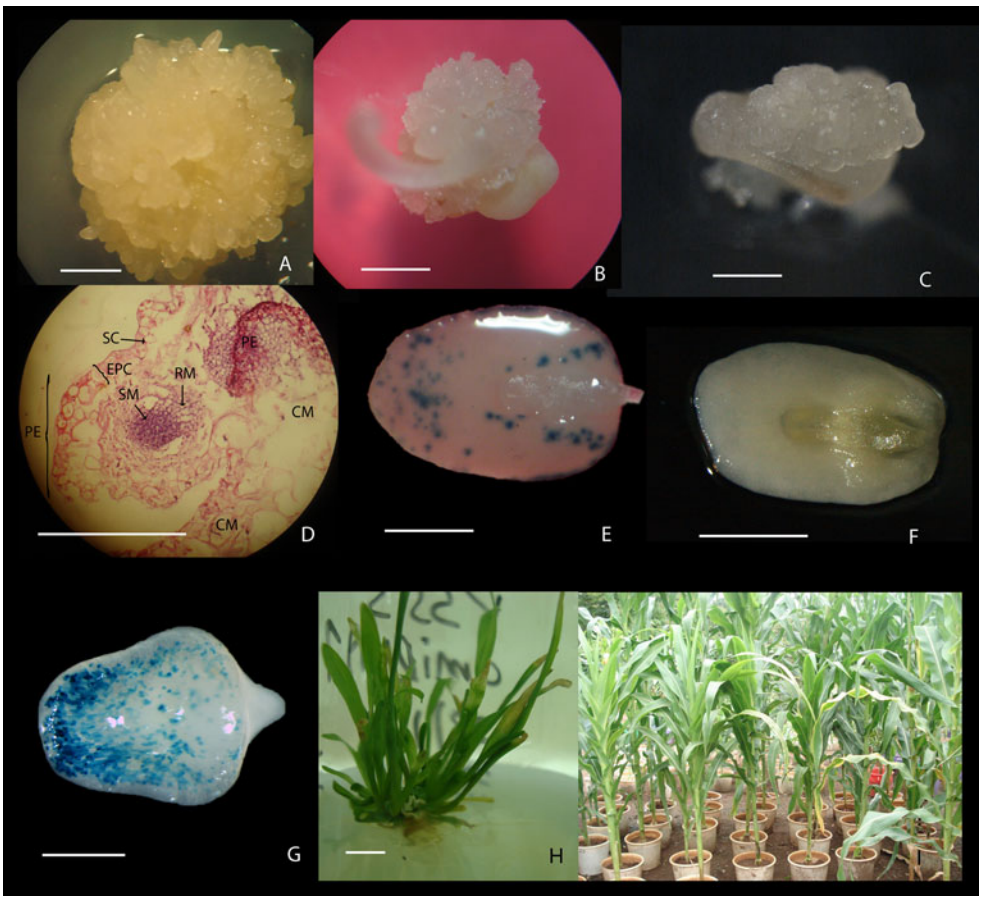

Fig. 2 Somatic embryogenesis in tropical maize inbred genotype CML216 and temperate maize inbred line B104. **a** Proliferating embryogenic callus from tropical maize immature embryos, 2 weeks after culture on CMM. **b** Callus induction at the embryo axis site in tropical maize CML216. **c** Callus induction on top of the scutellum in temperate maize genotype B104. **d** Histological section through a tropical maize somatic embryo. **e** Transient GUS activity in an immature embryo of tropical maize CML216. **f** Immature embryo of tropical maize genotype negative for transient GUS activity when an intron-containing GUS vector (pXBb7-SI-UBIL) was used in co-cultivation. **g** Temperate maize B104 with the intron-containing vector pXBb7-SI-UBIL. **h** Regeneration of tropical maize shoots on hormone-free SIM. **i** Normal viable tropical maize regenerants in pots in the field. *CM* callus mass, *EPC* epidermal cells, *PE* pro-embryo cells, *RM* root meristem, *SC* suspensor cells, *SM* shoot meristem

Table 3 Competence of tropical and temperate maize immature embryos to *Agrobacterium* infection

Genotype	Vector	pH	Medium	Embryos	GUS-positive embryos	Infection efficiency (%)	Dead embryos
B104	pXBb7-SI-UBIL	5.2	MS	88	12	14	76
B104	pBbm42GW7_GUS	5.2	MS	91	24	26	71
B104	pXBb7-SI-UBIL	5.8	LS	103	103	100	0
B104	pBbm42GW7_GUS	5.8	LS	160	160	100	0
CML216	pXBb7-SI-UBIL	5.2	MS	50	0	0	0
CML216	pBbm42GW7_GUS	5.2	MS	25	25	100	0
CML216	pXBb7-SI-UBIL	5.8	LS	55	0	0	0
CML216	pBbm42GW7_GUS	5.8	LS	54	54	100	0

in 24 and 14 shoots, respectively. The in vitro rooted plants were acclimatized by transfer to peat moss-containing pots covered with plastic sheets to maintain high moisture conditions for 3 days. The moisture was gradually reduced over a period of 15 days and plantlets were transferred to large soil-filled 20-liter pots in the field (Fig. 2i).

Table 4 Influence of 2,4-D on callus induction of TL26 inbred line and its single-cross hybrids

2,4-D (mg l^{-1})	TL26		CML244 × TL26		CML216 × TL26	
	Embryos	Nonembryogenic callus induction (%)	Embryos	Callus induction (%)	Embryos	Callus induction (%)
0	40	0	62	0	39	0
2	46	96	61	100[a]	42	100*
4	48	73	51	64	42	95
6	44	0	59	26	42	100
8	42	0	95	20	34	100
10	40	0	38	21	42	100

[a] Embryogenic callus

Phenotypic characteristics of regenerated plantlets at the reproductive stage

It is well known that some tissue culture conditions induce morphological abnormalities in R0 shoots that are (non)-heritable in the R1 progeny, so-called somaclonal variations. We observed abnormal phenotypes in R0 regenerants during the reproductive phase, including emergence of both the tassel and silk from the tassel stalk resulting in tassel seeds (Fig. 3a). Other somaclonal variations occurring at low frequency included multiple ears, leaves emerging from the same node (Fig. 3b, c), and a reduced number of tassels (Table 6). These defects were more pronounced in the regenerants from the hybrids than from inbred lines, suggesting that genetic factors might be responsible for the major differences between hybrids and inbreds because heterochromatic knobs might undergo alterations in culture leading to mitotic disturbance, a possible genotype-dependent response (Fluminhan and de Aguiar-Perecin 1998) or, alternatively, result from the activation of transposable elements in the hybrids (Williams et al. 1990). The higher the level of 2,4-D in the medium, the more severe the malformations. The morphological defects in the R0 shoots were not inherited in the R1 progeny and might have originated from temporary hormone imbalance caused by the growth regulators in the media.

Discussion

Efficient genetic transformation for plant improvement allows the introduction of useful agronomic traits without altering the features of the cultivar, but necessitates the development of in vitro systems for the genetic transformation and plant regeneration. Indeed, regeneration and transformation in tropical and temperate maize have been achieved by both organogenesis and somatic embryogenesis (O'Connor-Sánchez et al. 2002; Sairam et al. 2003; Al-Abed et al. 2006; Valdez-Ortiz et al. 2007). T0 shoots regenerated from somatic embryos are usually clonal because these origininated from single somatic cells as compared to T0 shoots regenerated through organogenesis that are often chimeric because of a multicellular origin. Tropical and temperate maize transformation has been facilitated by protoplast transformation (Golovkin et al. 1993) and particle bombardment (Gordon-Kamm et al. 1990; Shepherd et al. 2007), but, the preferred method is *Agrobacterium*-mediated transformation (Frame et al. 2006; Valdez-Ortiz et al. 2007) that results in low-copy numbers of intact transgenic events, which are stable and less prone to silencing (Ishida et al. 1996; Zhao et al. 1998). In vitro regeneration systems for tropical genotypes have been reported in the literature, but routine regeneration systems have not been established (Prioli and da Silva 1989; Bohorova et al. 1995; Carvalho et al. 1997; Oduor et al. 2006) and, additionally, no regeneration response of tropical maize genotypes upon co-cultivation with *Agrobacterium* has been reported. The availability of an in vitro regeneration system for tropical maize is a prerequisite for effective genetic transformation.

The composition of the culture medium is known to affect the embryogenic response of maize tissue culture (Armstrong and Phillips 1988). Here, the highest frequency

Table 5 Regeneration and survival during acclimatization of tropical inbred lines and their single-cross hybrids

Genotype	Embryos	Regenerants	Acclimatized plants	Plantlet survival efficiency (%)[a]
CML216	192	167	40	24
CML244	25	33	8	24
CML216 × TL26	24	24	20	83
CML244 × TL26	14	14	4	29

[a] Seedlings that survived during acclimatization

Fig. 3 Somaclonal variations in tropical maize R0 regenerants. **a** Silks emerged from the tassel, hence tassel seeds. **b** Two ears emerging from the same node. **c** Leaves of regenerants emerging from the same node. **d** Dwarf phenotype of a regenerant without tassel and with leaves emerging from the bottom of the ears

Table 6 Reproductive structures of R0 regenerants

Genotype/cross breed	Pedigree	Ears/plant	Ears silking/plant	Tassel branches/plant
TL26	Inbred	3	1	40
CML244	Inbred	2	1	9
CML216	Inbred	3	1	11
CML244	Regenerants	1	1	4
CML244	Regenerants	1	1	1
CML216	Regenerants	2	1	4
CML216	Regenerants	2	1	2
CML216 × TL26	Regenerants	2	1	12
CML244 × TL26	Regenerants	3	1	12

of embryogenic callus formation was recorded on LS medium supplemented with 2 mg l^{-1} 2,4-D. Compared to N6, the LS medium is highly enriched with additional micronutrients that include cobalt (II) chloride hexahydrate, copper sulphate 5-hydrate, sodium molybdate dihydrate, and the macronutrient ammonium nitrate and, in addition, has 19.34% more total reduced nitrogen in the form of ammonia and 70.7% nonreduced nitrogen in the form of nitrates. In contrast, N6 has 235% more combined phosphates and 261% sulphates than LS, suggesting that nitrates might be more critical in the initiation of callus than phosphates and sulphates. Similar results have been reported for the tropical CIMMYT inbred lines from Mexico that induced more callus on MS than on N6 medium (Shohael et al. 2003). In fact, the composition of LS and MS media is similar, except for the organic supplements.

Here, a single cross between the tropical inbred lines CML216 × TL26 and CML244 × TL26 enhanced the regeneration capability in tissue culture of the TL26

genotype. Maternal inheritance of tissue culture regeneration capacity has been described in maize by Tomes and Smith (1985). As the effect of this trait is additive, F1 embryos of the crosses should be used as explants rather than the F2 generation. The regeneration efficiency of the inbred lines CML216 and CML244 was higher than that of the single-cross hybrid with the TL26 inbred line, perhaps attesting to the additive effect of the regenerability trait (Tomes and Smith 1985). Recalcitrance to regeneration under tissue culture conditions that is genotype specific (Hodges et al. 1986), as observed in the TL26 genotype, might have a genetic basis and hint at the presence of genes or alleles that repress the formation of somatic embryos.

Somatic embryogenesis from immature embryos in temperate maize tissue cultures occurs from scutellum cells (Lu et al. 1982; Fig. 2c). Our results showed that in the tropical maize genotypes, somatic embryos and plant regeneration were induced from the embryo axis side, below the scutellum, coinciding with the presence of *Agrobacterium* when an intronless *GUS* construct was used, but not with the intron-containing *GUS* construct. The *Agrobacterium* infection process might be inhibited at some point, either during the initial stages of the infection, such as during the complex interaction between host and pathogen, during which the bacterial virulence genes are induced by plant phenolic compounds, or later during the T-DNA transfer or integration into the plant genome (Schläppi and Hohn 1992; Escudero et al. 1996). Genetic transformation of tropical genotypes by using particle bombardment (Bohorova et al. 1999, 2001; Shepherd et al. 2007) suggests that integration of T-DNA into the tropical maize genome is not the limiting factor and indicates that the early steps in *Agrobacterium* infection might be problematic. Hence, genetic transformation in tropical genotypes requires optimization of co-cultivation conditions in order to improve competence for *Agrobacterium* infection or, alternatively, particle bombardment should be used. Many factors that influence the *Agrobacterium*-mediated transformation of monocotyledonous plants have been investigated and plant host genes have been identified involved in T-DNA integration (for review see Cheng et al. 2004; Endo et al. 2006), amongst which the genes coding for histone H2A, H4, and H3-11 that enhanced transgene expression by protecting incoming transgene DNA during the initial stages of transformation and, hence, increased the efficiency of *Agrobacterium*-mediated transformation in *Arabidopsis thaliana* (Tenea et al. 2009) and in rice (*Oryza sativa*) (Zheng et al. 2009). Thus, allelic variation in host genes might contribute to the competence of plant genotypes to *Agrobacterium* infection.

In conclusion, efficient and highly reproducible shoot regeneration via somatic embryogenesis from tropical maize genotypes has been demonstrated. We show that the regenerative callus induction from immature embryos of tropical maize resides in the embryo axis, as supported by histological data. Regeneration recalcitrance can be overcome by single crosses with genotypes that regenerate efficiently. The results will assist in the achievement of genetic transformation in tropical maize that will accelerate germplasm development and fundamental research.

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