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Agrobacterium Mediated Transformation of Maize (*Zea mays* L.)

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Abstract

Agrobacterium tumefaciens mediated transformation may offer a better alternative than the biolistic gun for genetic transformation of maize plants. This gene delivery system results in a greater proportion of stable, low-copy number transgenic events than does the biolistic gun, and is highly efficient. In the present work, we studied maize transformation using *A. tumefaciens* by identifying some important factors that affect transformation efficiency subsequent tissue culture and regeneration of transgenic plants. Although, all genotypes produced embryogenic calli, S61 and A188 lines had higher percent (75% and 71% respectively) of regeneration than the other ones. Transformant events obtained when immature embryos (1.5-2 mm) were inoculated with *A. tumefaciens* LBA4404 harboring a standard binary vector pCAMBIA3301 after 72 h pretreatment culture of the embryos. Polymerase chain reaction (PCR) confirmed the presence of the *gus* and *bar* genes in the genome of regenerated plants. The transformation frequency (the number of independent, PCR-positive transgenic plants per 100 embryos infected) was 6.45% for S61 genotype. Therefore, our results identified suitable genotype (S61), embryo size (1.5-2 mm), *A. tumefaciens* strain (LBA4404), pretreatment culture, and appropriate antibiotic (Timentin) for *Agrobacterium* mediated transformation of *Z. mays*.

Keywords: *Agrobacterium tumefaciens*; Embryo; Transformation; *Zea mays*

Introduction

Maize is one of the most important crops around the world because of its importance as food and feed in man life; thus, breeding technology in this crop has been the subject of intense efforts resulting in several biotechnology approaches applied mainly in order to incorporate desiderable traits on several maize lines [1, 2]. Among the biotechnology tools, those related to transferring DNA have received special attention, leading to several strategies such as biolistic [3, 4] or *Agrobacterium tumefaciens* [5-7]. Particle bombardment and *Agrobacterium* mediated transformation are two popular methods currently used for producing transgenic cereals [8]. The application of *Agrobacterium* mediated transformation to monocotyledonous species, including rice and maize, has been recently reported. The main characteristics of the *Agrobacterium*

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system in these species are: i) high frequency of transformation; ii) proper integration of the foreign gene into the host genome; iii) low copy number of the gene inserted, resulting in most cases in a correct expression of the transgene itself. A limitation of the system is represented by the strict interaction between the genotype of the plant and the *Agrobacterium* strain, and the need to identify and to supplement specific signal molecules for the *vir* genes induction during the co-cultivation period (acetosiryngone or sinapinic acid) [8].

Although not being natural hosts for *A. tumefaciens*, monocotyledonous species seem to be in some instances susceptible to the infection. Studies on *Agrobacterium* infection of maize were first reported by Grimsley et al. [9] and Gould et al. [10], but the first evidences of the possibility of application of *Agrobacterium* mediated transformation of cereal species come from the works of Chan [11] and Hiei [12], who first obtained transgenic rice plants by means of transformation of immature embryos with *A. tumefaciens*. Most recently, the technique has been successfully applied to maize, and transgenic maize plants obtained at high frequency [5]. Ishida and co-workers [5] reported on the efficient transformation of maize inbred A188, and of some crosses between A188 and other inbreds. *Agrobacterium* mediated transformation method has been used to transform tissue culture amenable genotypes including the Hi II hybrid [13, 6, 14] or inbred lines A188 and H99 [5, 15, 16]. A limited number of proprietary [17] or public inbred lines [15, 7, 18], and various recalcitrant inbred lines crossed to A188 [19, 20] have also been transformed using this method.

The present work was developed with the aim to extend the methodology of transformation with *A. tumefaciens* for some maize genotypes which culture in Iran, and to identify some parameters need for transformation, and subsequent tissue culture and regeneration of transgenic plants.

Materials and Methods

Plant Material

Four inbred lines (S61, B73, Mo17 and A188) and two hybrid genotypes (HiIIB and HiIIC) of *Zea mays* supplied by ABRII (Agricultural Biotechnology Research Institute of Iran). Ears harvested 8-13 days after self-pollination in the field, sterilized with 70% ethanol for 2 min, followed by 5% sodium hypochlorite (NaClO) and 1% Tween20 for 20 min. Then they rinsed 3 times with sterile distilled water. Immature embryos (0.5- 5 mm) were aseptically dissected from seeds with a tiny loop.

Plant Tissue Culture and Regeneration

In order to identify suitable lines for genetic transformation, different size embryos of the lines were cultured on callus inducing medium. Some embryos were longitudinally sliced into halves as a treatment. Callus inducing medium contained modified N_6 basal salt, 1.5 mg/l 2, 4-D, 700 mg/l L-proline, 500 mg/l MES, 30 g/l sucrose in pH 5.8 and 3 g/l gelrite. Filter-sterilized N_6 vitamins, 400 mg/l Cycteine and 0.85 mg/l Silver nitrate ($AgNO_3$) were added after autoclaving [6]. The media were sterilized by autoclaving at 121°C for 20 min. The explants cultures were grown at 28°C for 3 weeks in darkness. The embryogenic calli were transferred to the first regeneration medium, incubated at 28°C in the 16/8 h (light/dark) photoperiod at 7000 *lux* light intensity for 2 weeks. The first regeneration medium was contained MS salts and vitamins, 100 mg/l myo-inositol, 60 g/l sucrose in pH 5.8 and 3 mg/l gelrite. The cultures were maintained at 25±2 °C under high light intensity (18000 *lux*) in the 16/8 h photoperiod for 4 weeks in the secondary regeneration medium until the roots of the plantlets reached to 10 cm. The secondary regeneration medium components were the same as the first medium but sucrose 30 g/l. The number of regenerated plants determined for each line.

Agrobacterium Strains and Vectors

Agrobacterium tumefaciens strains LBA4404 [21], EHA101 [22] and EHA105 (a derivative of the EHA101 strain), harboring the binary vector pCAMBIA3301 (CAMBIA Co. Australia) were used for transformation (Fig 1). The Plasmid pCAMBIA3301 was contained a P35S-bar selectable marker gene cassette (phosphinothricin acetyl transferase gene driven by the cauliflower mosaic virus [CaMV35S] promoter) and a P35S-*gus*-int reporter gene cassette (β-glucuronidase [GUS] gene with an intron driven by the CaMV 35S promoter) in the T-DNA and the broad host origin of replication (pVS1) and kanamycin-resistant marker gene for bacterial selection.

Figure 1. Schematic structure of the T-DNA region of the binary vector pCAMBIA3301. LB, left border; RB, right border; bar, phosphinothricin acetyltransferase gene; gus-int, β-glucuronidase gene containing an intron; P35S, CaMV 35S promoter; T35S, CaMV 35S terminator; TN, nopaline synthase terminator.

A. tumefaciens strains LBA4404, EHA101 and EHA105 (all harboring an standard binary vector pCAMBIA3301) were streaked out from a –80 °C glycerol stock onto an LB agar plate [23] containing appropriate antibiotics (for LBA4404 50 mg/l rifampicin and 50 mg/l kanamycin; whereas EHA101 and EHA105 50 mg/l kanamycin, 100 mg/l both of rifampicin and streptomycin). The plates were incubated at 28 °C for 3 days until single colonies developed. This stock plate was used on a weekly basis for up to a month. A single colony was streaked on LB medium containing the same antibiotics as stock plates. *Agrobacterium* were grown at 19 °C for 3 days for all transformation experiments.

Choice of Antibiotic against Bacterial Overgrowth

Prior to transformation experiments, different antibiotics were compared for their ability to control *Agrobacterium* growth. Three compositions of antibiotics were tested. For this, immature embryos of S61 line were infected with overnight cultures of three Agrobacterium strains. The infected embryos were blotted by sterile filter paper and cultured on callus inducing medium containing each of 250 mg/l Cefotaxime or Timentin, or 200 mg/l Vancomycin + 50 mg/l Jentamycin. The embryos transferred to a fresh medium every week. After three subcultures, the efficiency of Agrobacterium elimination determined for each antibiotics.

Embryo Size and Pretreatment Culture Study

The effects of embryo size and pretreatment culture on transformation frequency studied in S61 line as an efficient regenerable genotype of *Zea mays*. The transformation procedure used by Frame *et al.* [6] was basically followed with minor modifications. Embryos with 0.5 to 5 mm length were dissected from sterilized seeds. The embryos divided to three groups based on their size: 0.5- 2 mm, 2-3 mm and 3-5 mm. These embryos were inoculated with *A. tumefacience* LBA4404 (harboring pCAMBIA3301) suspension cultures. In all experiments, the bacterial cell densities were adjusted to OD_{550}=0.4-0.5 in liquid infection medium (Inf) medium using a spectrophotometer immediately before infection. The Inf medium consisted of modified N_6 basal salts and vitamins [24], 1.5 mg/l 2, 4-D, 700 mg/l L-proline, 68 g/l sucrose, 38 g/l fructose. The pH was adjusted to 5.2 and the medium was filter-sterilized. Bacterial suspensions used in transformation experiments were one full loop of a large colony from 72-h-old cultivation at 19°C in 5 ml. Inf medium supplement with 100µM Acetosyringone (AS) in a 50 ml falcon tube. The tube was fixed horizontally to a bench-top shaker on low speed (75 rpm) at 37°C for 5h. This pre- induction step was carried out for all experiments.

For inoculation, 15 to 25 immature zygotic embryos were washed twice in the Inf medium supplemented with 100 µM AS in a 2 ml tube. Then 1.5 ml of *A. tumefaciens* suspension was added to the tubes. Infection was accomplished by gently inverting the tube 20 times before resting it upright for 20 min. In order to facilitate *Agrobacterium* infection of embryo explants, we wounded some embryos by means of a toothed dissecting forceps during the infection.

Some embryos were transferred on a pretreatment medium for 72 h before infection. The pretreatment medium was consisted of MS salts and vitamins [25], 250 mg/l MES (2-morphlinoethanesulfonic acid), 1.5 mg/l 2, 4-D, 1 mg/l $CaCl_2$, 700 mg/l L-proline, 68 g/l sucrose and 8 g/l agar. The pH of the medium was adjusted to 5.8 with 0.5 N KOH and 0.5 N HCl prior to the addition of agar.

The infected embryos were blotted dry on paper filter and then were oriented with the embryo-axis side in contact with the co-cultivation medium (scutellum side up). The cultures incubated at 22±1 °C for 3 days in darkness. The co- cultivation medium contained of modified N_6 basal salt, 1.5 mg/l 2, 4-D, 700 mg/l L-proline, 30 g/l sucrose in pH 5.8 and 3 g/l gelrite. Filter-sterilized N_6 vitamins, 100 µM AS, 400 mg/l Cycteine and 0.85 mg/l Silver nitrate ($AgNO_3$) were added after autoclaving [6]. The explants were transferred onto rest medium at 28°C for 7 days in darkness. The rest mediums were the same as co- cultivation medium, except that, AS was eliminated and 500 mg/l MES and 250 mg/l Timentin were added for elimination of *Agrobacterium*. Thereafter, the explants were transferred to selection media. The first, second and third selective mediums were supplemented with 1.5, 3 and 5 mg/l PPT (L- Phosphinothricin) respectively and pH was adjusted to 5.2. Induced calli were maintained at 28 °C for 2 weeks at darkness in each selection medium. After two weeks, embryogenic calli tested for transformation efficiency by histochemical GUS assay.

Then, the embryogenic calli were transferred to regeneration media, as explained earlier. In all case, the regeneration media supplemented with 250 mg/l antibiotic and 5 mg/l PPT as selection agent. Thereafter, the plantlets with healthy roots were removed from culture, rinsed in water to remove media, and were transplanted to pots containing a mixture of equal parts of sterilized soil, peat moss and perlite. The pots were covered with plastic film and kept at 27±1 °C in a 16/8

h photoperiod. After 3 weeks of maintenance, the plantlets were uncovered and transferred to larger pots and then to the greenhouse.

Histochemical Assay

A histochemical GUS assay was conducted as described by Jefferson et al. [26]. The tissues were incubated overnight at 37°C in an X-Gluc solution, containing 100 mM sodium phosphate, 50 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclo-hexylammonium salt, 0.1% β- mercaptoethanol, and 0.1% Triton X-100 in pH 7.2. After staining, explants were soaked in 70% ethanol for bleaching. Assayed tissues were observed under a microscope and then photographed.

PCR

Putative transgenic plants were screened by the polymerase chain reaction (PCR) for the presence of the bar and *gus* genes. For this, genomic DNA was extracted from 15-20 mg of leaf tissue as described by Dellaporta et al. [27]. DNA extracted was dissolved in 20 µl of TE buffer containing RNAse. After 30 min, the DNA was completely dissolved and store at -20°C.

PCR reaction mixture contained 75 ng genomic DNA, 2.5 µl buffer 10X, 4 µl 2.5 mM dNTP, 1 µl 10 mM $MgCl_2$, 0.5 µl 20 µM of each primer and 0.8 unit Taq DNA polymerase in a 20 µl final volume. The primers used to detect the sequences of *gus* gene were: F-Gus: 5′- GGTGGGAAAGCGCGTTACAAG -3′ and R-Gus: 5′- TGGATTCCGGCATAGTTAAA -3′ [28]. Cycling conditions were one cycle of 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 60°C for 1min, 72 °C for 1 min ; a final extension at 72°C for 5 min (one cycle). The primers used to detect the sequences of *bar* gene were: 5′-CTCGAGTCAAATCTCGGTGACGGG-3′ and 5′-CGAGTCTACCATGAGCCCAGAACG-3′. Cycling conditions were one cycle of 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 64°C for 1min, 72 °C for 1.5 min; a final extension at 72°C for 5 min (one cycle). The PCR products were analyzed on an Agarose gel (1%) electrophoresis.

Results and Discussion

The main prerequisite for an efficient transformation system is the ability to regenerate complete plants from target tissues. In this sense the maize embryo-scutellum having a lot of competent cells for somatic embryo-genesis, is a proved primary explant from which fertile plants can be regenerated at high frequency [29, 30, 31], and has been selected as a target tissue for transformation by electroporation [32] and biolistic [3, 4, 30;] and *Agrobacterium* [15, 8, 7, 18, 2]. In the present work we used four inbred lines (S61, B73, Mo17 and A188) and two hybrid genotypes (HiIIB and HiIIC) for regeneration studies and selection of suitable line for genetic transformation. All lines tested were able to induce loose, soft and yellowish primary callus from the immature embryos within 5-6 days on the callus induction medium. The induction frequency of primary callus ranged from 64% to 100% depending on the genotype (Table 1). All the genotypes induced embryogenic calli after 9-10 weeks (Fig 2). S61 and A188 lines had highest regeneration as whole plants (75 and 71%, respectively). Longitudinal sliced immature embryos did not induce any embryogenic calli (data not shown). This is probably due to the fact that the immature embryonic axis injured by slicing. The same results reported by Ishida et al. [15] for A188. Therefore, S61 and A188 can be used as suitable genotypes in genetic transformation of maize on this culture medium.

Table 1. Callus induction, embryogenic callus formation and plant regeneration frequency in different lines of *Zea mays*

Genotype	Primary Callus Induction (%)	Embryogenic Callus (%)	Regeneration (%)
S61	100	80	75
B73	64	15	2
Mo17	68	11	0
A188	96	77	71
HiIIB	82	66	55
HiIIC	79	60	49

Table 2. The antibiotics efficiency to control of Agrobacterial pollution. Cef: Cefotaxime; Tim: Timentin; Van: Vanco-mycin; Jen: Jentamycin

Agrobacterium Strain	Antibiotic	Pollution Control Efficiency
LBA4404	Cef	Medium
	Tim	High
	Van+ Jen	Low
EHA101	Cef	Low
	Tim	Medium
	Van+ Jen	Low
EHA105	Cef	Low
	Tim	Medium
	Van+ Jen	Low

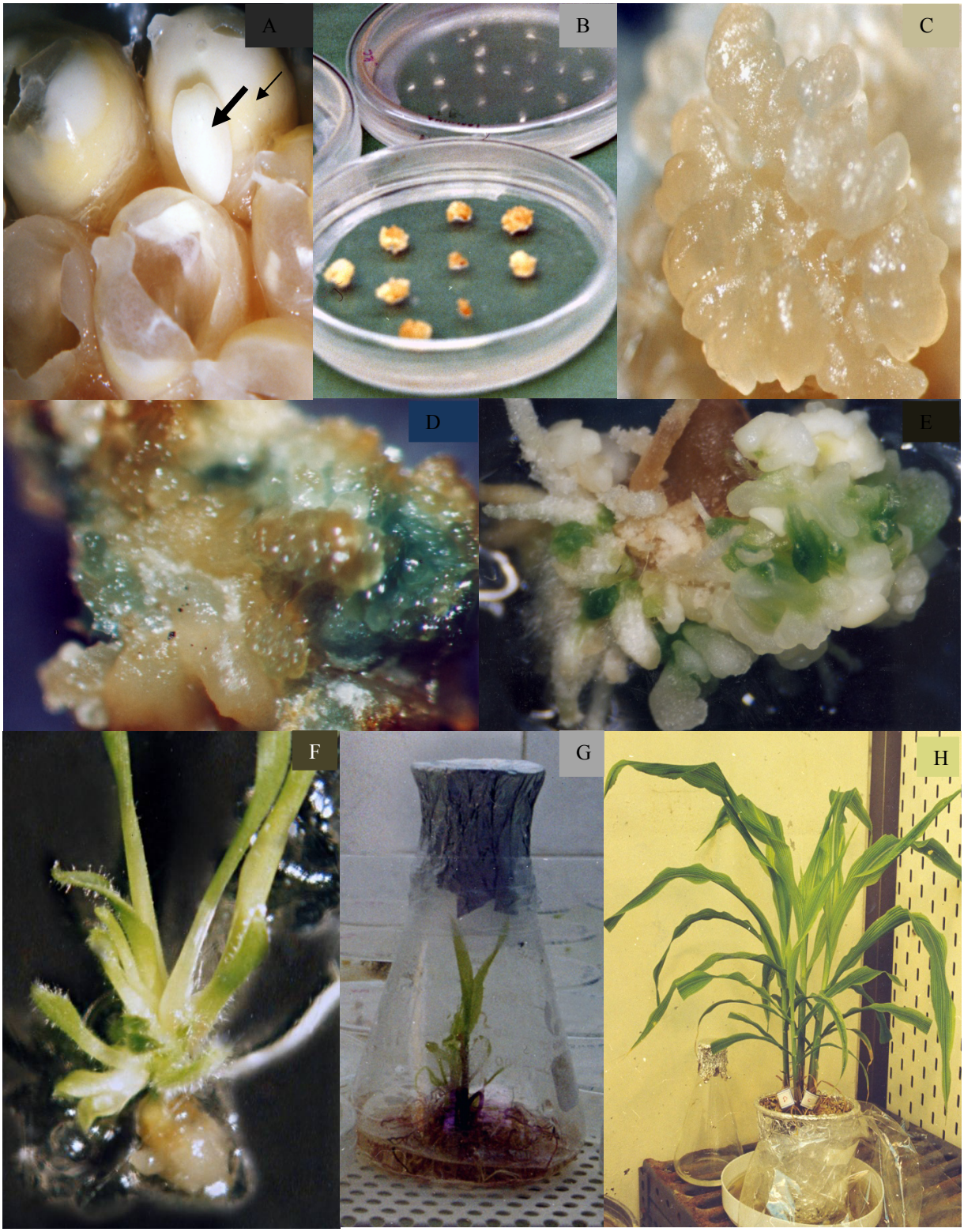

Figure 2. Transformation of maize plants using *A. tumefacience* harboring pCAMBIA3301. A: Dissected immature embryo (thick arrow) of maize seed (thin arrow). B: Callus induction on callus inducing medium. C: Embryogenic callus. D: Histochemical GUS assay of embryogenic callus. E: First step in plant regeneration of embryogenic callus. F: Regenerated transgenic maize plant. G: Regenerated plant in rooting medium. H: Rooted transgenic plant in the pot.

To select the most effective antibiotic for preventing bacterial overgrowth, three different antibiotics were tested. There are some reports on positive effects of Timentin on shoot regeneration from leaf discs, inhibitory effects of Cefotaxime on cotyledon explants regeneration and rooting of shoots [33, 34]. On the other hand, it was also shown that Timentin provided better protection against bacterial overgrowth than Cefotaxime and Carbenicillin [34-37]. In the present work, Timentin (a mixture of Ticarcillin and Clavulanic acid) was more effective than the others (Cefotaxime and Vancomycin + Jentamycin) to suppress the overgrowth of three Agrobacterial strains and tissue necrosis was decreased by the use of Timentin (Table 2).

Timentin is stable in solid agar medium and remained effective for at least 70 days [34]. Then using Timentin instead of Cefotaxime or Vancomycin + Jentamycin, the costs associated with antibiotic utilization are reduced.

Therefore, it could be concluded that the influence of Timentin on tissue culture was negligible or positive as compared to those of Cefotaxime or other antibiotics. Then we recommended Timentin as a suitable antibiotic to elimination of *Agrobacterial* contamination in transformation process. On the other hand, however there are some reports on high efficient transformation using EHA105 strain but we could not control the overgrowth of the strain by these antibiotics. EHA105 is a supervirulant bacterium, which result in frequent bacteria overgrowth. The use of 250 mg/l of Timentin or Cefotaxime was effective in killing bacterial cells of strain LBA4404, but not those of strains EHA105 and EHA101. This bacterial overgrowth may be controlled by more concentration of the antibiotics. Therefore, we used LBA4404 as a suitable *Agrobacterium* strain in the following transformation experiments.

In another experiment, we studied the effects of embryo size and pre-treatment culture on transformation frequency in S61 line as an efficient regenerable genotype of *Zea mays*. Embryo size has significantly effects on callus induction and plant regeneration in maize genotypes [38-40]. Therefore, genetic transformation of *Zea mays* may be related to embryo size explants. Our results demonstrated significant effects of embryo length/age on T-DNA delivery, callus induction and plant regeneration (Table 3). This may be attributed to physiological age of embryos. Small embryos with a proper physiological age have more embryogenic potential than the large ones, suggesting that embryos lose embryogenic competence with age. Moreover, changes in endogenous hormonal levels during embryogenesis sages may influence the control of cell cycle, and the co-ordination of cell division and DNA replication in immature embryos, and hence, transformation efficiency [41]. Therefore, the developmental stage of the embryo is an important factor in determining the success of maize transformation. However, calli obtained from larger embryos (>2.5mm) were showed significantly higher transient GUS expression levels but had lower regeneration frequencies than the smaller ones (<1.5 mm) (Fig 2).

The effect of wounding on gene transfer efficiency was analyzed by the evaluation of PPT resistant calli and histochemical analysis of transient GUS expression in embryos. The results showed that the wounded embryos produce more PPT resistant and GUS- positive calli than intact embryos (data not shown). The wounding allows the *Agrobacterium* efficiently infect the tissues by increasing in exposed surface, and stably transformed maize embryogenic tissue [42]. The wounding may also result in the active division of the cells and the accompanying DNA synthesis may enhance the incorporation of the T-DNA into the plant genome.

Histochemical GUS assays were carried out on PPT resistant callus events to determine whether those expressing the *bar* gene also expressed the *gus* reporter gene. Because the *gus* gene in this construct contains an intron (Fig. 1), blue staining was indicative of plant rather than *A. tumefaciens* expression of the transgene. Therefore, we concluded that 1.5-2.5 mm embryos are more suitable as an explant in *Agrobacterium* mediated transformation of *Zea mays*.

On the other hand, a 7-day preculture treatment significantly increased the number of green calli in selection medium (Table 3). The phase of the cell cycle influences stable transformation [43]. The formation of new and thin cell walls probably has effects on transformation efficiency, and may have influence in specific attachment capacity to *Agrobacterium* [44, 45].

Table 3. The effects of embryo size and pretreatment culture on callus induction and plant regeneration in *Zea mays*

Embryo size (mm)	Pretreatment Culture	Callus Induction	Plant Regeneration
0.5-2	+	Medium	Medium
	-	Low	Low
2-3	+	High	High
	-	Medium	Medium
3-5	+	High	Low
	-	Medium	Low

Table 4. The efficiency of *Agrobacterium*- mediated transformation using immature embryos of maize plants

Genotype	No. of Embryogenic Calli:(A)	No. of Plants tested for *GUS*	No. of positive PCR (*gus*/*bar*) Plants: (B)	Frequency (A /B) %
S61	517	9	4	6.45
A188	638	3	-	-
HiIIB	422	2	-	-

Therefore, preculture in a high hydrocarbonates and auxin medium can enhance transformation rate.

Finally, based on the preliminary results, we studied the transformation of three lines (S61, A188 and HiIIB) of the maize. We used 1.5 - 2.5 mm embryos, *A. tumefacience* LBA4404 harboring pCAMBIA3301, pretreatment culture and Timentin antibiotic for pollution eliminations in transformation process. S61, A188 and HiIIB PPT resistant lines were regenerated in selection medium. Nine PPT resistant plants were regenerated from 517 embryogenic calli of S61 line. The numbers of regenerated PPT resistant plants were three for A188 and two for HiIIB lines (Table 4).

DNA extracted from leaves of PPT resistant plants was used for amplification with primers to the *bar* and *gus* genes. The PCR reaction revealed the presence of the GUS fragment with expected length of 320 bp (Fig. 3) and the BAR fragment with expected length of 520 bp (Fig. 4) in the genomic of each putative transgenic plants. No amplified products were detected in non-transformed control plants (Figs. 3, 4). The transformation frequency (the number of independent, PCR-positive transgenic plants per 100 embryos infected) was 6.45% for S61 genotype (Table 4). Ishida and et al. [5] reported a transformation frequency between 5- 30% for A188 line using a super-binary vector. However, Frame et al. [18] reported 0% transformation frequency for A188 genotype. Therefore, based on the present results, S61 is a suitable line for genetic transformation of maize using *A. tumefacien*s.

In conclusion, the major factors limiting *Agrobacterium* application for genetic transformation of maize are low frequency of plant regeneration from cultured tissues and a weak virulence of *Agrobacterium* in relation to cereals. The present study illustrated some prerequisites for *Agobacterium* mediated transformation of *Z. mays*. Our results demonstrated suitable maize genotype (S61), embryo size (1.5 - 2 mm), *A. tumefaciens* strain (LBA4404), pretreatment culture, and appropriate antibiotic (Timentin) for *Agrobacterium* mediated transformation of *Z. mays*. The present results will be used for genetic transformation of maize using biotic and abiotic resistance genes.

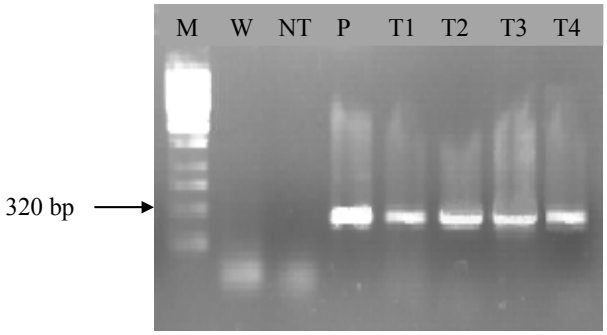

Figure 3. The representative PCR analysis of genomic DNA to detect the presence of the *gus* gene in putative transgenic maize plants S61 line. PCR amplification of the 320-bp fragment of the *gus* gene. Lane M, Molecular weight marker; Lane W, Water (negative control); Lane NT, DNA from untransformed plant (negative control); Lane P, pCAMBIA3301 plasmid DNA (positive control); Lane T1-T4, DNA from independently transformed plants.

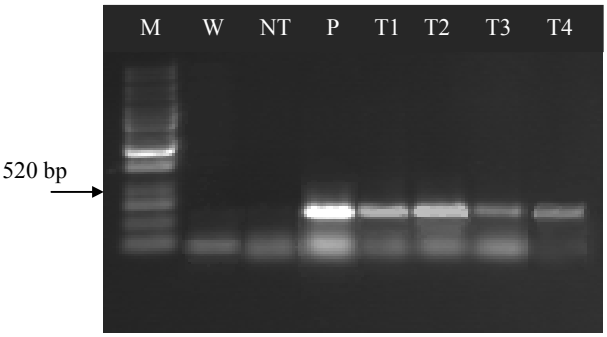

Figure 4. The representative PCR analysis of genomic DNA to detect the presence of the *bar* gene in putative transgenic maize plants S61 line. PCR amplification of the 520-bp fragment of the *bar* gene. Lane M, Molecular weight marker; Lane W, Water (negative control); Lane NT, DNA from untransformed plant (negative control); Lane P, pCAMBIA3301 plasmid DNA (positive control); Lane T1-T4, DNA from independently transformed plants.

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