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Successful *Agrobacterium*-mediated genetic transformation of maize elite inbred lines

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Abstract

An efficient transformation system was developed for maize (*Zea mays* L.) elite inbred lines using *Agrobacterium*-mediated gene transfer by identifying important factors that affected transformation efficiency. The hypervirulent *Agrobacterium tumefaciens* strain EHA105 proved to be better than octopine LBA4404 and nopaline GV3101. Improved transformation efficiencies were obtained when immature embryos were inocubated with *Agrobacterium* suspension cells (A_{600} = 0.8) for 20 min in the presence of 0.1% (v/v) of a surfactant (Tween20) in the infection medium. Optimized cocultivation was performed in the acidic medium (pH5.4) at 22 °C in the dark for 3 days. Using the optimized system, we obtained 42 morphologically normal, independent transgenic plants in four maize elite inbred lines representing different genetic backgrounds. Most of them (about 85%) are fertile. The transformation frequency (the number of independent, PCR-positive transgenic plants per 100 embryos infected) ranged from 2.35 to 5.26%. Stable integration, expression, and inheritance of the transgenes were confirmed by molecular and genetic analysis. One to three copies of the transgene were integrated into the maize nuclear genome. About 70% of the transgenic plants received a single insertion of the transgenes based on Southern analysis of 10 transformed events. T_1 plants were analyzed and transmission of transgenes to the T_1 generation in a Mendelian fashion was verified. This system should facilitate the introduction of agronomically important genes into commercial genotypes.

Abbreviations: BA – 6-benzyladenine; bar – phosphinothricin acetyltransferase gene; 2,4-D – 2,4-dichloro phenoxyactic acid; GUS – β-glucuronidase; IBA – indole-3-butyric acid; MES – 2-(N-morpholino) ethanesulfonic acid; PCR – polymerase chain reaction; PPT – phosphinothricin; *uid*A – β-glucuronidase gene from *Escherichia coli*; X-gluc – 5-bromo-4-chloro-3-indolyl-β-glucuronide

Introduction

Maize (*Zea mays* L.) is one of the three most important cereals on a world production level. Plant genetic engineering opens tremendous avenues for genetic improvement of and fundamental research on maize. The ability to transfer genes into this agronomically important crop may enable improvement of the species with respect to some desirable characters, such as nutritional quality, high yield production, resistance to herbicides, diseases, viruses, and insects, and tolerance to drought, cold, salt, and flooding. These improvements in maize will directly enhance global production and human health. However, at one time, maize was considered to be one of the most recalcitrant crops for genetic transformation. It took more than a decade of

arduous effort for scientists to develop a universally efficient transformation system for genetic improvement of maize. At present, transgenic maize plants have been unequivocally produced using several techniques including PEG-mediated (Golovkin et al., 1993), electroporation (D'Halluin et al., 1992), silicon carbide whisker-mediated (Frame et al., 1994; Petolino et al., 2000), microprojectile bombardment (Gordon-Kamm et al., 1990; Wan et al., 1995) and *Agrobacterium*-mediated (Ishida et al., 1996; Negrotto et al., 2000; Zhao et al., 2001; Frame et al., 2002). Among the various methods available, *Agrobacterium*-mediated transformation offers several advantages, such as the defined integration of transgenes, preferential integration into transcriptionally active regions of the chromosomes, and potentially single or low copy number with rearrangement being relatively rare (Birch, 1997; Hiei et al., 2000). Highly efficient *Agrobacterium*-mediated transformation systems for maize have been reported by several laboratories. Ishida et al., (1996) reported that transformants of maize inbred A188 were efficiently produced from immature embryos cocultivated with *Agrobacterium* that carried 'super-binary' vectors. Frequencies of transformation were between 5 and 30%. Negrotto et al. (2000) used phosphomannose-isomerase as a selectable marker to recover transgenic maize via *Agrobacterium*-mediated transformation. Zhao et al. (2001) developed a high throughput genetic transformation system in maize with *Agrobacterium* mediated T-DNA delivery. With optimized conditions, stable callus transformation frequencies for Hi-II (A188-derived cross) immature embryos averaged approximately 40%. Frame et al. (2002) had achieved routine transformation of maize (the hybrid line Hi II) using an *Agrobacterium* standard binary (non-superbinary) vector system and the average stable transformation efficiency was 5.5%. However much of the work has been restricted to a model inbred line A188 and A188-derived crosses, in which the A188 was a genotype with poor agronomical value. If the *Agrobacterium*-mediated transformation were limited to this inbred line, the introgression of useful genes into elite inbred lines would need to follow crosses of transgenic A188 and the genotype of interest and selection of segregating progeny, a time-consuming and costly procedure.

Accordingly, the present work was developed with the aim

– to identify some key parameters that may potentially enhance transformation and,
– to extend the methodology of transformation with *Agrobacterium* to several maize elite inbred lines of agronomically importance.

This may be useful for effectively broadening the application of the *Agrobacterium*-mediated transformation to maize.

Materials and methods

Plant materials and media

Four inbred lines of maize with outstanding agronomic characters, which are not related to A188, were considered for *Agrobacterium*-mediated transformation. The inbred line 9046, Mo17 and 414 was derived from a cross between 7922×5003, $C103 \times C187\text{-}2$ and 478×3144, respectively. The inbred line Qi319 was derived from second cycle line from PN78599. The seeds of 9046 were kindly supplied by Dandong Academy of Agricultural Science, Liaoning Province. The seeds of Mo17, Qi319 were kindly supplied by Institute of Maize, Shandong Academy of Agricultural Science, Shandong Province. The seeds of 414 were kindly supplied by Institute of food crops, Jiangsu Academy of Agricultural Science, Jiangsu Province. Immature embryos used for these experiments were from plants grown in the field during summer seasons in 2002 and 2003. Immature ears were harvested 10–15 days post pollination and sterilized 0.1% mercuric chloride ($HgCl_2$) for 10 min. The sterilized ears were rinsed six times with sterilized distilled water. Immature embryos of 1.0–2.0 mm in length were aseptically excised from kernels and used for transformation experiment. To minimize the variation introduced by the explants, a large number of immature embryos isolated from different ears were subdivided onto different treatments in each test. The media used in the study are listed in Table 1.

Agrobacterium *strains, plasmids, and growth conditions*

Vir helper strains of *Agrobacterium* used in this study include GV3101 (Koncz and Schell, 1986),

Table 1. Media used for bacterial culture, tissue culture and transformation of maize

Media	Composition
YEP	10 g l^{-1} peptone, 5 g l^{-1} NaCl, 10 g l^{-1} yeast extract, 1.5% (w/v) agar pH 7.0
Inf	N6 (Chu et al., 1975) salts and B5 (Gamborg et al., 1968) vitamins, 2.0 mg l^{-1} glycine, 2.5 mg l^{-1} 2,4-D, 0.69 g l^{-1} L-proline, 68.5 g l^{-1} sucrose, 36 g l^{-1} glucose, 50 mg l^{-1} ascorbatic acid, pH 5.2. Add 200 μM acetosyringone[a,b] before using.
CM	Inf without glucose, reduced sucrose to 30 g l^{-1} and supplemented with 0.5 g l^{-1} MES buffer, 0.85 mg l^{-1} silver nitrate, 8.0 g l^{-1} agar, pH 5.4 (unless otherwise specified).
SM	CM without acetosyringone, and supplemented with 250 mg l^{-1} carbenicillin[a,c], 400 mg l^{-1} cefotaxime[a,d] and various concentrations of L-phosphinothricin[a], pH 5.8
DM	N6 salts and B5 vitamins, 2.0 mg l^{-1} glycine, 1.5 mg l^{-1} BA, 0.5 mg l^{-1} IBA, 60 g l^{-1} sucrose, 5.0 mg l^{-1} phosphinothricin[a], 250 mg l^{-1} carbenicillin[a], 400 mg l^{-1} cefotaxime[a], 8.0 g l^{-1} agar, pH 5.8
RM	1/2 MS salts and B5 vitamins, 2.0 mg l^{-1} glycine, 1.0 mg l^{-1} IBA, 30 g l^{-1} sucrose, 5.0 mg l^{-1} phosphinothricin[a], 250 mg l^{-1} carbenicillin[a], 400 mg l^{-1} cefotaxime[a], 6.0 g l^{-1} agar, pH 5.8

[a]Filter-sterilized.
[b]Sigma-Aldrich products.
[c]Cabenicillin disodium salt, Sigma products.
[d]Cefotaxim sodium salt, Sigma products.

EHA105 (Hood et al., 1993), and LBA4404 (Hoekema et al., 1983). pCAMBIA3301 (CAMBIA, Canberra, Australia) was used as the binary vector. This vector contains the *uid*A coding region with an intron and the phosphinothricin (PPT) acetyltransferase gene (*bar*) conferring PPT resistance for selection (Figure 1). This binary vector has been transformed into all *vir* helper strains of *Agrobacterium* mentioned above by freeze-thaw method (Höfgen and Willmitzer, 1988). All binary *Agrobacterium* strains are maintained on solid YEP medium (Chilton et al., 1974; Table 1) supplemented with 100 mg l^{-1} kanamycin sulfate (Sigma). A single colony was transferred to 5 ml YEP liquid medium containing the same selective antibiotics and the culture was allowed to shake overnight at 200 rpm and 28 °C. The overnight culture was transferred into 50 ml YEP medium containing the same selective antibiotics. The culture was grown overnight under the same conditions as described above. When the culture was at log phase, which corresponded to an absorbance 600 nm (A_{600}) of 1.4–1.6, cells were pelleted by centrifugation at 3400 × *g* for 10 min and then resuspended in infection medium (Inf). After adjusting A_{600} to 0.4, 0.8 or 1.2, the *Agrobacterium* suspension was used for infection. In the experiment examining the effects of a surfactant, 0.01, 0.1 or 1% (v/v) of Tween20 (Wako Pure Chemical Industries, Osaka, Japan) was added to the Inf medium.

Transformation

The transformation process was divided into 5 sequential steps as described by Zhao et al. (2001): bacterium inoculation, cocultivation, resting, selection and plant regeneration. The freshly isolated immature embryos were immersed in *Agrobacterium* suspension for 5, 10, 20, 40 or 60 min to begin

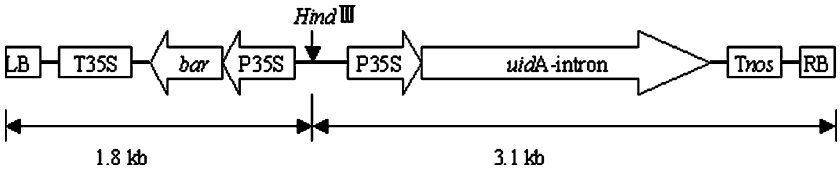

Figure 1. Schematic representation of the T-DNA (4.9 kb) of pCAMBIA3301 containing the *uid*A and *bar* genes (total size: 11.3 kb). LB/RB – left/right T-DNA border sequences; P35S/T35S – CaMV 35S promoter/terminator; *bar* – coding region of the phosphinothricin acetyltransferase gene; T*nos* – nopaline synthase terminator; *uid*A -intron -*uid*A gene coding region with intron sequence.

the bacterium inoculation process. For the subsequent steps, approximately 30 embryos were cultured with the scutellar side up (facing away from the medium) on each petri dish (10 × 2 cm) containing 35 ml cocultivation medium (CM). The petri dishes were sealed with Parafilm and placed in the dark at 22 °C (unless otherwise specified) for 1–6 days for the cocultivation step (Figure 2a). For the resting step, the embryos were cultured on medium SM at 28 °C in the dark for 7 days (each petri dish contained 10 embryos). Following the resting step, the embryos were cultured on SM containing 5 mg l^{-1} L-PPT (Meiji Seika Kaisha, Japan) at 28 °C in the dark for two weeks to begin the selection process. Next, these embryos were moved to SM containing 10 mg l^{-1} L-PPT and were sub-cultured every 2 weeks. The cultures were kept in the dark at 28 °C for approximately two months until PPT-resistant callus proliferated (Figure 2b). For the regeneration step, the PPT-resistant calli were transferred to 100-ml Erlenmeyer flask containing 60 ml regeneration medium (DM) at a density of five calli per container. Each culture was sealed with a cotton plug and covered with aluminum foil. They were maintained at 27 °C under a 16-h photoperiod with cool white fluorescent lights (40 μmol m^{-2} s^{-1}) for approximately 2 weeks to initiate shoots. Growing, PPT-resistant calli with small shoots were subsequently moved to rooting medium (RM) and cultured at 25 °C under a 16-h photoperiod with cool white fluorescent lights (80 μmol m^{-2} s^{-1}). After 2–4 weeks, the regenerated plants with healthy roots (Figure 2c) were then transferred to soil in pots and grown in a greenhouse.

Histochemical analysis of transient and stable GUS expression

Histochemical GUS assays (Jefferson, 1987) were used to assess transient expression of the *uid*A gene in immature embryos 2 day after the 3-day cocultivation (5 days after infection, or as indicated otherwise). Explants were incubated at 37 °C for 16 h in buffer containing 1 mM X-Gluc, 100 mM sodium phosphate buffer (pH 7.0), 10 mM Na_2EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide and 0.1% (v/v) Triton X-100. T-DNA delivery was assessed by counting the number of embryos with GUS foci (Figure 2d). Histochemical GUS assays were also used to assess stable expression of the *uid*A gene in leaf tissue of transgenic plants in the T_0 and T_1 generations (Figure 2e). Tissues were submerged in the substrate, vacuum infiltrated (20 inch Hg) for 10 min, and incubated at 37 °C for 16 h and, if necessary, for a further 1–2 days at 25 °C. Blue staining cells were visualized by soaking tissues in 75% followed by 95% (v/v) ethanol to remove chlorophyll and tissues scored as positive or negative for GUS expression.

Herbicide leaf painting assay

Healthy leaves of non-transformed control and transformed (T_0) plants were selected for leaf painting. Using a writing brush, 200 mg l^{-1} L-PPT with 0.1% Tween20 was applied to paint the upper surface of the distal half of the selected leaves. Leaves were scored for herbicide damage ten days after application.

Polymerase chain reaction analysis

Putative transformants were screened by the polymerase chain reaction (PCR) for the presence of the *bar* and *uid*A genes. DNA was extracted from approximately 10–20 mg of leaf tissues according to the method of Wang et al. (1993). The 440-bp coding region of *bar* gene was amplified using 20-bp oligonucleotide primers (5′-GCACCATCGTCA ACCACTAC-3′ and 5′-GAAGTCCAGCTGCCA GAAAC-3′). The 1060-bp coding region of *uid*A gene was amplified using 20-bp oligonucleotide primers (5′-CACCGTTTGTGTGAACAACG-3′ and 5′-GTACCTTCTCTGCCGTTTCC-3′). PCR amplification reactions contained 50 ng of template DNA, 0.4 μM of each primer, 200 μM of a dNTP mixture, 1 × *Taq* DNA polymerase reaction buffer and 2 U *Taq* DNA polymerase (Takara, Japan) in a 50 μl final volume. The amplification reaction was carried out using a thermal cycler (Perkin Elmer, Foster City, Calif.) under the following conditions: one cycle of 94 °C for 5 min; 30 cycles of 94 °C for 45 s (denaturation), 58 °C for 30 s (annealing), 72 °C for 1 min (extension); a final extension at 72 °C for 10 min (one cycle). To ensure that reagents were not contaminated, DNA from non-transformed (control) plant was included in the experiments. The amplified products were separated by electrophoresis on a 1.2% agarose gel and visualized with ethidium bromide.

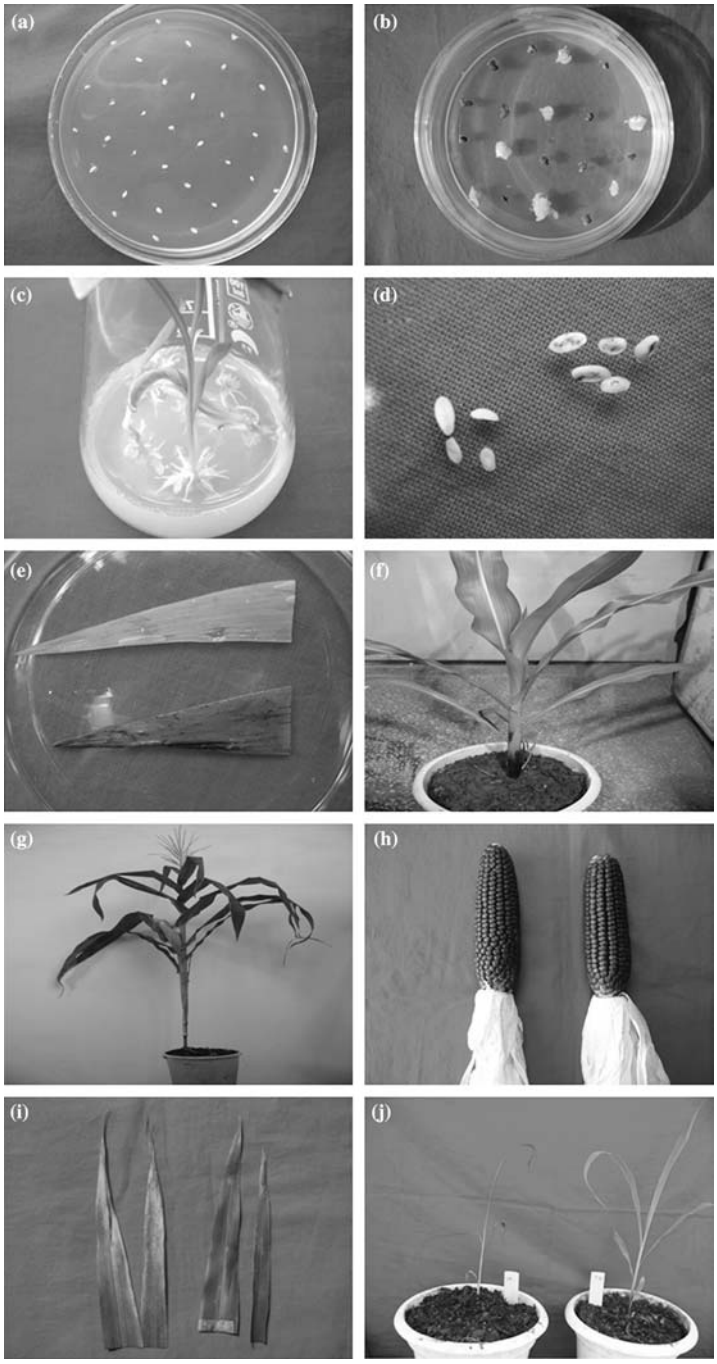

Figure 2. Production of transgenic maize plants via *Agrobacterium*-mediated transformation of immature embryos. (*a*) Immature embryos during cocultivation. (*b*) PPT-resistant calli growing on the selection medium. (*c*) PPT-resistant plantlets with healthy roots growing on the rooting medium. (*d*) GUS histochemical assay of transformed (right) and non-transformed control (left) immature embryos after 3 days of cocultivation. (*e*) GUS histochemical assay of leaves segments of transformed (lower) and non-transformed control (upper) plant. (*f*) A putative transgenic plant growing in pot. (*g*) A transformed plant at flowering stage. (*h*) Harvested ears of a transformed plant (right) and a non-transformed control plant (left). (*i*) PPT spot paint test in leaves of non-transformed control plant (left) and transformed plant (right). (*j*) Test of the progeny for resistance to Basta. Seed-derived young plants of transformed (right) and non-transformed (left) plant were sprayed with 0.5% Basta solution. The photograph was taken 10 days after the application of the herbicide.

Southern blot

Genomic DNA was extracted from leaves of non-transformed control and transformed plants, as described by Dellaporta et al. (1983). Thirty micrograms of high-molecular-weight DNA was completely digested with *Hin*dIII (a single *Hin*dIII restriction site exists within the plasmid). Digested DNA fragments were separated by electrophoresis in 0.8% agarose gel, then transferred onto a Hybond-N^+ nylon membrane (Amersham, Buckinghamshire, England) according to Sambrook et al. (1989). The 1060-bp PCR-fragment containing the *uid*A coding region and the 440-bp PCR-fragment containing the *bar* coding region of pCAMBIA3301 were labeled with $\alpha[^{32}P]$-dCTP using a ramdom primer DNA labeling system (Takara, Japan), and were used as probes for hybridization. Prehybridization, hybridization and washing membrane were carried out according to Sambrook et al. (1989). Hybridized membranes were exposed to Kodak XAR-5 film at −70 °C for 2–3 days.

Progeny segregation analysis for bar gene expression

A Basta (Hoechst Aktiengesellschaft, Frankfurt, Germany) leaf-spray test was used to establish segregation ratios for expression of the *bar* gene in progeny. A 0.5% Basta (200 g l^{-1} of the active ingredient, glufosinate, the ammonium salt of L-PPT), along with 0.1% (v/v) Tween20 was applied. Beginning 15 day after planting, seedlings were sprayed twice at 2-day intervals with a freshly prepared Basta solution. Ten days after herbicide application, sensitivity to Basta was scored.

Experimental design and statistical analysis

Six factors were evaluated for their effects on transformation efficiency:

(1) the effects of *Agrobacterium* strains;
(2) *Agrobacterium* cell density and inoculation time;
(3) length of cocultivation period;
(4) the pH in the cocultivation medium;
(5) cocultivation temperature;
(6) effect of surfactant in the infection medium.

Different treatments were compared for each factor. For each treatment, three replicates with a minimum of 30 explants per replicate were used (with the exception of the effects of *Agrobacterium* strains). To test the effects of *Agrobacterium* strains, at least 100 immature embryos of each inbred lines were infected by the *Agrobacterium* strains EHA105, LBA4404 and GV3101, respectively. The data were analyzed by ANOVA (analysis of variance). The means were compared using the Duncan's New Multiple-Range Test at $p < 0.05$.

Results

Factors influencing Agrobacterium*-mediated transformation of maize*

In order to optimize conditions for maize transformation, the effects of several parameters known to influence *Agrobacterium*-mediated DNA transfer were compared.

Agrobacterium strains play an important role in the transformation process, as they are responsible not only for infectivity but also for the efficiency of gene transfer. In this study, *A. tumefaciens* with different disarmed Ti plasmids but harbouring the same binary plasmid (pCAMBIA3301) were tested for their capacity to infect immature embryos of maize. Gene transfer was evaluated by the number of GUS-expressing explants 2 days after 3-day cocultivation (Figure 2d; Table 2). Of the four maize inbred lines tested under these conditions, the nopaline-type strain GV3101 was the least effective for infectivity. However, octopine-type strain LBA4404 and agropine-type hypervirulent strain EHA105 were effective and transformation with EHA105 yielded the highest efficiency (23.8%). EHA105 was used in subsequent transformation experiments.

Experiments were set up to determine the effects of variations in *Agrobacterium* cell density (A_{600}) and inoculation time on transformation of maize immature embryos. It was observed that the efficiency of T-DNA delivered into immature embryos was highly affected by *Agrobacterium* cell density and inoculation time (Figure 3). And an interaction between *Agrobacterium* cell density and inoculation time was significant. The lower cell density ($A_{600} = 0.4$) produced fewer PPT-resistant calli with 5 min inoculation. However, increasing the duration of inoculation to 40 min, increased frequency of PPT-resistant calli. The higher cell

Table 2. Effects of *Agrobacterium* strains on transformation of maize inbred lines[a]

Agrobacterium strains	Inbred lines	No. of immature embryos inoculated	No. of GUS-positive explants[b]	Frequency of transient GUS expression (%)
EHA105	9046	135	36	26.7
	Qi319	146	31	21.2
	414	111	28	25.2
	Mo17	104	23	22.1
Means[c]				23.8a
LBA4404	9046	150	28	18.7
	Qi319	165	26	15.8
	414	125	30	24.0
	Mo17	115	17	14.8
Means				18.3b
GV3101	9046	108	8	7.4
	Qi319	131	5	3.8
	414	125	4	3.2
	Mo17	154	2	1.3
Means				3.9c

[a]Immature embryos were inoculated with *Agrobacterium* (without Tween20 in the infection medium) and cocultivated (pH 5.8) at 25 °C for 3 days.
[b]Blue foci were counted after cocultivation.
[c]Different letters indicate means are significantly different according to the Duncan's New Multiple-Range Test at $p < 0.05$.

density (A_{600} = 1.2) produced higher PPT-resistant calli frequency, but this was obtained with a 5 min inoculation. When inoculation time was increased for this cell density, PPT-resistant calli frequency decreased significantly. With intermediate cell density (A_{600} = 0.8) and 20 min inoculation, the frequency of PPT-resistant calli was the highest (13.3%). Therefore, this condition (A_{600} = 0.8 and 20 min inoculation) was used in our subsequent transformation experiments.

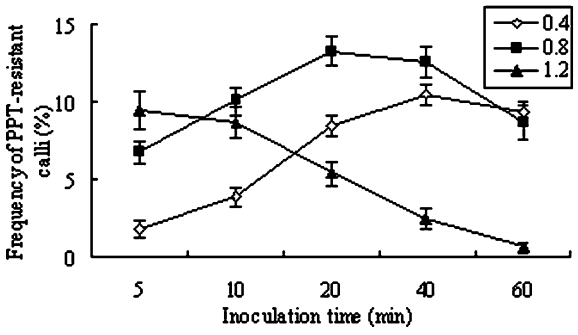

Figure 3. The effects of *Agrobacterium* cell density and inoculation time on the transformation efficiency of maize. Immature embryos of inbred line 9046 were inoculated with *Agrobacterium* strain EHA105 (without Tween20 in the infection medium) and cocultivated (pH5.8) at 25 °C for 3 days. Frequency of PPT-resistant calli (%) was defined as the number of immature embryos that generated PPT-resistant calli at the end of 2 months of culturing on selection medium relative to the total number of immature embryos inoculated with *Agrobacterium*. Three replicates for each treatment with at least 30 immature embryos per replicate were used. Vertical *bars* represent the standard error.

The periods of cocultivation differed according to plant species in the literature. Longer periods of cocultivation seem effective for efficient transfer of the Ti plasmid to plant cells. However, it was more difficult to eliminate *Agrobacterium* after longer periods of cocultivation. Cocultivation for 2–7 days is generally considered to be suitable for *Agrobacterium*-mediated transformation, as reported for many plant species. We investigated the effect of varying the periods of cocultivation with transient expression of the *uid*A gene and stable expression of the *bar* gene. Figure 4 shows the influence of the cocultivation period on transformation of maize immature embryos. When the explants were transferred to selective medium immediately after inoculation with *Agrobacterium* (no cocultivation), no transformation was observed. The transformation frequency was very low after 1-day cocultivation, but increased rapidly when the cocultivation was prolonged to 2 days, reaching a maximum at the 3rd day. However, transient GUS expression did not vary

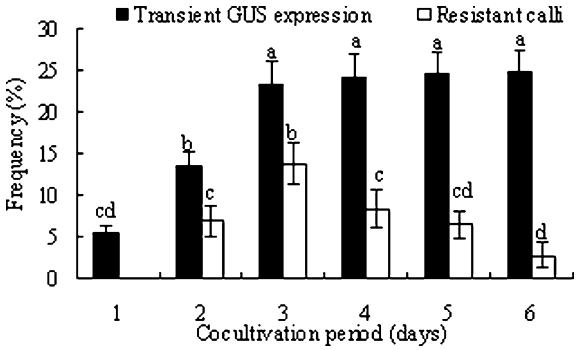

Figure 4. The effect of cocultivation time on transformation efficiency of maize. Immature embryos of inbred line 9046 were inoculated with *Agrobacterium* strain EHA105 (without Tween20 in the infection medium) at A_{600} = 0.8 for 20 min and cocultivated (pH5.8) at 25 °C. Frequency of transient GUS expression (%) was defined as the number of immature embryos with GUS expression after cocultivation relative to the total number of immature embryos inoculated with *Agrobacterium*. Frequency of PPT-resistant calli (%) was defined as the number of immature embryos that generated PPT-resistant calli at the end of 2 months of culturing on selection medium relative to the total number of immature embryos inoculated with *Agrobacterium*. Three replicates for each treatment with at least 30 immature embryos per replicate were used. Columns denoted by different letters are significantly different according to the Duncan's New Multiple-Range Test at $p < 0.05$. Vertical bars represent the standard error.

significantly over 3- to 6-day cocultivation. But prolonged cocultivation periods of more than 3 days resulted in abundant proliferation of the bacteria and a subsequent decrease in the PPT-resistant calli frequency of transformed immature embryos. Therefore, a 3-day cocultivation was routinely used.

To investigate the influence of the pH of the cocultivation medium on gene transfer, we cocultivate immature embryos in the cocultivation medium with pH ranging from 5.0 to 5.8. Cocultivation medium with a pH of 5.4 produced the highest PPT-resistant calli frequency (Figure 5).

To determine the influence of temperature during cocultivation, we performed experiments at temperatures ranging from 19 to 28 °C. The highest transient GUS expression was observed at 22 °C, in which 35% of total immature embryos showed GUS activity (Figure 6). Transient GUS expression markedly decreased when the temperature was increased to 25 °C. Lower levels of GUS expression were observed at 28 and 19 °C.

In the present study, the effect of the inclusion of a surfactant, Tween20 in the infection

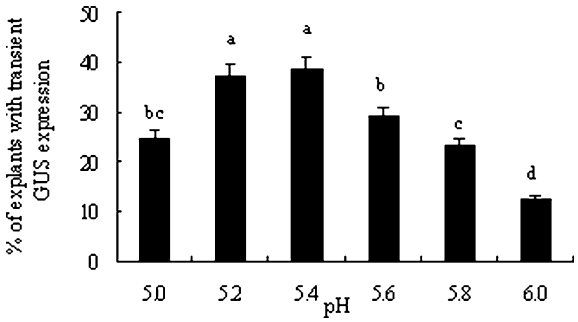

Figure 5. The effect of pH in the cocultivation medium on transformation efficiency of maize. Immature embryos of inbred line 9046 were inoculated with *Agrobacterium* strain EHA105 (without Tween20 in the infection medium) at A_{600} = 0.8 for 20 min and cocultivated at 25 °C for 3 days. Three replicates for each treatment with at least 30 immature embryos per replicate were used. Columns denoted by different letters are significantly different according to the Duncan's New Multiple-Range Test at $p < 0.05$. Vertical bars represent the standard error.

medium was also examined. Tween20 proved to have a positive effect on transient expression of the *uid*A gene and the production of PPT-resistant calli (Figure 7). Tween20 at 0.1% gave the highest frequency of transient GUS expression and resistant calli. Although a comparable effect (transient GUS expression) was obtained with 1% Tween20, most of the immature embryos treated with this concentration of

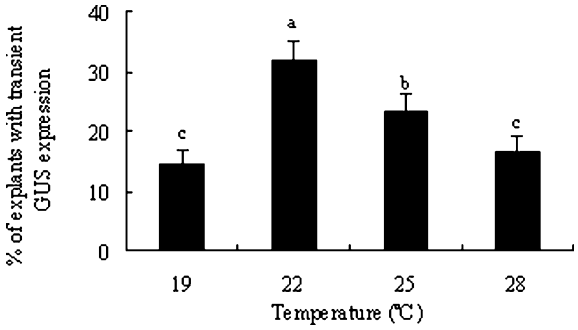

Figure 6. The effect of cocultivation temperature on transformation efficiency of maize. Immature embryos of inbred line 9046 were inoculated with *Agrobacterium* strain EHA105 (without Tween20 in the infection medium) at A_{600} = 0.8 for 20 min and cocultivated (pH 5.8) for 3 days. Three replicates for each treatment with at least 30 immature embryos per replicate were used. Columns denoted by different letters are significantly different according to the Duncan's New Multiple-Range Test at $p < 0.05$. Vertical bars represent the standard error.

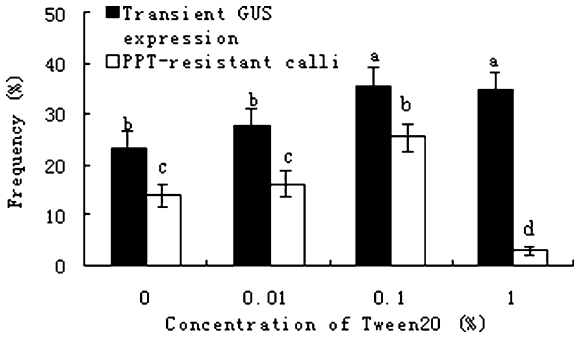

Figure 7. The effect of Tween20 in the inoculation medium on the transformation efficiency of maize. Immature embryos of inbred line 9046 were inoculated with *Agrobacterium* strain EHA105 at A_{600} = 0.8 for 20 min and cocultivated (pH5.8) at 25 °C for 3 days. Frequency of transient GUS expression (%) was defined as the number of immature embryos with GUS expression after cocultivation relative to the total number of immature embryos inoculated with *Agrobacterium*. Frequency of PPT-resistant calli (%) was defined as the number of immature embryos that generated PPT-resistant calli at the end of 2 months of culturing on selection medium relative to the total number of immature embryos inoculated with *Agrobacterium*. Three replicates for each treatment with at least 30 immature embryos per replicate were used. Columns denoted by different letters are significantly different according to the Duncan's New Multiple-Range Test at $p < 0.05$. Vertical bars represent the standard error.

Tween20 turned rapidly brown and eventually died on selection medium after cocultivation. Based on these results, 0.1% Tween20 was added to the infection medium.

Transformation of multiple inbred lines

Several factors were assayed individually in a series of experiments in this study. When these optimized transformation conditions were used for stable transformation experiments in the four maize elite inbred lines, a significant increase of the transformation efficiency was achieved. Before optimization, one to two independent transformants could be produced in one transformation experiment with 300 immature embryos of inbred line 9046. When the improved procedure was used, seven independent transgenic plants were obtained from a single experiment with 135 immature embryos of inbred line 9046. Data obtained from these experiments are summarized in Table 3. A total of 53 independent, PPT-resistant plantlets from four inbred lines were grown in a greenhouse. Almost all of the plants were normal in morphology (Figure 2f and g) and the majority (about 85%) produced seeds by self-pollination in quantities comparable with those of non-transformed control plants (Figure 2h). Although genotypic differences in the transformation efficiency were observed among the four inbred lines, these data showed that the protocol with transformation frequency, ranging from 2.35 to 5.26%, could be used to introduce useful genes into maize elite inbred lines.

Table 3. Efficiency of *Agrobacterium tumefaciens*-mediated transformation[a] of maize elite inbred lines

Experiment No.	Inbred lines	Number of immature embryos						Frequency (B/A, %)
		Inoculate-d (A)	Produced PPT-resistant callus	Regenerated plantlets	Produced positive plants[b] (B)	Produced PCR-positive for *bar* plants	Produced PCR-positive for *uid*A plants	
1	9046	175	78	8	6	6	6	3.43
2		138	72	9	7	7	6	5.07
3		95	45	5	5	5	5	5.26
4		120	58	6	4	4	4	3.33
1	Qi319	114	50	6	4	4	4	3.51
2		137	55	5	4	4	4	2.92
1	414	162	61	5	4	4	4	2.47
2		90	35	3	3	3	3	3.33
1	Mo17	108	39	3	3	3	3	2.78
2		85	28	3	2	2	2	2.35

[a]All immature embryos were inoculated with *Agrobacterium* strain EHA105 (with 0.1% (v/v) of Tween20 in the infection medium) at A_{600} = 0.8 for 20 min and cocultivated (pH5.4) at 22 °C for 3 days.
[b]Plants contained at least one transgene as determined by PCR.

Analysis of the putative transformants

All of the 53 putative transgenic plants derived from PPT-resistant calli from four inbred lines were checked by PCR. Forty-one plants showed the expected both 440-bp band (for *bar* gene) and 1060-bp band (for *uid*A gene) (Figure 8). And one plant (lane 14 in Figure 8) showed only 440-bp band and no 1060-bp band. No 440-bp band and 1060-bp band were amplified in the non-transformed plant. These PCR results confirm that most of (approximately 80%) the regenerated plants contain the transgenes derived from the pCAMBIA3301 plasmid. The production of negative plants could be due to non-transformed calli surviving in the selection medium or to the transferred gene being not stably integrated into plant genome.

Healthy leaves of the 41 PCR-positive for *uid*A and *bar* plants were painted with 200 mg l^{-1} L-PPT. All of the plants showed resistance to PPT painting, while the non-transformed controls showed necrosis (Figure 2i). These 41 plants were also tested for *GUS* expression in leave tissues and all of them had visible GUS activity in leave tissues (Figure 2e). These results verified the functional expression of *uid*A and *bar* genes in the transgenic plants.

Ten randomly sampled PCR-positive for both *uid*A and *bar* plants were further subjected to Southern blot analysis (Figure 9). Extracted DNA from leaves was digested with *Hin*dIII which cleaves pCAMBIA3301 once between the *bar* and *uid*A gene cassettes within the T-DNA and allowed to hybridize with *bar* and *uid*A probes. Both genes were detected in all of the T_0 plants analyzed, whereas no hybridization signal was detected in the non-transformed plants. As expected from the T-DNA map of pCAMBIA3301, digestion of the DNA with *Hin*dIII yielded various band sizes longer than 1.8 kb that hybridized to the *bar* probe, and various band sizes longer than 3.1 kb that hybridized to the *uid*A probe. The number of hybridizing bands reflected the number of insertion locus of the transgenes in the plant genome, which varied from one to three (Figure 9). Seven of the ten plants contained a single *uid*A and *bar* genes locus. The frequency of single inserts was similar to that observed in maize by Ishida et al. (1996) (60–70%). Two plants showed two *uid*A and *bar* genes loci and one showed three loci. The results of Southern analysis were in accordance with those of the PCR analysis and histochemical GUS assay and PPT-resistant analysis, thus confirming the presence, integration and expression of the transgene in transformants.

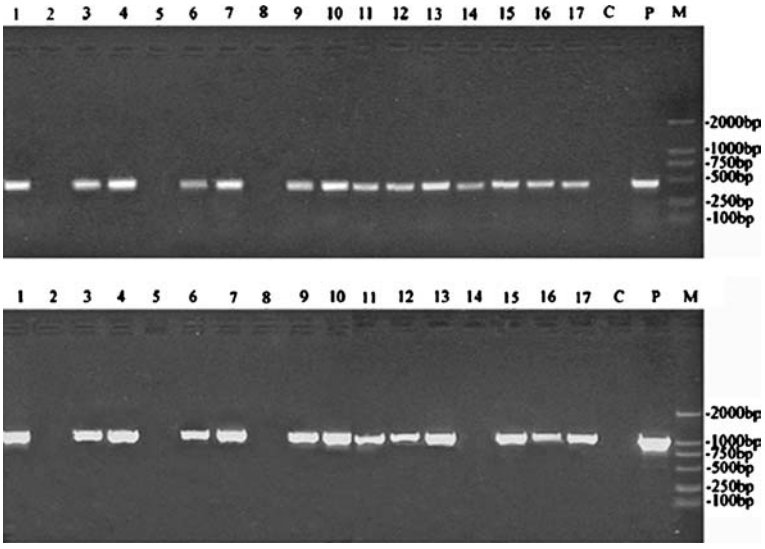

Figure 8. The representative PCR analysis of genomic DNA to detect the presence of the *uid*A and *bar* genes in putative transgenic maize plants. (*a*) PCR amplification of the 440-bp fragment of the *bar* gene. (*b*) PCR amplification of the 1060-bp fragment of the *uid*A gene. *Lane M* Molecular weight marker, *Lane P* pCAMBIA3301 plasmid DNA (positive control), *Lane C* DNA from untransformed plant (negative control), *Lane 1–17* DNA from independently transformed plants.

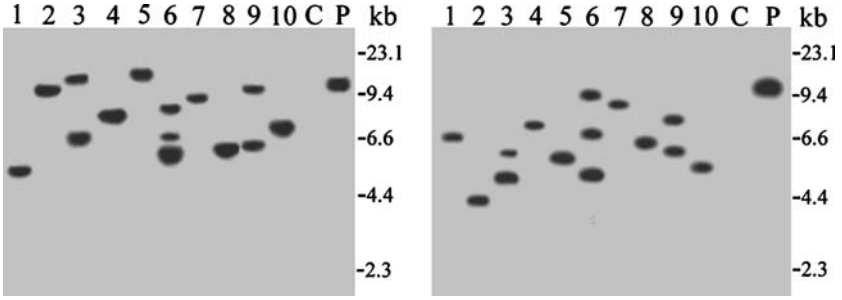

Figure 9. Southern blot analysis of PCR-positive for *uid*A and *bar* transformed plants (T_0 generation). Plasmid and genomic DNA were digested with *Hind*III and allowed to hybridize to the *uid*A (left) or *bar* (right) probe. *Lane P* Plasmid DNA of pCAMBIA3301 (10 pg), *Lane C* DNA from non-transformed plant of inbred line 9046 (30 µg), *Lanes 1–10*, DNA from transformed plants (No. 51, 45, 41, 35, 30, 26, 17, 9, 3, and 2) regenerated from PPT-resistant calli, which were derived from independent immature embryos of inbred lines Mo17 (lane 1), 414 (lanes 2, 3), Qi319 (lanes 4, 5), and 9046 (lanes 6–10) infected with EHA105, respectively (30 µg).

Inheritance of marker genes

To confirm the transmission of the transgenes to the next generation, the self-fertilized progeny of 3 T_0 plants grown in the greenhouse were examined for PPT resistance and GUS expression. The results are detailed in Table 4. The sensitive seedlings died within 10 days after herbicide treatment, while the resistant plants were as healthy as untreated control plants (Figure 2j). PPT resistance and GUS expression were strongly linked and a segregation ratio of 3:1 for both traits (resistant:sensitive and positive:negative) was observed in two lines (T_0-17 and T_0-30). A line (T_0-3) showed a segregation pattern of 15:1. These results indicated the *bar* and *uid*A gene were inherited in a normal Mendelian fashion by the progeny.

DNA was extracted from the T_1 progeny of transformants T_0-3 and T_0-17 shown in (Figure 9), and analyzed by Southern hybridization. The *bar* gene and the *uid*A gene were present in the PPT-resistant, GUS-positive progeny and absent from the sensitive, negative progeny (Figure 10). The hybridizing bands detected in T_1 plants were identical in size to those in the respective parent T_0 plants.

Discussion

Several reports have documented a difference in transformation efficiency when using different *A. tumefaciens* strains (Chan et al., 1992; Sunilkumar and Rathore, 2001; Ko et al. 2003). However, some reports indicate that the efficiency of plant

Table 4. Progeny analysis of independent T_0 transformants[a]

Trans formant (T_0)	No. of T_0 seed set	No. of progeny assayed	Segregation pattern									
			*uid*A gene expression					*bar* gene expression				
			Pos[b]	Neg[c]	Ratio	χ^2	*p*-value	Res[d]	Sen[e]	Ratio	χ^2	*p*-value
T_0-3	350	46	42	4	15:1	0.470	0.493	42	4	15:1	0.470	0.493
T_0-17	389	58	43	15	3:1	0.023	0.879	43	15	3:1	0.023	0.879
T_0-30	294	38	25	13	3:1	1.885	0.170	25	13	3:1	1.885	0.170

[a]Transgenic plants were self-pollinated.
[b]Pos, GUS assay positive (*uid*A -expresser).
[c]Neg, GUS assay negative (*uid*A non-expresser).
[d]Res, Resistant to Basta spray (*bar*-expresser).
[e]Sen, Sensitive to Basta spray (*bar* non-expresser).

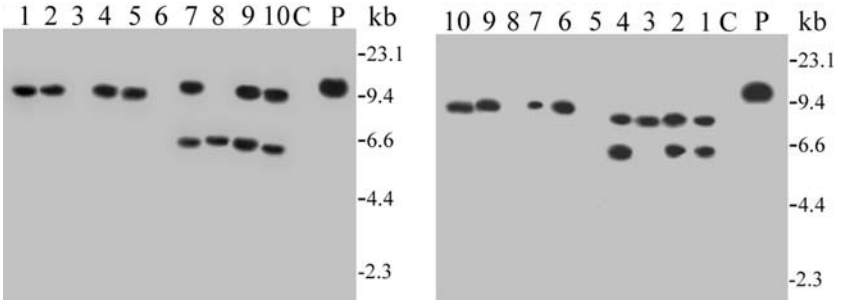

Figure 10. Southern blot analysis of the T_1 progeny of transformed plants No.3 and No.17 shown in Figure 9. Plasmid DNA (10 pg) and genomic DNA (30 µg) were digested with *Hind*III and allowed to hybridize to the *uid*A (left) or *bar* (right) probe. *Lane P* Plasmid DNA of pCAMBIA3301, *Lane C* DNA from non-transformed plant of inbred line 9046, lane 1, 2, 4, DNA from PPT-resistant and GUS-positive T_1 progeny of transformant No.17, lane 3, DNA from PPT-sensitive and GUS-negative T_1 progeny of transformant No.17, lane 5, DNA from T_0 plant No.17, lane 6, DNA from PPT-sensitive and GUS-negative T_1 progeny of transformant No.3, lane 7–9, DNA from PPT-resistant and GUS-positive T_1 progeny of transformant No.3, lane 10, DNA from T_0 plant No.3.

transformation mediated by *Agrobacterium* is not affected by different strains (reviewed in Hiei et al., 1997). To our knowledge, all *Agrobacterium*-mediated transgenic maize plants have been produced using strain LBA4404. In the present study, our results indicate that EHA105 is superior to LBA4404 in transformation of maize. EHA105 has also been found to be more suitable for transformation of other cereals (Rashid et al., 1996). The hypervirulence of EHA105 derives from the disarmed pTiBo542 (Hood et al., 1993), in which the virG and the virA genes increase the induction of the vir genes, necessary for T-DNA transfer (for review Gelvin, 2000; Gelvin, 2003). Thus, the improved plant transformation efficiency observed with EHA105 is probably related to the increased induction of the *vir* genes. Inoculation intensity which may be defined to include, either individually or in various combinations, the factors: cell density of the inoculum, the duration of inoculation and/or cocultivation, plays a critical role in determining the transformation efficiency (Amoah et al., 2001). Our observations (Figures 3 and 4) showed that increasing the inoculation intensity either by increasing the cell density of the inoculum, by longer inoculation and/or cocultivation time, increases the response obtained in the form of transient GUS expression. However, our results also suggested that beyond a threshold value, further increases in the inoculation intensity tended to cause decreases in the transformation efficiency, perhaps as a result of a decrease in cell viability. This appears to lend credence to the hypothesis that each plant cell binds to a finite number of bacteria and beyond this threshold, it appears that cell viability may be compromised, resulting in lower transformation efficiency (Gutlitz et al., 1987).

The influence of the pH of the cocultivation medium on transformation efficiency is well-documented in literature. Mondal et al., (2001) observed that the pH 5.6 in the cocultivation medium had positive effects on transformation of tea. The importance of pH for the cocultivation has also been confirmed in dendrobium orchids (Yu et al., 2001). An acidic pH of 5.5 is generally considered to be suitable as acidic pHs may induce the *vir* (virulence) genes (Stachel et al., 1986; Al-Moerbe et al., 1988). Acetosyringone is known to activate the virulence genes of the Ti plasmid at pH 5.0–5.5 and to initiate the transfer of the T-DNA (Stachel et al., 1986). In the present study (Figure 5), it appears that the pH of the cocultivation medium (pH 5.2–5.4) favored the induction of the *Agrobacterium tumefaciens vir* genes and might have contributed to the high efficiency of transgenic maize plants obtained.

Cocultivation with *Agrobacterium* at lower temperature has been shown to improve *Agrobacterium*-mediated gene transfer to plant cells. Dillen et al. (1997) indicated that temperature plays an important role in transformation with *Agrobacterium tumefaciens*. In their study, the best transformation efficiency was obtained at 22 °C in both

Phaseolus acutifolius and *Nicotiana tabacum*, irrespective of the type of helper plasmid. We investigated the effect of temperature during cocultivation in maize transformation and found under these conditions, the optimum temperature for cocultivation to be 22 °C (Figure 6). Similar results were obtained when the effect of temperature was investigated in garlic transformation (Kondo et al., 2000). The highest transient GUS expression in garlic calli was observed at 22 °C, whereas the ratio of GUS-stained calli to total calli decreased by 85% at 20 °C and by 69% at 24 °C. In the published cotton transformation reports (Sunilkumar and Rathore, 2001), cocultivation of cotyledon discs at 21 °C, compared to 25 °C, consistently resulted in higher transformation frequencies. Fullner et al. (1996) showed that low temperatures promoted pilus assembly leading to increased number of pili on the cell surface. It has been proposed that the low-temperature increase in transformation efficiency may be due to better functioning of the VirB-VirD4 part of the T-DNA transfer machinery (Fullner and Nester, 1996; Fullner et al., 1996).

The surfactant Silwet L-77 has been shown to improve the *Agrobacterium*-mediated transformation efficiency of wheat (Cheng et al., 1997; Wu et al., 2003) and floral-dip methods in radish (Curtis and Nam, 2001). In the present study, a surfactant, Tween20, added into the inoculation medium, proved to be one of the most important factors improving transformation efficiency of maize (Figure 7). Our results with Tween20 are consistent with the previous report of Suzuki and Nakano (2002) which showed improvement in transformation efficiencies in the presence of 0.1% Tween20 in the inoculation medium. The possible explanation for the positive effect of a surfactant on enhancing T-DNA delivery might be that surface-tension-free cells favor *Agrobacterium* attachment, as suggested by Cheng et al. (1997) and/or the elimination of certain substances which inhibit the attachment of *Agrobacterium* to plant cells or the growth of *Agrobacterium*.

In summary, we have established an efficient *Agrobacterium*-mediated transformation system and have identified factors that allow enhanced generation of transformed embryos and subsequent plant development. The factors included in: hypervirulent *Agrobacterium tumefaciens* strain EHA105; immature embryos inoculated with *Agrobacterium tumefacien* at $A_{600} = 0.8$ for 20 min; inclusion 0.1% Tween20 in the infection medium; cocultivation in the acidic medium (pH 5.4) at 22 °C for 3 days. Stable integration, expression, and inheritance of the transgenes were confirmed by molecular and genetic analysis. Thus, this gene transfer system could enable the development of elite maize clones with engineered traits of economic importance.

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