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Full Length Research Paper

Effects of basal media, salt concentrations, antioxidant supplements and co-effects on the *Agrobacterium*-mediated transformation efficiency in maize

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Transformation efficiency enhancement in maize *Agrobacterium*-mediated transformation was tested using four different basal media, five levels of N6 salts, two antioxidants and copper sulfate. In the absence of the antioxidants L-cysteine and dithiothreitol (DTT), the frequencies of transient GUS expression was higher using Linsmaier and Skoog (LS) and Murashige and Skoog (MS) media as an alternative to Chu (N6) and Ducan (D). N6 basal medium exhibited better performance in the presence of antioxidants than MS, LS and D basal media. Five different levels of N6 medium salts (10, 30, 50, 70 and 100%) were tested, and the highest transformation efficiency was 15.9% under a 50% salt concentration, followed by 6.4% transformation efficiency with 70 and 3.2% under 100% salt conditions. More than 95% of infected immature embryos exhibited GUS staining under 10 and 30% salt concentrations, however none of the embryos developed into embryogenic callus, indicating that low salt levels favored T-DNA delivery, but not stable transformation. Additions of DTT or L-cysteine, or a combination of L-cysteine and DTT, showed a significant improvement in the frequency of transient GUS expression, however increases were not observed with independent $CuSO_4$ treatments. Polymerase chain reaction (PCR) and Southern-blot analysis confirmed T-DNA integration into the maize genome.

Key words: Maize (*Zea mays* L.), *Agrobacterium tumefaciens*, transformation.

INTRODUCTION

The maize genome project has been completed and the draft sequence represents a vast repository of genomic data that is now readily available (Gore et al., 2009).

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Abbreviations: **N6,** Chu medium; **MS,** Murashige and Skoog medium; **LS,** Linsmaier and Skoog medium; **D,** Ducan medium; **2,4-D,** 2,4-Dichlorophenoxyacetic acid; **MES,** 2-(N-Morpholino) ethanesulfonic acid; **DTT,** Dithiothreitol; **NAA,** α-Naphthalene acetic acid; **6-BA,** N6-benzylaminopurine; **CTAB,** Cetyltrimethyl ammonium bromide; **DIG,** Digoxin; **PVP,** Polypyrrolidone; **GUS,** β-Gluceronidase gene; **Bar,** Bialaphos resistance gene.

However, research continues to annotate sequences to ascertain gene function in maize. Genetic transformation is considered one of the most direct and effective strategies to determine gene function in plants. Microprojectile bombardment (Gordon-Kamm et al., 1990) and *Agrobacterium tumefaciens*-mediated transformation (Ishida et al., 1996) are two popular methods that are currently used in maize transformation. Many studies have shown that high-copy number and extensive rearrangement of foreign DNA are frequently identified in transgenic plants by microprojectile bombardment, which often leads to transgenic silencing and unstable inheritance (Register et al., 1994; Shou et al., 2004). Therefore, high transformation frequency of maize mediated by *A. tumefaciens* has been reported as the method of choice for the delivery of

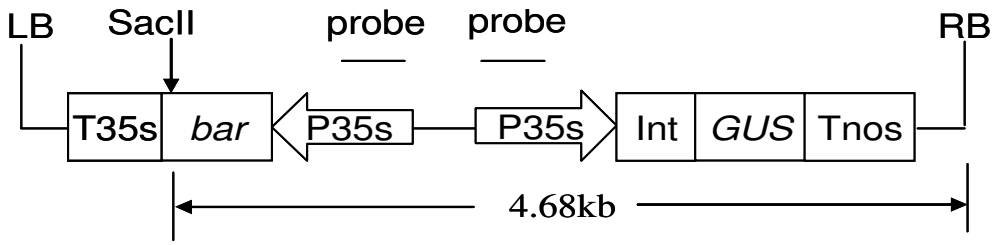

Figure 1. pCAMBIA3301. LB and RB diagram: left and right borders of the T-DNA; P35s: CaMV35s promoter; T35s and Tnos: CaMV35s terminator and nopline synthase gene terminator; *bar*: bialaphos resistance gene; *GUS*: β-gluceronidase gene.

exogenous genes into the maize genome.

A. tumefaciens-mediated transformation is the pre-eminent method for plant genetic transformation due to several advantages, including high efficiency, low-copy number, large DNA segments, low rearrangement rate and low cost (Ishida et al., 1996; Hansen and Wright, 1999). Initially, this method was only successfully applied in dicotyledonous plant genetic transformation, because monocotyledonous plants were not naturally susceptible to *A. tumefaciens*. Consequently, efforts to extend the host range of *Agrobacterium* to monocotyledonous plants were achieved and *A. tumefaciens*-mediated transformation was applied in monocots (Hernalsteens et al., 1984; Hooykaas-Van Slogteren et al., 1984). Subsequently, it was successfully implemented in several economically important monocotyledonous plants, such as rice (Hiei et al., 1994), wheat (Cheng et al., 1997), maize (Ishida et al., 1996) and barley (Tingay et al., 1997). High throughput genetic transformation systems in maize were developed using super binary vector systems and transformation frequency was approximately 40% (Zhao et al., 2001). However, transformation frequency was still low relative to standard binary vector systems, even under the improved co-culture conditions in maize (Frame et al., 2002). Therefore, further improvements in transformation efficiency in standard binary vector systems are still necessary.

Several factors can affect *A. tumefaciens*-mediated transformation efficiency, including co-cultivation period and temperature, co-cultivation medium pH, host genotype, explant type and source, *Agrobacterium* strain and vector, and components of the medium (Huang and Wei, 2005; Zhang et al., 2001; Frame et al., 2002; Zhao et al., 2001; Ishida et al., 1996; Frame et al., 2006). Two of the key factors in transformation efficiency are host genotype and medium components. Maize remains highly recalcitrant to *A. tumefaciens*-mediated transformation, but genotype A188, Hi hybrid and H99 are three genotypes recommended for successful transformation (Zhao et al., 2001; Ishida et al., 1996; Negrotto et al., 2000). Although there is an increasing interest in elite genotypes or hybrids, inbred line A188 or derivatives of A188 remain the line of choice for *A. tumefaciens*-mediated transformation. This is because type or friable embryogenic callus is easily produced from these lines. Furthermore, A188 is a model genotype widely used in *A. tumefaciens*-mediated transformation, with a reported transformation frequency of 5 to 50% (Ishida et al., 1996; Ishida et al., 2007).

Medium components are also key factors in successful transformations. Many basal media, including N6, MS, LS and D media have been used in maize transformation (Vega et al., 2008; Frame et al., 2006; Ishida et al., 2007; Yang et al., 2001). Previous studies determined that antioxidant supplemented media, such as L-cysteine, DTT and PVP (polypyrrolidone), and low salt concentration media, improved *A. tumefaciens*-mediated transformation frequency in several crops species (Perl et al., 1996; Olhoft and Somers, 2001; Paz et al., 2004). However, few reports of transformation efficiency using multiple factors and the interactive effects of these factors are available for maize.

In the present study, the effects of antioxidants, basal media, salt concentration and copper sulfate on transformation efficiency was evaluated, and focused was also on optimizing these factors to establish a high-frequency transformation system in maize.

MATERIALS AND METHODS

Plant material

The maize inbred line, A188, was used as initial material and cultivated on the Agronomy farm at China Agricultural University, Beijing, China. Following self-pollination at approximately 9 - 12 days, the ears were collected and sterilized with 70% ethanol for 5 min. Kernels of normal and healthy morphology were sampled for the study. Immature embryos approximately 1.0 to 1.5 mm in length were isolated.

Agrobacterium strain and vector

Agrobacterium strain LBA4404 (Hoekema et al., 1983) was used to harbor a standard binary vector pCAMBIA3301 (CAMBIA, Australia) that contained a CaMV35s promoter-*bar* and a CaMV35s promoter-*gus*-intron reporter gene cassette (Figure 1). *Bar* was used as the selectable marker gene and *uidA* (GUS) included an intron as the reporter gene in the binary vector. The pCAMBIA3301 vector was mobilized into *Agrobacterium* by a direct DNA transfer method (An et al., 1988) and its integrity in *Agrobacterium* cells was confirmed

Table 1. Media composition used in *Agrobacterium*-mediated transformations.

Infection medium[a]	N6, MS, LS , D medium basal salt and their vitamin, 68.5 g/l sucrose, 36.0 g/l glucose, 0.7 g/l L-proline, 0.1 g/l myo-inositol, 0.5 g/l MES, 2.0 mg/l 2,4-D, pH 5.2. Add 100 µM acetosyringone[a] before using.
Co-cultivation medium	Infection medium without glucose, reduce sucrose to 30 g/l and supplemented with 0.05 µM copper sulfate, 0.4 g/l L-cysteine[a], 0.15 g/l DTT[a], 8 g/l agar, 100 µM acetosyringone[a], pH 5.8.
Resting medium	Cocultivation medium without copper sulfate, L-cysteine[a] and DTT[a], supplemented with 0.85 mg/l silver nitrate[a] and 0.1 g/l carbenicillin[a], 2.5 g/l gelrite, pH 5.8.
Selection medium	Resting medium supplemented with 2.0 g/l bialaphos, pH 5.8.
Regeneration medium	MS basal salt and vitamin, supplemented with 30 g/l sucrose, 0.1 g/l myo-inositol, 3.5 mg/l 6-BA, 3.0 g/l gelrite, pH 5.8.
Rooting medium	MS basal salt and vitamin, supplemented with 25 g/l sucrose, 0.5 mg/l NAA, 1.2 g/l gelrite, pH 5.8.

[a] Infection medium, acetosyringone, L-cysteine, DTT, silver nitrate carbenicillin and bialaphos were filter sterilized.

by restriction enzyme analysis.

Medium

N6, MS, LS and D media were chosen and the compositions are listed in Table 1. Methods followed Ishida et al. (2007) and Vega et al. (2008) with several modifications, including: (1) The addition of $CuSO_4$, L-cysteine and DTT into the co-cultivation medium; (2) Replacing cefotaxime by carbenicillin in the resting and selection media; (3) Supplementing with silver nitrate in co-cultivation, resting and selection media; (4) Autoclaving basal salt, sucrose and glucose; and (5) Filter-sterilizing infection medium, antioxidants, vitamins and antibiotics.

Transformation procedure

Agrobacterium cells were incubated at 19℃ for 3 days in YP solid medium. The pre-infection preparation, bacterium inoculation, co-cultivation, selection and plant regeneration were conducted according to Ishida et al. (2007). Isolated immature embryos were immersed in 1.5 ml liquid infection medium in a 2.0 ml micro-centrifuge tube. The medium was removed and replaced with 1.5 ml fresh liquid infection medium when approximately 200 immature embryos were collected. The tube with collected immature embryos was centrifuged at 20,000 g at 4℃ for 10 min. The supernatant was subsequently replaced by 1 ml *Agrobacterium* suspension (OD660 = 1.0). The tube was vortexed and the isolated immature embryos were incubated for 5 min at room temperature. Infected embryos were plated with scutellum side up on co-cultivation medium, which was overlaid by a piece of sterile filter paper. Following 3 days of incubation at 19℃ in the dark, the embryos were transferred to a resting medium and incubated at 25℃. The following 7 days, all embryos were transferred into a selection medium containing 1.5 mg/l bialaphos. After 3 weeks, bialaphos was increased to 2.0 mg/l for 6 weeks to strengthen selection, followed by 2.5 mg/l bialaphos for 6 weeks. After 2 months, emergent resistant calli were transferred to a regeneration medium and incubated at 25℃ under a 16/8h (light/dark) photoperiod. Plantlets regenerated from the resistant callus within 2 - 3 weeks were transferred to a tube containing rooting medium. Plantlets with fully-grown roots were transplanted into soil and grown under greenhouse conditions.

Histochemical GUS assays

GUS assays were conducted on infected immature embryos after 3 days of co-cultivation (Jefferson et al., 1987). One hundred infected immature embryos were randomly chosen for each assay and three replicates were conducted. Ten resistant calli and shoots which developed from resistant callus were also submitted for assay. The frequency of transient GUS expression (%) was determined as the percentage of the number of infected immature embryos that exhibited GUS staining after 3 days of co-cultivation. The transformation frequency (%) was the percentage of the number of positive transgenic plants against the total number of immature inoculated embryos.

Transgene analysis

Total genomic DNA from regenerated plant leaves was extracted following the CTAB isolation procedure (Saghai-Maroof et al., 1984), and used for polymerase chain reaction (PCR) and Southern blot analysis. PCR primers for the *bar* genes were forward primer: 5′-ACTTCAGCAGGTGGGTGTAGAGCGT-3′ and reverse primer: 5′-GCACCATCGTCAACCACTACATCGA-3′. The PCR parameters included one cycle of 95℃ 3 min; 35 cycles of 95℃ 30 s, 61℃ 30 s, 72℃ 45 s and one cycle of 72℃ 10 min; samples were stored at 4℃. PCR products were 273 bp and separated on 2% (w/v) agarose gels. 20 mg of each DNA sample was digested with Sac at 37℃ overnight. Digested products were fractionated on 0.8% agarose gels and subsequently transferred to nylon membranes. DNA was fixed to the membrane at 80℃ for 2 h. The CaMV35s promoter probe was DIG labeled applying PCR and forward primer: 5′-TACCCGAGCAATAATCTCCAGG-3′ and reserve primer: 5′-CGGCAGAGGCATCTTCAACGA -3′. Southern blot analysis was conducted according to the Roche Southern blot Kit protocol (Roche Applied Science, Mannheim Germany).

Transgenic plants regenerated from resistant calli were selfed or back-crossed to the wild-type A188 plants. For progeny analysis, five sets of progeny were analyzed using PCR assays to examine the *bar* gene segregation patterns. Progeny segregation analysis of

Table 2. The effects of media, antioxidants and N6 salts levels on infection frequency.

Type of medium	Treatment		Number of infected immature embryos	Number of GUS staining embryos	Average frequency of transient GUS expression	Number of infected immature embryos	Number of resistant callus	Number of regeneration plants	Number of positive plants	Transformation frequency
MS	1		300	113	37.7±5.0	723	8	7	0	0.0
	2		300	188	62.6±4.5	1132	39	35	23	2.0
LS	1		300	121	40.3±4.7	847	13	10	0	0.0
	2		300	170	56.6±4.1	1498	57	51	33	0.9
D	1		300	101	33.6±4.5	426	5	5	0	0.0
	2		300	163	54.3±4.7	792	31	26	14	1.8
N6	1		300	82	27.3±3.5	551	6	6	0	0.0
	2	10%	300	295	98.3±0.6	877	0	--	--	--
		30%	300	287	95.6±1.5	784	0	--	--	--
		50%	300	278	92.6±3.2	653	112	107	104	15.9
		70%	300	246	82±4.0	574	47	44	37	6.4
		100%	300	217	72.3±3.7	872	44	41	28	3.2

Treatment 1 = Absence of 0.4 g/l L-cysteine and 0.15 g/l DTT.
Treatment 2 = Presence of 0.4 g/l L-cysteine and 0.15 g/l DTT.
10, 30, 50, 70 and 100% mean levels of N6 medium basal salts.

the *bar* gene was statistically analyzed for goodness-of-fit to simple Medelian expectations using a Chi-square test.

RESULTS

Effects of basal medium and antioxidants

The absence of 0.4 g/l L-cysteine and 0.15 g/l DTT resulted in a decreased frequency of transient GUS expression in N6 medium (27.3%) compared with LS (40.3%), MS (37.7%) and D media (33.7 %) (Table 2). GUS activity increased with the addition of both 0.4 g/l L-cysteine and 0.15 g/l DTT. Among the four different basal media, transient GUS expression improved most with N6 basal medium; 72.3% infected immature embryos displayed GUS staining, approximately three fold higher than in the absence of L-cysteine and DTT (27.3%). Consequently, 0.4 g/l L-cysteine and 0.15 g/l DTT were added to the N6 salt co-cultivation medium for subsequent experiments.

Transformation frequency was also affected by basal medium and antioxidants. The inclusion of 0.4 g/l L-cysteine and 0.15 g/l DTT in the co-cultivation medium increased the transformation frequency of the N6 medium (3.2%) compared to MS (2.0%), LS (0.9%) and D (1.8%) media (Table 2).

Effects of N6 medium under different salt concentrations

The differences in the frequency of transient GUS expression were exhibited among the five different basal salt levels of N6 medium. The following 3 days of co-cultivation, over 95% of infected immature embryos displayed GUS staining in 10 and 30% N6 basal salts medium. Unfortunately, most infected immature embryos did not develop further and died, indicating that low salt concentrations in the medium could have negative effects on the embryogenic capacity of immature maize embryos, although salt concentration facilitated high frequency of T-DNA delivery. The frequency of transient GUS expression reached 92.7% with 50% N6 basal salts, but with the increased salt concentration, transient GUS expression decrea-sed by 82.0% in 70% basal salt and 72.3% in 100 % basal salts (Table 2). Therefore, salt concentration in basal medium likely affected the transformation frequency and high salt concentration resulted in a low transformation rate.

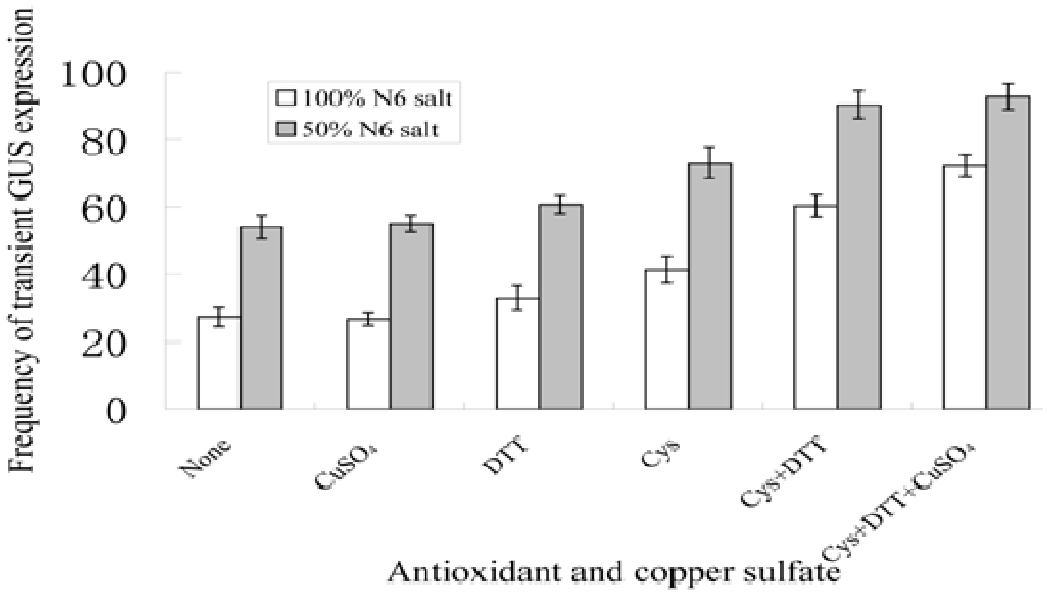

Figure 2. The effects on infection frequency of copper sulfate and antioxidants added to the co-cultivation medium. Data shown are mean ± SE of three experiments; each experiment consisted of N = 100. L-cysteine at 0.4 g/l, DTT at 0.15 g/l and copper sulfate at 0.05 µM.

The application of 50% N6 salt concentration resulted in a transformation frequency increase as high as 15.9%, followed by 6.4% with a 70% salt level and 3.2% with a 100% salt concentration (Table 2). These results suggested that basal salt concentration greater than 50% was not favorable for the transformation process, probably suppressed infection and subsequent integration of T-DNA into the plant genome. As a result, a 50% salt concentration, 0.4 g/l L-cysteine, 0.15 g/l DTT and 0.05 µM $CuSO_4$ for *A. tumefaciens*-mediated transformation in the system was used.

Effects of individual and combinations of antioxidants and copper sulfate

In N6 basal salt and absence of copper sulfate co-cultivation medium, 27.3% of the immature embryos displayed GUS staining. However, a copper sulfate supplement of 0.05 µM decreased the frequency of transient GUS expression to 26.7% (Figure 2). Addition of DTT alone resulted in a higher level of GUS expression with 33% in 100% N6 salt and 60.7% in 50% salt. L-cysteine separately showed an increase in transient GUS expression by 41.3% in 100% N6 salt and 73% in 50% N6 salt, indicating that L-cysteine was more favorable to DTT alone in the co-cultivation medium. The combined use of both L-cysteine and DTT in the co-cultivation medium demonstrated a rise in the level of GUS expression to 60.3% in 100% N6 salt and 90.3% in 50% N6 salt. Including copper sulfate with the combined use of L-cysteine and DTT resulted in higher GUS activity with a transient GUS expression of 72.3% at a 100% salt level and 92.7% at 50% N6 salt.

Transient GUS expression was not increased at 100% N6 salt and $CuSO_4$ alone. However, a significant improvement in transient GUS expression was observed when DTT was added alone ($p < 0.05$) (Table 3). Addition of L-cysteine alone and the combination of L-cysteine and DTT in the co-cultivation medium resulted in significant increase in transient GUS expression ($p < 0.01$) (Table 3). Similar results were obtained when 50% N6 salt concentration was applied to the co-cultivation medium (Table 3).

Transgenic plant recovery and molecular and genetic characterization

Resistant calli began to emerge from the immature infected embryos following approximately two months of bialaphos selection (Figure 3). The plants regenerated from these calli appeared normal morphologically (Figures 4a and b). Ten resistant calli and plantlets were randomly chosen for GUS assays, respectively and all samples displayed blue GUS staining (Figures 5a - c). These results indicated the *uidA* gene was integrated and expressing in the calli and plantlets.

Total genomic DNA was extracted from leaf material of 332 putative plantlets for PCR analysis. Primer pairs specific to detect the *bar* gene were employed. 239 of 332 putative plantlets tested positive with 72.0% selection efficiency (Figure 6), suggesting our three-step selection regime (beginning with 1.5 mg/l for 3 weeks; increased to

Table 3. T-test analysis: Effects of different antioxidants and copper sulfate on the frequency of transient GUS expression.

N6 Salt concentration	Treatment	Average frequency of transient GUS expression	Variance	P value	Significance level
100%	none	27.33 ± 3.51	12.33		
	$CuSO_4$	26.67 ± 2.51	6.33	0.401241	NS
	DTT	33.00 ± 2.65	7.00	0.044696	*
	L-cys	41.33 ± 4.51	20.33	0.006618	**
	DTT+L-cys	60.33 ± 4.04	3.00	0.000218	**
	DTT+L-cys+$CuSO_4$	72.33 ± 3.79	14.33	5.62E-05	**
50%	none	54.00 ± 3.00	9.00		
	$CuSO_4$	55.00 ± 2.00	4.00	0.328023	NS
	DTT	60.67 ± 3.51	12.33	0.033383	*
	L-cys	73.00 ± 4.00	16.00	0.0013794	**
	DTT+L-cys	90.33 ± 3.21	10.33	6.92258E-05	**
	DTT+L-cys+$CuSO_4$	92.67 ± 3.21	10.33	5.42E-05	**

100 and 50% N6 basal salts were used in co-cultivation medium for every treatment.
Data shown are mean ± SE of three experiments; each experiment included N = 100.
L-cysteine at 0.4 g/l, DTT at 0.15 g/l and copper sulfate at 0.05 mM.

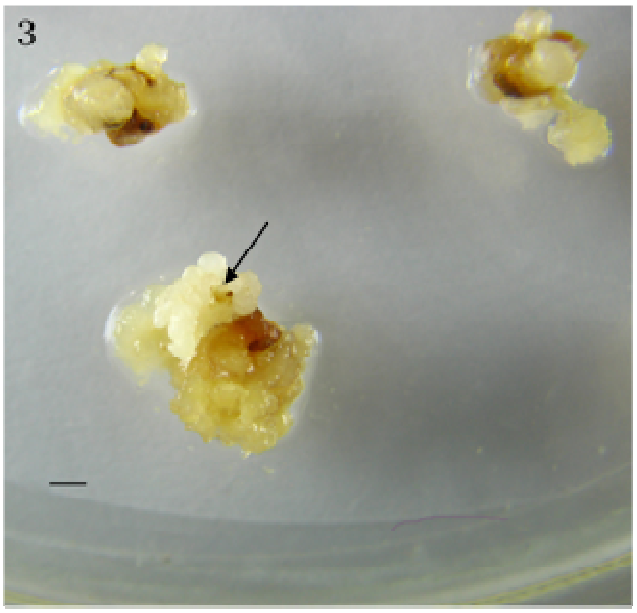

Figure 3. Resistant embryogenic callus emergent from an immature maize embryo A188 showing vigorous growth on selective medium with 2.5 mg/l bialaphos (scale bar = 1 mm).

2.0 mg/l for 6 weeks and 2.5 mg/l for 6 weeks) resulted in a low escape rate.

Six genomic DNA samples were obtained from PCR positive plants and used for Southern blot analysis. Figure 7 indicates that four transgenic plants possessed a single copy of the transgene; one possessed two copies of the transgene, revealing that the T-DNA was successfully integrated into the genome, and the different banding patterns suggested each was from independent transgenic events.

Five transgenic events with large numbers of seeds were chosen to examine *bar* gene segregation in the progeny. Two transgenic events (T83 and T141) were selfed and three events (T127, T189 and T206) were backcrossed to the wild-type A188 plants. T83 and T141 displayed a 3:1 segregation ratio and T127, T189 and T206 displayed a 1:1 ratio, which indicated the presence of single locus integration in all five transgenic lines (Table 4).

DISCUSSION

Maize transformation frequency has primarily been affected by basal medium in *A. tumefaciens*-mediated transformation (Ishida et al., 1996; Frame et al., 2006). However, to date the reason for this limitation is not well understood. The production of type or friable embryogenic callus from maize immature embryos is clearly related to the nature of the basal medium, and the efficiency of embryogenic callus formation is different when various basal media are used (Armstrong and Green, 1985; Ducan et al., 1985; Carvalho et al., 1997). The present results demonstrated that for maize A188 inbred line, LS medium performed better than N6 medium when L-cysteine and DTT were absent from the co-cultivation medium. This result is consistent with that reported by Ishida et al. (1996). However, when a 50%

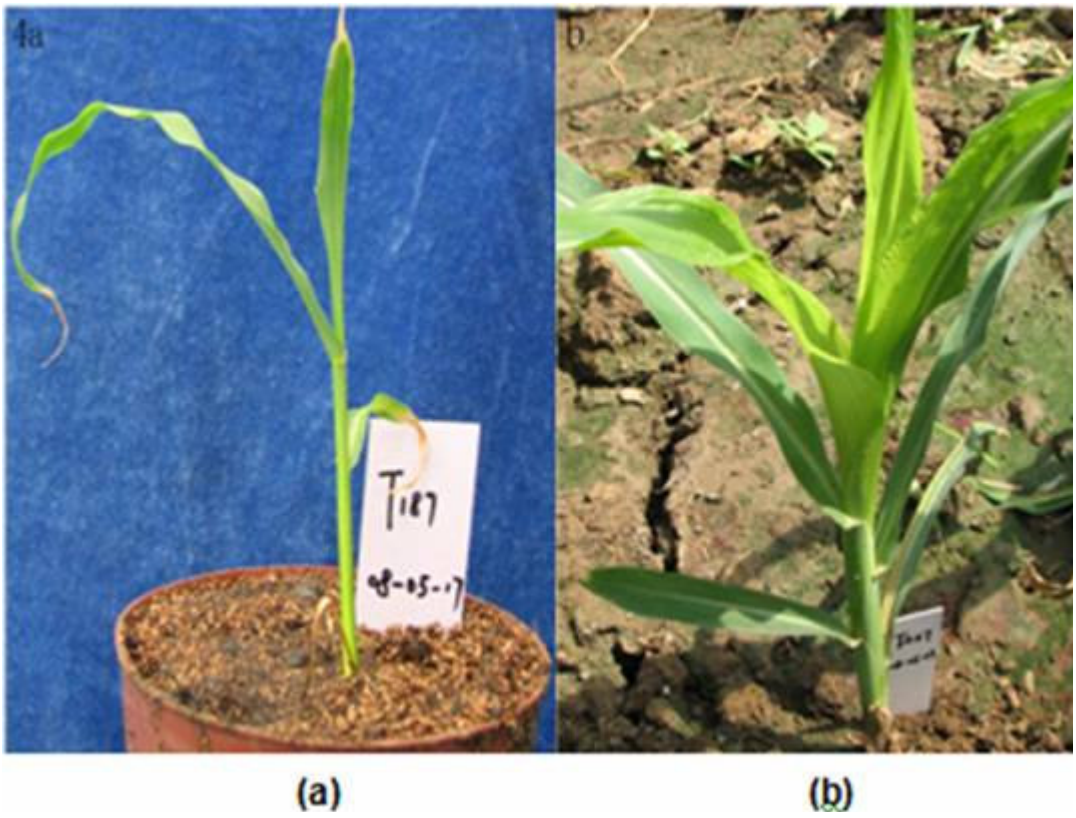

Figure 4. The regenerated transgenic plants transferred to soil. **a:** Transgenic plant (T_0) transferred from tissue culture to pot. **b:** Transgenic plant grown in the field and under greenhouse conditions.

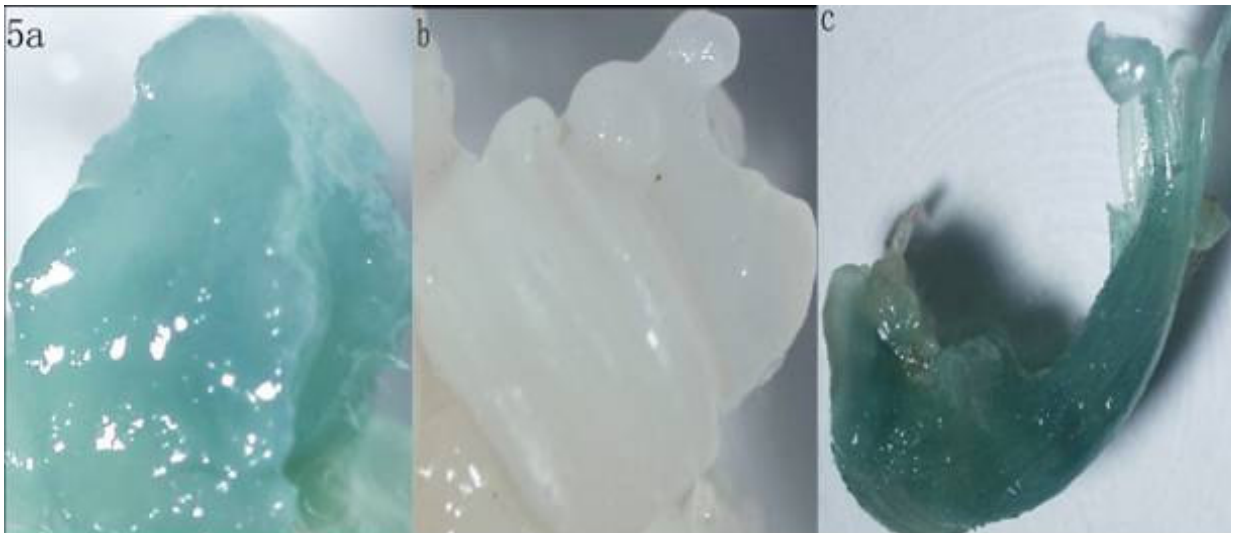

Figure 5. Stable GUS expression was observed in resistant callus and shoots. **a:** Bialaphos resistant callus. **b:** Normal A188 callus (control). **c:** Shoot regenerated from resistant callus.

instead of full strength basal salt was used in conjunction with both 0.4 g/l L-cysteine and 0.15 g/l DTT in the co-cultivation medium, a high frequency of transient GUS expression was observed and transgenic plants were obtained from N6 basal medium. These results are incongruent from those reported by Ishida et al. (1996), who showed that no positive transgenic plants were obtained when using N6 as a basal medium. In addition,

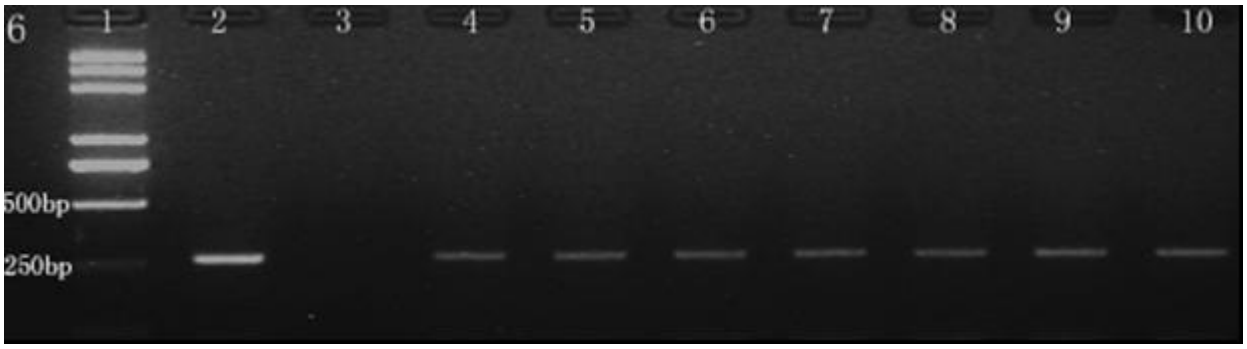

Figure 6. PCR amplification using bar primers. Lane 1: DL2000 plus DNA maker. Lane 2: Plasmid DNA. Lane 3: Non-transgenic plant. Lanes 4 - 10: transgenic plants.

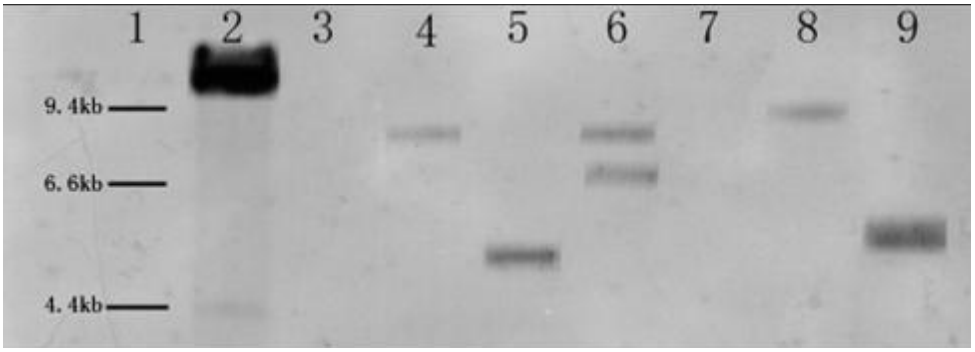

Figure 7. Southern-blot analysis of transformed plants. Lane 1: λDNA/Hind maker. Lane 2: pCAMBIA3301 digested with Sac. Lane 3: Non-transgenic plant. Lane 4 - 6 and 8 - 9: Different PCR-positive plants. Lane 7: False PCR-positive plant.

Table 4. Progeny segregation analysis of primary transgenic A188 maize events.

Events	Total progeny	Selfing or back crossing	Positive	Negative	Segregation ratio	χ^2
T83	39	Selfing	28	11	3:1	0.214
T127	52	Back crossing	24	28	1:1	0.308
T141	26	Selfing	19	7	3:1	0.051
T189	91	Back crossing	44	47	1:1	0.099
T206	63	Back crossing	29	34	1:1	0.397

$\chi^2 = 3.84$ (0.05, 1 df).

antioxidants were integral in *A. tumefaciens*-mediated transformation of maize (Frame et al., 2002, 2006; Vega et al., 2008). The role of antioxidants may minimize cell death caused by a hypersensitive response (Frame et al., 2002). This may enable increased survival and embryogenesis of infected embryogenic-competent cells, resulting in improved transformation frequency. In the system used in this study, 0.4 g/l L-cysteine and 0.15 g/l DTT as a supplement to the N6 co-cultivation medium resulted in increased transformation frequency.

Gao and Bao (2004) found that salt concentration influenced gene transfer by influencing *A. tumefaciens* growth. However, the reason transformation frequencies improved under low concentration remains unclear. Most GUS staining appeared on the embryo axis sides and the edges when a 100% N6 basal salt concentration was used in the co-cultivation medium. However, more than 50% GUS staining displayed in the scutellum area when the salt concentration was below 50% (data no show). Callus is typically derived from the scutellum area of immature embryos and rarely from the axis side or edges. This likely explains why the transformation frequency was improved in low salt concentrations. However, very low salt concentrations of 10 and 30% exhibited negative effects on transformation frequency. Infected immature embryos severely damaged, did not

grow further into embryogenic calli, and consequently no resistant calli were recovered. N6 medium with 50% salt concentration showed the highest frequency of transient GUS expression, and also facilitated further development of infected immature embryos without negative impact, resulting in increased transformation efficiency.

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