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GENETIC TRANSFORMATION AND HYBRIDIZATION

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Improved *Agrobacterium*-mediated transformation of three maize inbred lines using MS salts

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Abstract Transformation technology as a research or breeding tool to improve maize is routinely used in most industrial and some specialized public laboratories. However, transformation of many inbred lines remains a challenging task, especially when using *Agrobacterium tumefaciens* as the delivery method. Here we report success in generating transgenic plants and progeny from three maize inbred lines using an *Agrobacterium*-mediated standard binary vector system to target maize immature embryos. Eleven maize inbred lines were pre-screened for transformation frequency using N6 salts. A subset of three maize inbred lines was then systematically evaluated for frequency of post-infection embryogenic callus induction and transformation on four media regimes: N6 or MS salts in each of two distinct media backgrounds. Transgenic plants recovered from inbred lines B104, B114, and Ky21 were analyzed for transgene integration, expression, and transmission. Average transformation frequencies of 6.4% (for B104), 2.8% (for B114), and 8% (for Ky21) were achieved using MS salts. Availability of *Agrobacterium*-mediated maize inbred line transformation will improve future opportunities for maize genetic and functional genomic studies.

Keywords *Agrobacterium tumefaciens* · *Zea mays* · Inbred maize · MS salts · N6 salts · Type I callus

Introduction

Agrobacterium tumefaciens-mediated transformation is the preferred method for plant genetic transformation because it generates a high proportion of independent events with single or low transgene copy numbers (Zhao et al. 1998; Dai et al. 2001; Shou et al. 2004) which is expected to favor consistent transgene expression in progeny generations (Meyer and Saedler 1996). This method has been used to transform tissue culture amenable genotypes such as the Hi II hybrid (Zhao et al. 2001; Frame et al. 2002) or inbred lines A188 and H99 (Ishida et al. 1996, 2003; Negrotto et al. 2000). A limited number of proprietary (Gordon-Kamm et al. 2002) or public inbred lines (Ishida et al. 2003; Huang and Wei 2005), and various recalcitrant inbred lines crossed to A188 (Lupotto et al. 2004; Zhang et al. 2003) have also been transformed using this method.

In diverse transformation studies targeting scutellar cells of maize immature embryos, success in recovering transgenic events has been attributed, in part, to the induction and maintenance of a high embryogenic callus induction frequency (ECIF, Songstad et al. 1996; Brettschneider et al. 1997; Ishida et al. 2003; Lupotto et al. 2004; Lowe et al. 2004). Efforts to improve ECIF of tissue culture-recalcitrant inbred maize genotypes have focused on media manipulations (Armstrong and Green 1985; Tomes and Smith 1985; Duncan et al. 1985; Hodges et al. 1986; Close and Ludeman 1987; Vain et al. 1989; Songstad et al. 1991; Bohorova et al. 1995; Carvalho et al. 1997); crossing “responsiveness” from A188 into the background of choice (Tomes and Smith 1985; Hodges et al. 1986; Lupotto et al. 2004) and back crossing with marker-assisted breeding (Armstrong et al. 1992; Lowe et al. 2004).

In studies comparing the effect of basal salts used in tissue culture media on embryogenic callus induction frequency in maize, both response frequency and range of responding inbreds was higher on MS (Murashige and Skoog 1962) than on N6 (Chu et al. 1975) salts but remained strongly influenced by maize genotype (Tomes and Smith 1985; Hodges et al. 1986). *Agrobacterium*-mediated transformation frequency was improved when Hi II (Armstrong et al. 1991) embryos were cultured on N6 salts or a combination of N6 and MS salts instead of MS salts (Zhao et al. 2001); however, transformation of inbred line A188 was achieved using LS salts (synonymous with MS salts) and

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LS vitamins (Linsmaier and Skoog 1965) but not N6 salts and vitamins (Ishida et al. 1996). Using silver nitrate in solid-culture steps and replacing the antibiotic cefotaxime with carbenicillin for bacteria counter-selection increased the transformation frequency of Hi II embryos on both N6 and MS media (Zhao et al. 2001) and inbred line H99 on MS medium (Ishida et al. 2003).

This study was undertaken to identify a media regime for improved transformation frequency (TF) of one or more maize inbred lines using the *Agrobacterium* standard binary vector system previously described (Frame et al. 2002). Eleven maize inbred lines were pre-screened for transformation using N6 salts. A subset of three maize inbred lines was then systematically evaluated for post-infection ECIF and TF on four media regimes: N6 or MS salts in each of two distinct media backgrounds. Evidence is presented that transformation frequency for inbred lines B104, B114, and Ky21 was improved when MS instead of N6 salts were used in tissue culture medium.

Materials and methods

Plant material

Seed for inbred embryo donor plants was obtained, with our thanks, from researchers at Iowa State University (A Hallauer for B73, B104 and B114; M Lee for H99 and Mo17; P Schnable for Ky21; and M James for W64 and Oh43), University of Minnesota (R Phillips for A188), Louisiana State University (Z Chen for Mp420 and GT-Mas:gk), University of North Carolina (B Thompson for M37W), and University of Wisconsin–Madison (H Kaeppler for W22). Inbred lines were included in this study for various reasons, as follows: model laboratory lines for inbred germplasm transformation (A188, H99); relevance in maize genome projects (B73, W22); targeted for specific research needs (Ky21, Oh43, Mp420, GT-Mas:gk, W64); or favorable agronomic traits (B104, B114, Mo17, M37W). Immature embryos (1.2–2.0 mm) were aseptically

Table 1 Media formulations

					Media A		Media B	
Medium component	Source	Stock	Unit per litre	Infection media[a]	Cocultivation[b]	Resting or selection	Cocultivation	Resting or selection
Major and minor salts[c]	Phytotech Lab[d]	–	g	N6	N6 or MS[e]		N6 or MS[e]	
2,4-D	Sigma[f]	4.5 mM	mL	1.5	1.5	1.5	–	–
Dicamba	Sigma	30 mM	mL	–	–	–	0.5	0.5
Sucrose	Fisher[g]	–	g	68.4	30	30	30	30
Glucose	Fisher	–	g	36	–	–	–	–
MES	Fisher	–	g	–	–	0.5	–	0.5
Myo-inositol	Sigma	–	g	–	–	–	0.1	0.1
Casein hydrolysate	Sigma	–	g	–	–	–	0.1	0.1
Proline	Fisher	–	g	0.7	0.7	0.7	0.7	0.7
pH				5.2	5.8	5.8	5.8	5.8
Gelrite	Sigma	–	g	–	3.0	–	2.3	2.3
Purified agar	Sigma	–	g	–	–	8	–	–
					Autoclave (25 min, 121°C, 125 kPa)			
Vitamins[h]	Fisher or Sigma	1000 X	mL	N6	[e]	[e]	[e]	[e]
Silver nitrate	Fisher	50 mM	mL	–	0.1	0.1	1.8	1.8
L-Cysteine[i]	Sigma	100 mg/mL	mL	–	3.0	–	3.0	–
Acetosyringone	Sigma	100 mM	mL	1.0	1.0	–	1.0	–
Antibiotics	Phytotech Lab	variable	mL	–	–	[e]	–	[e]
Bialaphos	Duchefa[j]	1 mg/mL	mL	–	–	[e]	–	[e]

[a] Infection medium was filter sterilized

[b] Made from one to four days prior to use

[c] N6 salts (4.0 g/L); MS salts (4.3 g/L)

[d] Shawnee Mission, Kansas

[e] See “Materials and methods” section

[f] St Louis, Missouri

[g] Pittsburgh, Pennsylvania

[h] N6 or modified MS (1 mL/L) from 1000 × stock

[i] 300 mg/L in all experiments unless stated otherwise

[j] Duchefa Biochemie, the Netherlands

dissected from field-grown or greenhouse-grown ears harvested 10–16 days post-pollination.

Agrobacterium strains and vectors

Agrobacterium strains EHA101 (Hood et al. 1986), LBA4404 (Hoekema 1983), and C58Z707 (Deblaere et al. 1985) harboring the standard binary vector pTF102 (Frame et al. 2002) with the single 35S promoter-*bar*/35S promoter *gus*-intron cassette, or the standard binary vector pTF101.1 (Paz et al. 2004) containing the double 35S promoter-*bar* selection cassette and no visual marker gene were used. pTF102 serves as a test construct because it carries the *gus* gene, while pTF101.1 contains a multiple cloning site and is used as a vector for subcloning various genes of interest. Antibiotics in YEP media used to maintain these vectors were as follows: pTF102 and pTF101.1 in EHA101 (100 mg/L spectinomycin, 50 mg/L kanamycin sulfate, 25 mg/L chloramphenicol), in LBA4404 (50 mg/L rifampicin, 100 mg/L spectinomycin) and pTF102 in C58Z707 (50 mg/L kanamycin sulfate, 100 mg/L spectinomycin). Bacteria manipulations, maintenance, and preinfection preparation were identical to the protocol detailed in Frame et al. (2002). *Agrobacterium* solid cultures were grown at 19°C for 3 days in preparation for all transformation experiments.

Culture media

Infection medium

Liquid infection medium (Table 1) was used for preculture, washing and infection steps in all experiments, including those for which MS salts were compared with N6 salts in subsequent solid-culture steps. Acetosyringone (AS) from frozen stocks was added (to a final concentration of 100 μM) at use (Inf + AS).

Media backgrounds

Throughout this study, two distinct media were used in solid-culture steps (cocultivation, resting, and selection): Media A [after Zhao et al. (2001) with some modifications as detailed in Frame et al. (2002)]; and Media B (after Carvalho et al. (1997) with minor modifications). Formulations for Media A and B are outlined in Table 1. Within each media background, selection medium was identical to resting media but was supplemented with 1.5, 3, and finally 5 mg/L bialaphos.

Major and minor salts and other media variations

In preliminary transformation experiments, 11 inbred lines were screened for post-infection ECIF and TF using both Media A and B backgrounds, each containing N6 (Chu et al. 1975) basal salts (N6-A and N6-B). Two cocultivation media treatments (0 or 300 mg/L cysteine) were also compared (Table 2) and resting and selection media were supplemented with cefotaxime (100 mg/L) and vancomycin (100 mg/L) for eradicating bacteria. In a separate experiment, 13 inbred lines were also screened for pre-infection ECIF (i.e, without *Agrobacterium* infection), using MS basal salts in media backgrounds A and B (MS-A and MS-B, Fig. 3).

Media A and Media B, each supplemented with both N6 or MS (Murashige and Skoog 1962) basal salts were used

Table 2 Frequency (%) of embryogenic callus induction and stable transformation for 11 maize inbred lines

Inbred line	N6 media				Transformation frequency[c,d] (%)	Total no. of embryos infected
	A[a]	A[b]	B[a]	B[b]		
A188	31	39	68	43	0.0	767
B104	49	37	61	20	0.0	742
B114	**58**[c]	27	69	57	0.2	561
B73	2	2	6	3	0.0	709
GT-Mas:gk	36	33	72	57	0.0	858
H99	73	84	73	86	0.0	641
Ky21	30	**50**[c]	47	**64**[c]	2.0	246
Mo17	34	3	11	2	0.0	588
Mp420	5	12	22	41	0.0	674
Oh43	19	11	87	89	0.0	573
W22	37	64	47	69	0.0	755

Frequency (%) from nine ears (averaged over three ears per *Agrobacterium* strain). Each ear was split to four media treatments

[a] Cocultivation media treatment: 0 mg/L cysteine

[b] Cocultivation media treatment: 300 mg/L cysteine

[c] Bold numbers represent inbred line and media treatment from which stable transgenic events were recovered

[d] No. of bialaphos resistant callus events/total no. of embryos infected (× 100)

Table 3 Effect of salts complement and media background on stable transformation frequency of three maize inbred lines

Inbred line ID[a]	*Agrobacterium* vector (strain)	Transformation frequency (%)[b]			
		MS Salts[c]		N6 Salts[c]	
		Media A	Media B	Media A	Media B
B104	pTF102(EHA101)	0.0	0.0	0.0	0.0
	pTF102(LBA4404)	0.0	2.3	0.0	0.0
	pTF101.1(EHA101)	3.2	0.7	0.0	0.0
	Average frequency (%)	**1.0**	**1.0**	**0.0**	**0.0**
B104 (GH)[d]	pTF102(EHA101)	6.3	13	0.5	0.5
	pTF102(LBA4404)	1.5	7.4	6.8	0.0
	pTF101.1(EHA101)	0.0	15	0.0	0.0
	Average frequency (%)	**2.6**	**12**	**2.4**	**0.2**
B104 (all)[e]	**Average frequency (%)**	**1.8**	**6.4**	**1.2**	**0.1**
B114	pTF102(EHA101)	2.3	6.9	1.4	0.0
	pTF102(LBA4404)	0.0	1.6	0.0	0.0
	pTF101.1(EHA101)	2.8	0.0	0.0	2.3
	Average frequency (%)	**1.7**	**2.8**	**0.5**	**0.7**
Ky21	pTF102(EHA101)	1.7	16	0.0	2.1
	pTF102(LBA4404)	6.5	4.6	0.0	0.0
	pTF101.1(EHA101)	nt	3.2	0.0	0.0
	Average frequency (%)	**4.1**	**8.0**	**0.0**	**0.7**

[a] Nine field ears/inbred (87–257 embryos per treatment). Each ear was split to all four media (except nt)

[b] No. of bialaphos resistant callus events/no. of infected embryos (× 100)

[c] Media backgrounds A and B: See Table 1 and "Materials and methods" section

[d] Average from eight greenhouse (GH) ears (219–284 embryos per treatment). Each ear was split to all four media

[e] Average over field and greenhouse

to compare post-infection ECIF (Fig. 1) and TF (Table 3) for a subset of inbred lines (B104, B114, Ky21) on four different (N6-A, N6-B, MS-A, and MS-B) media. In these latter experiments, cocultivation media was supplemented with 300 mg/L cysteine and carbenicillin (250 mg/L) was used for counter selecting bacteria.

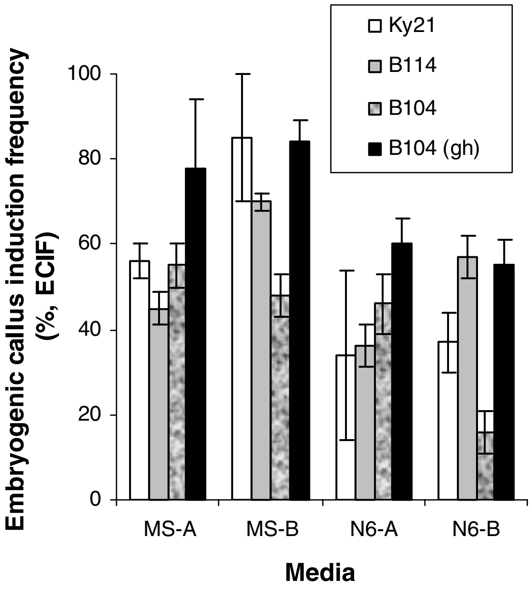

Fig. 1 Post-infection embryo response (% ECIF) of three inbred maize lines on MS or N6 salts in two media backgrounds (A and B). Field-derived immature embryos of maize inbred lines B114, Ky21, and field- or greenhouse-grown B104 were infected with pTF102 and pTF101.1 in EHA101 and pTF102 in LBA4404. Embryos were cocultivated on medium supplemented with 300 mg/L cysteine and cultured on two divergent media backgrounds (A and B), each supplemented with N6 or MS salts. All media contained carbenicillin (250 mg/L) for bacteria counter selection. Ten days after infection, embryos were assessed for embryogenic callus induction frequency using a dissection scope. Data is averaged over three *Agrobacterium* vector and strain combinations, and embryos from nine ears per field-grown inbred line (or eight ears from greenhouse-grown B104)

References to N6 or MS salts throughout this study imply that the corresponding N6 or modified MS vitamins, respectively, were also used.

Vitamin stocks

N6 vitamin stocks (1000 ×) contained 2.0 g/L glycine, 1 g/L thiamine HCl, 0.5 g/L each of pyridoxine HCl and nicotinic acid. Modified MS vitamin stock (1000 ×) contained 2 g/L glycine, 0.5 g/L each of thiamine HCl and pyridoxine HCl, 0.05 g/L nicotinic acid and no myo-inositol. Vitamins were added to the media (1 mL/L) after autoclaving (Table 1).

Regeneration media

MS salts and modified MS vitamins, 6% sucrose, 100 mg/L myo-inositol, no hormones (modified from Armstrong and Green 1985 and McCain et al. 1988), 0.3% gelrite, pH 5.8 and supplemented with 4 or 6 mg/L glufosinate ammonia (Pestanal®, Sigma) and 250 mg/L cefotaxime after autoclaving. Germination of somatic embryos was carried out on MS medium, 3% sucrose, 100 mg/L myo-inositol, 0.3% gelrite, pH 5.8, without glufosinate or cefotaxime.

Other

100 mm × 25 mm petri-plates were used for all solidified media, and all stocks were prepared as described in Frame et al. (2002).

Transformation procedure

Ear sterilization, bacteria preparation, infection, and cocultivation procedures were according to Frame et al. (2002). Briefly, *Agrobacterium* suspension precultured in Inf + AS (2 h) was diluted to OD_{550} 0.3–0.4 and added (1 mL) to the dissected embryos that were prewashed in bacteria-free Inf + AS medium. The tube was gently rocked 20 times and incubated (room temperature, dark) for 5 min. Infected embryos were plated, scutellum side up, on cocultivation medium with or without 300 mg/L cysteine, and incubated in the dark (20°C) for 3 days. All embryos were transferred to resting medium containing antibiotics but no bialaphos and incubated at 28°C (dark) for 7 or 10 days after which they were transferred to medium containing 1.5 mg/L bialaphos to begin the selection. Selection was enhanced to 3 mg/L and then to 5 mg/L bialaphos at 2-week intervals. Putative transgenic callus events emerged from selection as early as 6 weeks and as late as 12 weeks after infection and those that continued to proliferate on medium containing 5 mg/L bialaphos were considered to be transformed with the *bar* gene. Regeneration was carried out under continued selection pressure in the dark for 2–3 weeks followed by a germination step in the light (80 $\mu E/m^2/s$, 25°C, 16-h photoperiod) to produce plantlets. One-week-old plantlets were transferred directly from the petri-dish to soil in small pots and 3 weeks later were sprayed with 500 mg/L glufosinate before being transplanted to 2 gal pots for maturation and seed production in the greenhouse as previously described (Frame et al. 2002).

Transformation frequency (TF, %) was defined as the number of independent transgenic calli resistant to 5 mg/L bialaphos produced from the total number of embryos (responding and nonresponding) infected (× 100). All embryos were moved from cocultivation through selection regardless of their in vitro response rating.

Scoring for embryogenic callus induction frequency (ECIF)

Immediately prior to initiating selection (10 or 13 days after infection), each zygotic embryo was examined using a dissecting scope and scored for embryogenic callus induction. An embryo explant was scored as “responding” if embryogenic callus was visible emerging from its scutellum, while those on which no embryogenic callus was visualized were scored as “non-responding.” These numbers, as a percent of total embryos plated were used to calculate ECIF (%) for infected or uninfected embryos. Classification of embryo response at this early stage was reported to be an accurate assessment of regeneration potential (Tomes and Smith 1985).

Histochemical GUS assays

GUS assays (Jefferson 1987) were carried out on Type I callus of putative events (where applicable) after two cycles of selection on 5 mg/L bialaphos and on leaf pieces cut from the tip of the first leaf of 9-day-old T_1 progeny plants (germinated from seed of fertile transgenic events pollinated with nontransgenic pollen).

Glufosinate screening of T_0 plants and T_1 progeny for *bar* gene expression

Two to three weeks after transplant to soil in small pots, T_0 plants (regenerated from Type I callus) of putative transgenic events were sprayed with 500 mg/L glufosinate prepared from the herbicide Liberty®(Bayer CropScience, USA). This final in vivo screening ensured that all events taken to seed were expressing the introduced *bar* transgene.

T_1 progeny plants were screened for *bar*-gene expression by spraying germinated seedlings at 9 and 11 days post-emergence with a glufosinate (500 mg/L) solution. Plants were assessed for resistance (alive) or susceptibility (dead) to glufosinate 3 days after the final spray.

Southern blot analysis

Total genomic DNA from each T_0 plant was extracted from 200 mg of leaf tissue using the CTAB protocol (Saghai-Maroof et al. 1984). Ten micrograms of each DNA sample was digested with *Hind*III or *Xho*I at 37°C overnight. Digestion products were separated on a 1% (w/v) agarose gel. Southern blotting was performed as described in Sambrook and Fritsch (1989). The blot was hybridized with a P^{32} labeled *bar* or *gus* gene coding region. Negative control samples consisted of non-transgenic genomic DNA of B104 and Ky21 lines. For positive control samples, the same type of DNA was mixed with the equivalent of one copy of pTF102 plasmid DNA per maize diploid genome.

Statistical analysis

ECIF (%) and TF (%) data were arc sine transformed (Snedecor and Cochran 1980) and analyzed using ANOVA.

Results

Embryogenic callus induction frequency for 11 maize inbred lines and transformation of inbred lines B104, B114, and Ky21

Immature embryos from 11 field-grown maize inbred lines (A188, B104, B114, B73, GT-Mas:gk, H99, Ky21, Mo17, Mp420, Oh43, and W22) were (separately) infected with three strains of *Agrobacterium* carrying the standard binary vector pTF102 (EHA101, LBA4404, C58Z707). Embryos from the same ear were cocultivated on media containing either 0 or 300 mg/L L-cysteine in Media A or B backgrounds containing N6 salts. Resting and selection steps were carried out on the respective media background (N6-A or N6-B) supplemented with cefotaxime (100 mg/L) and vancomycin (100 mg/L).

Post-infection embryogenic callus induction frequency (ECIF, %) was assessed 13 days after infection. Inbred line response varied depending on media background (A or B) and cocultivation treatment (0 or 300 mg/L cysteine, Table 2). In the absence of cysteine during the cocultivation step, embryo response was higher on N6-B media for all lines except Mo17, for which it was lower, and H99 for which the response was consistent (and high) across both media backgrounds. In particular, Oh43 response dropped from over 80% on N6-B media to less than 20% on N6-A media. Average ECIF for embryos cocultivated on media containing 300 mg/L cysteine was higher (H99, Ky21, Mp420, W22), lower (B104, B114, and Mo17) or similar (A188, B73, GT-MAS:gk, Oh43) to non-cysteine cocultivated embryos (Table 2). No differential effect of *Agrobacterium* strain was observed on the frequency of embryogenic response (not shown). Initial callus phenotype on responding embryos was Type II (B73), Type II and Type I mixed (A188 and H99), or Type I (B104, B114, GT-MAS:gk, Ky21, Mo17, MP420, Oh43, and W22).

In this preliminary screening, transgenic events were recovered from inbred lines Ky21 (2%) and B114 (0.2%) from those media treatments highlighted in Table 2. Five Ky21 events were recovered from embryos cocultivated on media containing 300 mg/L cysteine, while the B114 event arose from an embryo cocultivated in the absence of cysteine (Table 2). All events derived from infections with strains EHA101 or C58Z707 (not shown). No stable events were recovered from B104 field embryos (Table 2), however, in parallel experiments, a consistent, but low stable transformation frequency (0.2%) was achieved when greenhouse-grown B104 embryos infected with numerous constructs based on pTF101.1 (EHA101) were cocultivated on media containing 300 mg/L cysteine and cultured on either N6-A or N6-B medium (data not shown).

Effect of four tissue culture media on post-infection ECIF and TF of inbred lines B104, B114, and Ky21

Using the three inbred lines successfully transformed on media containing N6 salts and vitamins, we systematically compared the effect of using N6 or MS salts in media backgrounds A and B on post-infection ECIF and stable transformation frequency (TF) of inbred lines B104, B114, and Ky21. Embryos were infected with *Agrobacterium* constructs pTF101.1 or pTF102 in EHA101 or pTF102 in LBA4404. All cocultivation media were supplemented with 300 mg/L cysteine, and carbenicillin (250 mg/L) was used to counter select bacteria in resting and selection media.

Average response of these inbred lines was higher on MS media (65%) than on N6 media (45%, Fig. 1), and for responding embryos of all three genotypes Type I callus production per embryo was more rapid on media containing MS rather than N6 salts (Fig. 2). On all four media, ECIF for greenhouse-grown B104 embryos was equal to or higher than that for Ky21 and B114 embryos (ranging from 55% on N6-B to 84% on MS-B), while B104 field embryos responded poorly by comparison (ranging from 16% on N6-B to 55% on MS-A, Fig. 1).

The beneficial effect of MS salts on the ECIF of infected embryos was associated with an average positive effect on the recovery rate of transgenic events from these inbred lines (Table 3). Average transformation frequency for each inbred line, in each media background (A and B), was higher for media containing MS salts than the N6 counterpart. TF for Ky21 was 4.1 and 8% for MS-A and MS-B media, respectively, and 0 and 0.7% for the corresponding

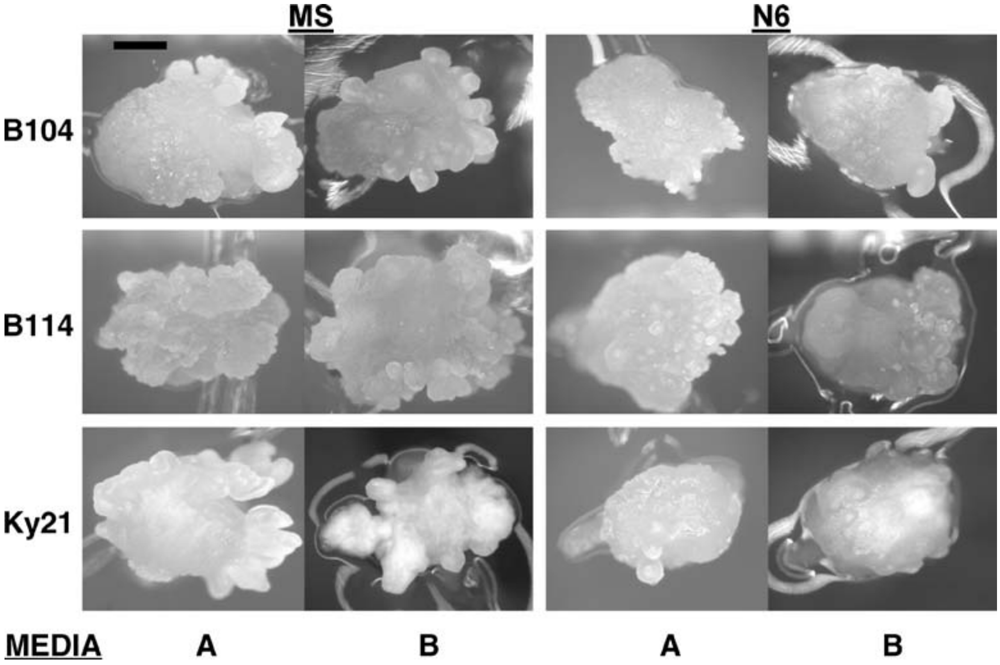

Fig. 2 Representative phenotypes of embryogenic callus induction from immature embryo scutellum of maize inbred lines B104, Ky21, and B114 infected with *Agrobacterium* and cultured for 10 days on four different media. Somatic embryogenesis on scutellum of immature zygotic embryos of field-grown inbred lines B104, Ky21, and B114 on four media (N6-A, N6-B, MS-A, MS-B, see Table 1 in "Materials and methods" section). Response phenotypes were recorded after infection with *Agrobacterium,* and 3 days cocultivation followed by 7 days resting on each media. Bar = 1 mm

N6-A and N6-B media. Similarly, for B114, averages of 1.7 and 2.8% TF were achieved on MS-A and MS-B media, while rates of only 0.5 and 0.7% were achieved when using N6-A or N6-B media, respectively. Again, no events were recovered from either N6 media for field-grown embryos of inbred line B104, while 1% efficiency was achieved on both MS-A or MS-B media. A similar trend was observed for B104 greenhouse-grown embryos, although a relatively high transformation frequency (12%) was achieved on MS-B media for the three experiments in which embryos from eight ears were cultured across all four media (Table 3). Average TF for B104 embryos (greenhouse + field) in this four-media comparison (Table 3) was 1.8% on MS-A, 6.4% on MS-B, 1.2% on N6-A, and 0.1% on N6-B medium.

The overall average transformation frequency achieved on media containing MS salts (4%) was higher ($P = 0.0001$) than for media containing N6 salts (0.5%). Although a higher overall average transformation rate was achieved on B medium (3%) than on the A medium background (1.4%), this difference was not statistically significant ($P = 0.21$).

Embryogenic callus induction frequency for 13 maize inbred lines cultured (without infection) on MS salts in two media backgrounds

Embryos from 10 additional inbred lines were cultured, without infection, on MS-A and MS-B resting media for 10 days and ECIF was assessed as a preliminary step to determine how effective these two media might be for targeting other maize inbred lines in future transformation studies. B104, B114, and Ky21 were included as controls. A188, B104, GT-Mas:gk, M37W, and Oh43 responded at high frequency on both these media (Fig. 3). Response was moderate or low but not divergent on both media for B114, Ky21, and W64, while inbred lines B73, H99, Mo17, Mp420, and W22 showed a differential response on these two media (Fig. 3). W22 embryos responded at a two-fold higher frequency on MS-B (75%) than on MS-A medium (37%).

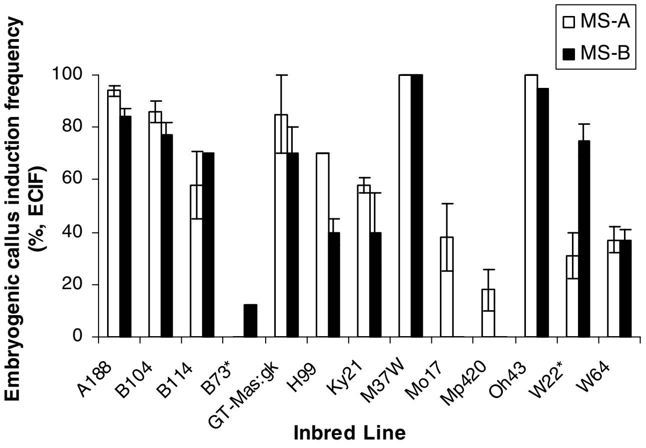

Fig. 3 Pre-infection in vitro response (% ECIF) for immature embryos from 13 maize inbred lines on media containing MS salts. Embryos from the 13 maize inbred lines were cultured, without infection, on media backgrounds A and B supplemented with MS salts. ECIF were assessed 10 days after embryo isolation. Percentages are the average of embryo response from two ears for each inbred line. All inbreds were field-grown except those marked with an asterisk (greenhouse-grown)

Regeneration and fertility of transgenic events

Of the 88 B104, 12 B114, and 19 Ky21 bialaphos resistant callus events for which regeneration was attempted, T_0 transgenic plants were produced from 84, 50, and 79 of the events, respectively (Table 4). All these plants survived spraying with 500 mg/L glufosinate prior to transplant to big pots in the green house. Seed sets for B114 and Ky21 were considerably poorer than those for inbred line B104 (Table 4), with as few as 9% (1/11) of Ky21 events producing >20 kernels compared to 71% (46/65) for B104. Ky21 and B114 transgenic events formed excessive roots and few shoots under the regeneration conditions used in this study and required extra in vitro pruning steps to recover plants. Conversely, T_0 plants were efficiently regenerated from B104 transgenic callus without the need for extra tissue culture manipulation. All B104 events taken to seed were male and female fertile and produced an average of 55 kernels per transgenic ear, while average seed set for Ky21 was only six kernels per ear (Table 4).

Histochemical GUS, Southern blot, and progeny analyses

Eighty-nine percent (75/84) of independent, Type I calli recovered from constructs pTF102 in EHA101 and LBA4404 and resistant to 5 mg/L bialaphos also expressed the *gus* marker gene (data not shown). T_0 leaf samples from 46 events were analyzed by Southern blot analysis using the *bar* and *gus* probes as illustrated in Fig. 4A. The *bar* gene probe was used to hybridize the DNAs digested with *Xho*I (one restriction site on the T-DNA) and the *gus* gene probe was used to hybridize the DNAs digested with *Hind*III (two restriction sites on the T-DNA, Fig. 4B and C) thereby allowing us to estimate transgene copy numbers by counting hybridization bands (one band was estimated as one transgene insertion copy). Insertion of the *bar*, *gus*, or both transgenes was confirmed in all the events analyzed. Although the *gus* transgene was not confirmed in event 755 (Fig. 4B), this event was among a subset of 23 events confirmed by Southern blot to contain the *bar* gene (Fig. 4C). Because event 755 was also histochemically GUS negative it is likely that the *gus* gene was lost during the integration process. A small number of bands (ranging from one to four) were evident for each event (Fig. 4B and C), which is consistent with a simple insertion pattern expected from *Agrobacterium*-mediated genetic transformation (Shou et al. 2004).

χ^2 analysis for the *bar* gene in progeny of 16 B104, 1 B114, and 1 Ky21 transgenic events indicate that the transgene was inherited in all events and was segregating

Table 4 Regenerability and fertility of transgenic events from three maize inbred lines

Inbred line	No. of bar^R calli to regen[a]	Regenerable T_0 event (%)[b]	Fertile T_0 events (%)[c]	No. of T_0 plants pollinated	Average no. kernels per T_1 ear
B104	88	84 (74/88)	71 (46/65)	85	55
B114	12	50 (6/12)	33 (2/6)	6	42
Ky21	19	79 (15/19)	9 (1/11)	16	6

[a] Includes regeneration of events from pTF101.1 and pTF102 constructs

[b] No. of events with plants/no. of events attempted in regeneration (× 100)—all T_0 plants survived two sprays with 500 mg/L glufosinate

[c] No. of events with >20 kernels (total) from one to two plants/no. of events pollinated (× 100)

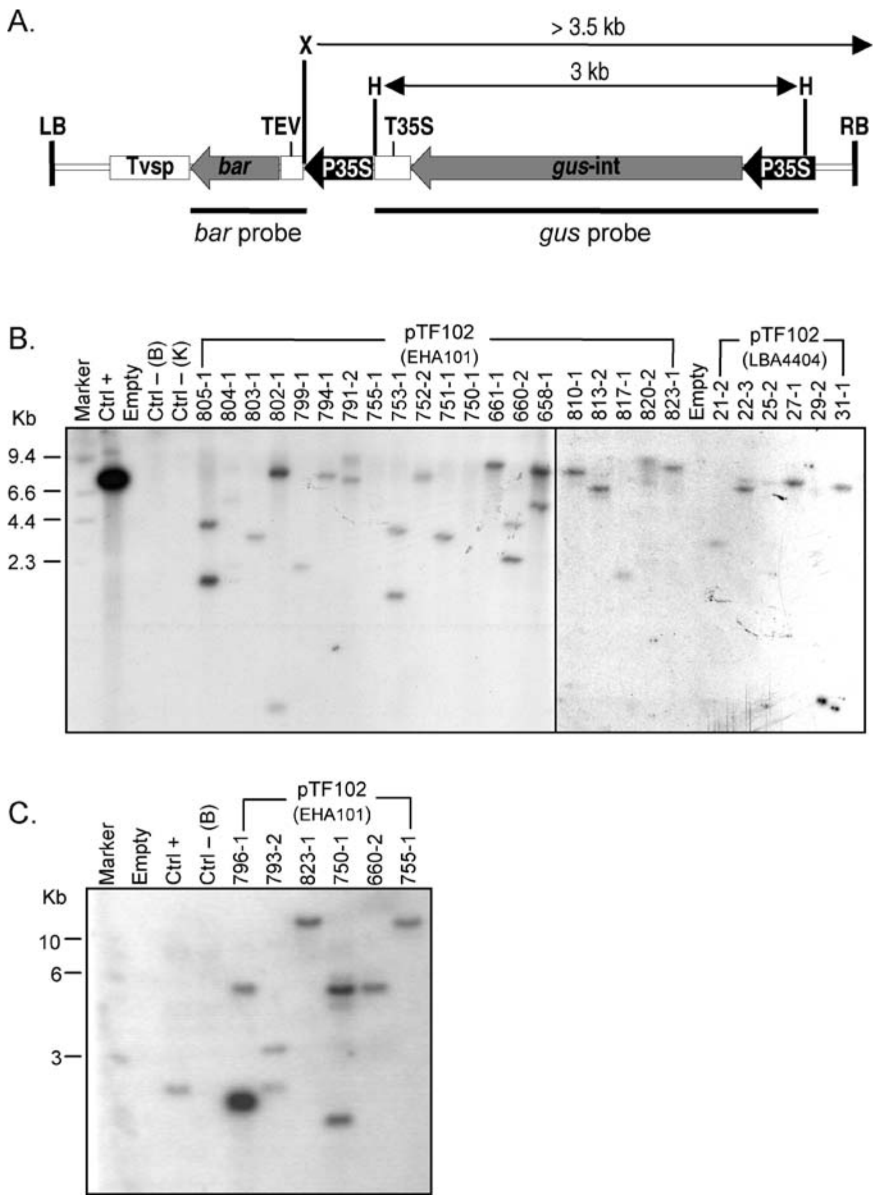

Fig. 4 pTF102 vector and Southern analyses of maize T_0 transformants. **A** T-DNA region of binary vector pTF102. LB, left border; RB, right border; Tvsp, soybean vegetative storage protein terminator; *bar*, phosphinothricin acetyltransferase gene; TEV, tobacco etch virus translational enhancer; P35S, CaMV 35S promoter; T35S, CaMV 35S terminator; *gus*-int, β-glucuronidase gene containing an intron; H, *Hind*III; X, *Xho*I. Ten micrograms of genomic DNA was digested with *Xho*I enzyme that cuts once in the T-DNA region of the plasmid and probed with the *gus* probe **B**, or *Hind*III that cuts at two restriction sites in pTF102 and probed with the *bar* probe **C**. Construct pTF102 was in *Agrobacterium* strain EHA101 or LBA4404 for the events shown. Negative controls (Ctr –) were nontransgenic B104 (B) and Ky21 (K) DNA. Positive control (Ctr +) was B104 genomic DNA spiked with 4.4 pg of pTF102 digested by *Xho*I. Transgenic event 750-1 and 29-2 hybridized poorly on the membrane in **B**, but were confirmed as transgenic on other membranes (**C** for 750-1 and not shown). Events 805-1 and 799-1 in **B** are representative of inbred lines Ky21 and B114. All other events listed in **B** and **C** are inbred line B104

in the expected 1:1 ratio for single locus integration for all but two events (#753 and #794, Table 5). GUS expression was also observed in all segregating progeny of the B104, B114, and Ky21 events tested (Table 5), confirming that both transgenes were stably inherited in the three inbred lines studied.

Discussion

Transformation frequency of maize inbred lines B104, B114, and Ky21 was improved when immature zygotic embryos were cultured on media containing MS instead of N6 salts. This beneficial effect of MS salts was observed in

Table 5 Segregation analysis for *gus* and *bar* gene expression in T_1 progeny plants

Vector	Inbred line	Event ID	Segregation ratio					
			Herbicide		X^2[f]	GUS		X^2[e]
			Res[a]	Sen[b]		Pos[c]	Neg[d]	
pTF102	Ky21	386	5	9	1.14	5	9	1.1
	B114	799	8	7	0.06	8	7	0.1
	B104	25	19	11	2.10	19	11	2.1
		658	14	15	0.03	15	13	0.2
		753	18	7	4.80	20	5	9.0
		794	4	26	16.1	14	15	0.1
pTF101.1	B104	35	19	11	2.13			
		78	11	19	2.13			
		79	18	11	1.69			
		84	16	9	1.96			
		85	15	15	0.00			
		88	14	15	0.03			
		89	16	14	0.03			
		133	13	17	0.53			
		134	16	14	0.13			
		137	11	12	0.04			
		148	12	17	0.86			
		213	14	11	0.36			

Transgenic plants were crossed as the female with pollen from nontransformed plants

[a] Res, resistant to glufosinate spray (*bar*-expresser)

[b] Sen, sensitive to glufosinate spray (*bar* nonexpresser)

[c] Pos, GUS assay positive (*gus*-expresser); *gus* gene not present in pTF101.1

[d] Neg, GUS assay negative (*gus* nonexpresser)

[e] $X^2 = 3.8$ (0.05, 1 df)

two divergent media backgrounds and was associated with improved post-infection ECIF and more rapid and sustained callus growth on individual explants. Ishida et al. (1996) reported a similar beneficial effect on transformation rate using a modified MS medium instead of N6 medium for *Agrobacterium*-mediated transformation of the non-elite inbred line, A188. While the reason for this effect is unclear, it is possible that nutrient components in MS salts optimize the morphological potential, and therefore the transformation competence, of targeted cells in these three inbred lines. MS salts contain a higher overall concentration of inorganic nitrogen but a lower ratio of nitrate to ammonium than do N6 salts (Armstrong and Green 1991; Elkonin and Pakhomova 2000). Differences in regeneration capacity between rice genotypes was shown to be associated with loci governing the break-down of toxic by-products from nitrate metabolism (Nishimura et al. 2005) suggesting that the genetic potential to undergo embryogenesis can be influenced by basal salt components in the tissue culture media as previously suggested by Hodges et al. (1986).

Initiation of Type I callus from cultured immature embryos is considered to be less genotype dependent than is the formation of friable Type II callus (Wan et al. 1995). MS salts were reported to promote preferential production of compact Type I callus, while N6 salts led to production of friable Type II callus from immature embryos of sorghum (Elkonin and Pakhomova 2000) and maize inbred line A188 (Armstrong and Green 1985). The three maize inbred lines successfully transformed in this study produced Type I callus on both MS and N6 media although callus grew more rapidly, with transgenic events being recovered sooner, from embryos cultured on MS rather than N6 media.

Because the thiamine HCl and nicotinic acid concentrations differed between the N6 and modified MS vitamins used, and vitamin complement varied consistently with the corresponding major and minor salt component in this study, the possibility that vitamin complement is responsible for the differences observed cannot be ruled out. Medium using N6 salts but containing B5 vitamins (Gamborg et al. 1968) has been used to successfully transform four maize inbred lines including Mo17 (Huang and Wei 2005).

Components of the A and B media backgrounds are divergent therefore no conclusions can be drawn as to what factors may be contributing to the higher transformation rate achieved using MS-B instead of MS-A media. It is possible that the higher concentration of silver nitrate in MS-B compared to MS-A may favor transformation frequency by inhibiting *Agrobacterium* regrowth on cultured embryos as previously suggested (Zhao et al. 2001; Cheng et al. 2004).

Greenhouse-grown embryos transformed at higher frequency than did field-grown embryos in two consecutive years in this study. This same trend was observed in a third season where transformation frequency for B104 embryos harvested from the greenhouse averaged 5% (reduced from as high as 12% the previous year), while field explants transformed at 1.5% (not shown). These data contradict observations by Zhao et al. (2000) for which higher transformation rates were observed from field-grown than greenhouse-grown sorghum immature embryos. The lower post-infection ECIF observed for B104 field embryos may account for its reduced transformation rate compared to greenhouse-derived embryos. High variability in embryo response of maize grown in two different field seasons or two locations and cultured on the same medium has been attributed to environmental conditions that may influence storage of food reserves in embryo scutellar tissue (Lu et al. 1983).

Transformation frequencies of 6.4, 2.8, and 8% were achieved for B104, B114, and Ky21; three inbred lines representing diverse pedigrees not related to the tissue culture-amenable lines A188 and H99. B104 derives from BS13(S)C5, an Iowa Stiff Stalk Synthetic (BSSS) population. Although slow drying at harvest, it is considered an agronomically superior inbred line (Hallauer et al. 1997). B114, a NonStiff Stalk line (NSS) developed from CIMMYT Pool 41-C15-19-2-1-1-1-1-1-1, is also considered an agronomically beneficial line and contributes to fast-dry down in crosses (Hallauer et al. 2000). Ky21 is a NSS line developed from the open pollinated population Boone County White but is no longer widely used in breeding programs. While Ky21 and B114 field embryos transformed at higher efficiency than B104 field embryos, they demonstrated inferior plant regeneration from tissue culture and low fertility in T_0 plants compared to B104. B104 immature embryo and pollen donor plants also performed consistently well in the field and greenhouse during this study, suggesting that it may serve as a model inbred line for maize agronomic trait improvement using *Agrobacterium*-mediated transformation.

Inclusion of 300 mg/L L-cysteine in cocultivation medium reduced post-infection ECIF for 3 of the 11 inbred lines compared to the non-cysteine control. A similar reduction in ECIF of embryos cocultivated on 400 mg/L cysteine was observed for a subset of proprietary inbred lines crossed with A188 (Lupotto et al. 2004), although anti-oxidants superoxide dismutase and catalase had no (positive or negative) effect on post-infection ECIF or TF from immature embryos of the maize inbred line H99 (Ishida et al. 2003). Transformation rate was increased, in spite of a reduced ECIF, for Hi II embryos cocultivated on 300 mg/L cysteine (Frame et al. 2002), while in the preliminary screen of this study using only N6 salts, B114 and Ky21 transgenic events were recovered from cocultivation treatments that produced the highest ECIF for both inbred lines (cysteine for Ky21 and noncysteine for B114). The relationship between ECIF and TF moderated by the presence of anti-oxidant in cocultivation media requires further investigation and is likely to vary by genotype.

Increased transformation frequency of inbred lines B104, B114, and Ky21, all cocultivated in the presence of 300 mg/L cysteine, was achieved using media that also enhanced post-infection embryogenic callus induction frequency and promoted vigorous callus growth on responding embryos. Preliminary results from prescreening noninfected embryos of 10 additional inbred lines on media containing MS salts demonstrate that these media may be useful in transforming other inbred lines using *Agrobacterium*. However, Oh43 exhibited excellent post-infection response on N6-B medium and is transformation competent using the biolistic gun (Wang et al. 2003), but no transgenic events were recovered from Oh43 in the present study. Although optimizing ECIF is necessary for transforming immature embryos, it is not a sufficient condition for achieving *Agrobacterium*-mediated transformation. The need to examine additional physical, biological or media parameters to overcome genotype specificity for *Agrobacterium*-mediated transformation of maize inbred lines remains.

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