Agrobacterium tumefaciens-Mediated Transformation of Maize Embryos Using a Standard Binary Vector System[1]

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We have achieved routine transformation of maize (*Zea mays*) using an *Agrobacterium tumefaciens* standard binary (non-super binary) vector system. Immature zygotic embryos of the hybrid line Hi II were infected with *A. tumefaciens* strain EHA101 harboring a standard binary vector and cocultivated in the presence of 400 mg L^{-1} L-cysteine. Inclusion of L-cysteine in cocultivation medium lead to an improvement in transient β-glucuronidase expression observed in targeted cells and a significant increase in stable transformation efficiency, but was associated with a decrease in embryo response after cocultivation. The average stable transformation efficiency (no. of bialaphos-resistant events recovered per 100 embryos infected) of the present protocol was 5.5%. Southern-blot and progeny analyses confirmed the integration, expression, and inheritance of the *bar* and *gus* transgenes in R_0, R_1, and R_2 generations of transgenic events. To our knowledge, this represents the first report in which fertile, stable transgenic maize has been routinely produced using an *A. tumefaciens* standard binary vector system.

Since fertile transgenic maize (*Zea mays*) was first produced using the biolistic gun (Gordon-Kamm et al., 1990), maize transformation technology has served as an important tool in germplasm development and research addressing fundamental biological questions through the study of transgenic maize (Armstrong, 1999). Recent reports have demonstrated that *Agrobacterium tumefaciens*-mediated maize transformation may offer a better alternative than the biolistic gun for delivery of transgenes to maize. This gene delivery system results in a greater proportion of stable, low-copy number transgenic events than does the biolistic gun (Ishida et al., 1996; Zhao et al., 1998), offers the possibility of transferring larger DNA segments into recipient cells (Hamilton et al., 1996), and is highly efficient (Ishida et al., 1996; Zhao et al., 1998). Reproducible protocols for *A. tumefaciens*-mediated maize transformation have used super binary vectors, in which the *A. tumefaciens* strain carries extra copies of *virB*, *virC*, and *virG* (Komari, 1990), to infect immature zygotic embryos of the inbred line A188 (Ishida et al., 1996; Negrotto et al., 2000) or the hybrid line Hi II (Zhao et al., 1998, 1999). We have successfully transformed Hi II immature zygotic embryos at an average efficiency of 5.8% using the *A. tumefaciens* super binary vector in strain LBA4404 (B. Frame, unpublished data). Because the cost of licensing this proprietary technology for use on a broader scale was prohibitive to our public sector laboratory, we focused instead on implementing an *A. tumefaciens* standard binary (non-super binary) vector system to transform maize Hi II immature zygotic embryos. Stable transformation of maize using a standard binary vector to infect shoot meristems was reported previously (Gould et al., 1991), but adoption of this method was hindered by its lack of robustness. Development of a reproducible and efficient method for transforming maize using a standard binary vector would not only provide researchers with the benefits already outlined, it would also facilitate vector construction when compared with the super binary vector. Final assembly of a super binary vector system involves co-integration of the gene of interest into a large plasmid (pSB1) in *A. tumefaciens* strain LBA4404 via homologous recombination (Ishida et al., 1996). Assembly of a standard binary vector does not require this additional step, making it a more efficient way to confirm the introduction of a gene of interest into an *A. tumefaciens* strain.

Using a protocol modified from Zhao et al. (1999), we infected Hi II immature zygotic embryos with a standard binary vector system containing a P35S-*bar*

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selectable marker gene cassette (phosphinothricin acetyltransferase gene driven by the cauliflower mosaic virus [CaMV] 35S promoter) and a P35S-*gus*-int reporter gene cassette (β-glucuronidase [GUS] gene with an intron driven by the CaMV 35S promoter) in *A. tumefaciens* strain EHA101 (Hood et al., 1986). Results from these initial experiments showed that the *gus* marker gene was preferentially expressed (in transient histochemical assays) in cells at the apical end and on the embryo axis side of infected embryos. In contrast, cells in the basal scutellum region, which were targeted for transformation because of their ability to produce embryogenic callus, showed limited to no expression. Lupotto et al. (1999) reported similar patterns of transient *gus* gene expression in inbred maize embryos infected with conventional *A. tumefaciens* binary vectors. Lack of T-DNA delivery to embryogenic-competent scutellum cells and correspondingly low rates of stable event recovery in these experiments led us to consider ways of promoting a more compatible interaction between the *A. tumefaciens* standard binary vector system and the targeted scutellum cells from which proliferating, embryogenic callus emerges after cocultivation.

In an incompatible host-pathogen interaction, pathogen invasion can be halted by localized plant cell death at the infection site. This resistance mechanism, or hypersensitive response, is mediated by an oxidative burst in which a rapid and transient production of large amounts of reactive oxygen species occur (Wojtaszek, 1997). Antioxidants used during explant germination, preculture, and infection (Enriquez-Obregon et al., 1999) or during and after cocultivation (Perl et al., 1996) were reported to favor stable transgenic event recovery in *A. tumefaciens*-mediated Japonica rice (*Oryza sativa* L. cv *R321*) and grape (*Vitis vinifera*) transformation experiments, respectively. Olhoft and Somers (2001) measured an increase in the frequency of T-DNA delivery (histochemical GUS assays) to targeted cotyledonary node explants of *A. tumefaciens*-infected soybean (*Glycine max* L. Merr. cv Bert) cocultivated on 400 mg L^{-1} of the antioxidant L-Cys. More importantly, this increase in transient *gus* gene expression observed 5 d after infection was correlated with a 2-fold increase in the rate of stable event recovery (independent, fertile transgenic soybean plants). The authors concluded that the increase in host-pathogen compatibility mediated by the presence of antioxidants during transformation moderated the detrimental effect of the hypersensitive response, which in turn lead to an increase in the survival rate of *A. tumefaciens*-infected cells and a corresponding rise in stable transformation efficiency.

Maize callus cells infected with *A. tumefaciens* were described as undergoing a rapid, hypersensitive type of cell death in a study characterizing *A. tumefaciens*-induced apoptosis in maize (Hansen, 2000). Use of antioxidants to moderate the *A. tumefaciens*-maize interaction has not been reported. We supplemented cocultivation medium with 400 mg L^{-1} Cys, carried out infection of Hi II immature zygotic embryos with the standard binary vector system, and measured the effect of this antioxidant treatment on three transformation criteria. First, analysis of transient GUS expression in infected embryos was performed to monitor T-DNA delivery to the targeted cells (no. and distribution of blue foci). Because recovery of cells targeted for transformation is critical to achieving stable transgenic events, we also assessed the proportion of embryos that gave rise to embryogenic Type II callus after cocultivation. Finally, stable transformation efficiency (the no. of bialaphos-resistant transgenic events per 100 embryos infected) was measured for Cys concentrations ranging from 100 to 400 mg L^{-1}.

Stable transformation efficiency was significantly increased upon addition of L-Cys to cocultivation medium. Using the protocol described, we have achieved routine production of fertile transgenic maize at an efficiency of 5.5%. To our knowledge, this marks the first report in which a reproducible method for maize transformation using an *A. tumefaciens* standard binary vector system has been demonstrated.

RESULTS

Transient GUS Expression

Immature zygotic embryos infected with *A. tumefaciens* strain EHA101 harboring the standard binary vector pTF102 (Fig. 1) were cocultivated as described in "Materials and Methods." Control embryos were cocultivated on medium without Cys to compare the amount and distribution of T-DNA delivery to targeted cells cocultivated in the presence or absence of 400 mg L^{-1} Cys. Histochemical GUS analysis was carried out on a subset of embryos cocultivated with Cys (138 embryos) or without Cys (146 embryos) across 10 independent experiments. Level of transient GUS expression (no. of blue foci on the scutellum side of each embryo) was determined as described in "Materials and Methods." Results are graphically presented in Figure 2A. The majority (56%) of embryos cocultivated on 400 mg L^{-1} Cys were moderate to high GUS expressers compared

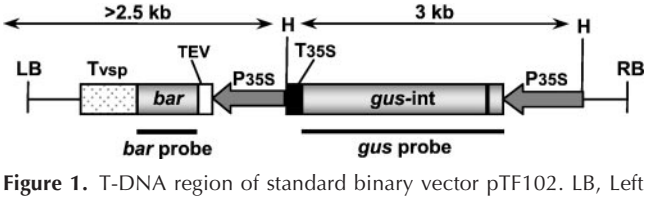

Figure 1. T-DNA region of standard binary vector pTF102. LB, Left border; RB, right border; *bar*, phosphinothricin acetyltransferase gene; *gus*-int, β-glucuronidase gene containing an intron; P35S, CaMV 35S promoter; TEV, tobacco etch virus translational enhancer; Tvsp, soybean vegetative storage protein terminator; T35S, CaMV 35S terminator; H, *Hin*dIII.

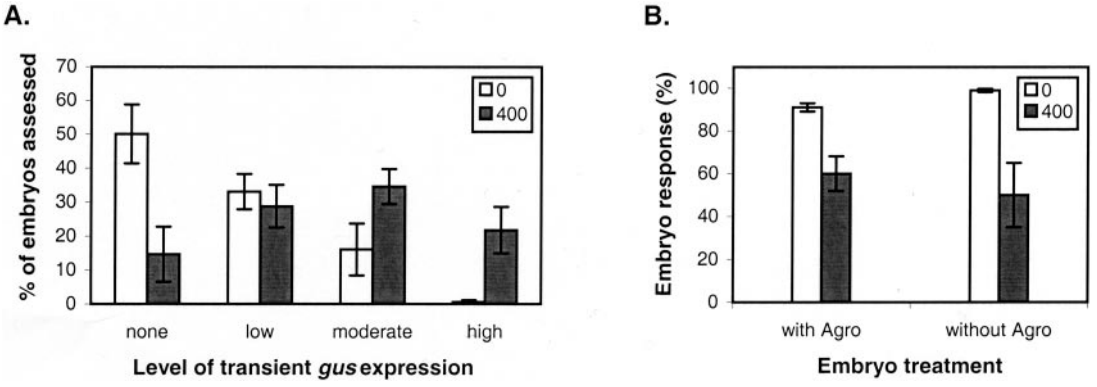

Figure 2. Effect of Cys (0 or 400 mg L^{-1}) in cocultivation medium on level of transient GUS expression in embryos (A) and on embryogenic callus response of *A. tumefaciens*-infected or noninfected embryos (B). Data from 10 experiments (A), eight experiments (B, with Agro), and three experiments (B, without Agro).

with those cocultivated in the absence of Cys (17%). Conversely, a high proportion (50%) of embryos treated with no Cys showed no blue foci on the scutellum side of the embryo compared with those embryos cocultivated on 400 mg L^{-1} Cys (15%).

The change in distribution of blue foci on embryos cocultivated in the presence of Cys was of particular significance. On embryos cocultivated in the absence of Cys, blue foci were generally localized to the embryo axis side and apical end of the embryo (Fig. 3a). Expression was rarely observed in the basal scutellum region of the embryo that, as the site of callus initiation on the immature zygotic embryo explant, was targeted for transformation. In contrast, in embryos cocultivated on 400 mg L^{-1} Cys, transient GUS expression was consistently observed not only at the apical end of the embryo, but also along the margins and in targeted cells at the base of the scutellum (Fig. 3b).

Post-Cocultivation Embryo Response

To assess the effect of 400 mg L^{-1} Cys in cocultivation medium on recovery of targeted cells, embryos were infected and cocultivated as described, and then transferred to resting medium for 4 to 7 d, after which they were assessed for Type II callus initiation. Although inclusion of 400 mg L^{-1} Cys in cocultivation had a positive impact on transient GUS expression in embryos, it reduced the proportion of embryos giving rise to embryogenic Type II callus on resting medium. Average percent embryo response (no. of embryos giving rise to embryogenic callus per 100 embryos treated) was assessed over eight independent experiments. Sixty percent of 341 *A. tumefaciens*-infected embryos cocultivated on 400 mg L^{-1} Cys produced embryogenic callus compared with 91% of 374 embryos cocultivated in the absence of Cys (Fig. 2B). Of 123 non-infected embryos cultured on cocultivation medium containing Cys, 52% produced embryogenic callus while 99% of 127 non-infected embryos incubated on cocultivation medium without Cys produced embryogenic callus (Fig. 2B).

Stable Transformation Efficiency

To test the effect of Cys in cocultivation medium on stable transformation, embryos infected with pTF102 were cocultivated for 3 d on media supplemented with 400 mg L^{-1} Cys (Fig. 3c) and then transferred to resting and selection media as described in "Materials and Methods." Beginning 5 weeks after infection, bialaphos-resistant callus events were identified by their sustained embryogenic growth on medium containing 3 mg L^{-1} bialaphos (Fig. 3, d and e) and were histochemically analyzed for stable expression of the *gus* gene (Fig. 3f).

Results from 15 independent experiments are summarized in Table I. Stable transformation efficiency (no. of bialaphos-resistant events recovered per 100 embryos infected) in these experiments averaged 5.5% and ranged between 1.1% and 22.2%. All but experiment 12/08/00 produced transgenic events, emphasizing the reproducibility of this method. Seventy-eight percent of bialaphos-resistant events also expressed the *gus* gene. Of the 51 callus events for which regeneration was attempted, all but one regenerated to plants (Table I).

Cocultivation on medium containing 400 mg L^{-1} Cys significantly increased stable event recovery compared with the control treatment in which no Cys was added to the cocultivation medium. In eight separate experiments, infected embryos were cocultivated at 23°C on medium containing either 0 or 400 mg L^{-1} Cys (Table II). The average percent stable transformation efficiency for embryos cocultivated on 400 mg L^{-1} Cys was higher (5.3%) than that for embryos cocultivated on 0 mg L^{-1} Cys medium (0.2%). This difference was statistically significant ($P < 0.001$). In addition, reducing cocultivation temperature from 23°C to 20°C did not alter the favorable effect of the Cys cocultivation treatment on stable

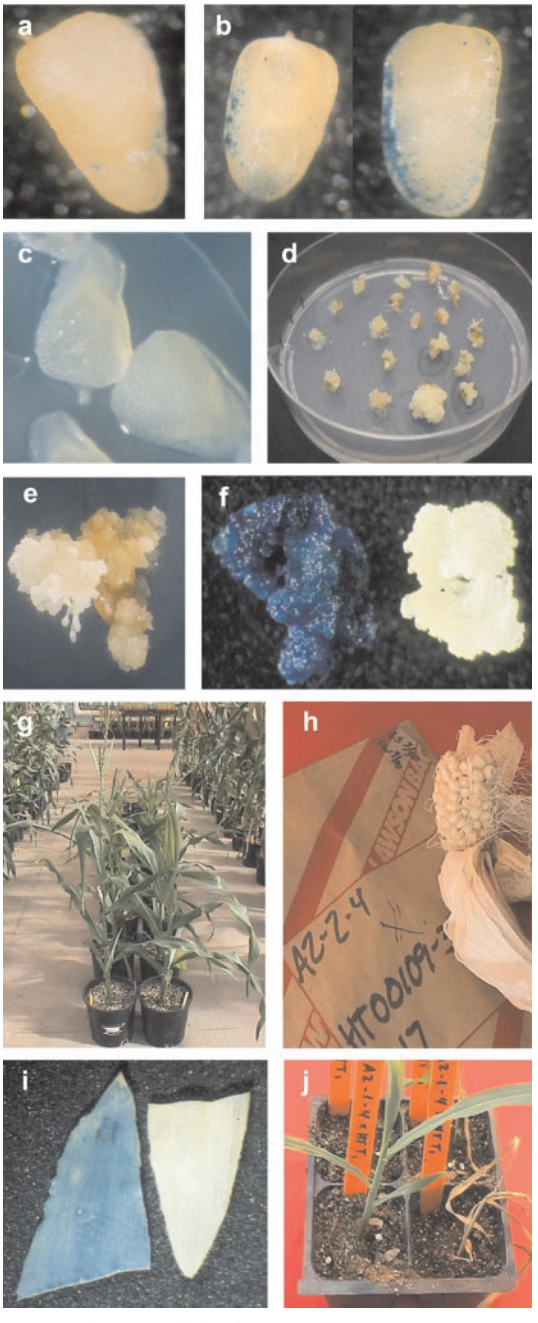

Figure 3. Distribution of blue foci (transient *gus* gene expression) in maize Hi II immature zygotic embryos infected with *A. tumefaciens* standard binary vector system, pTF102, and cocultivated on medium containing 0 mg L^{-1} (a) or 400 mg L^{-1} Cys (b). Embryos during cocultivation (c). Putative transformant growing on 3 mg L^{-1} bialaphos (d). Bialaphos-resistant, Type II embryogenic callus event emerging from a single embryo explant (e). Stable *gus* gene expression in pTF102 callus event (f, left). Callus on the right is not expressing the *gus* gene. Transgenic pTF102 plants (R_0) flowering in greenhouse (g). Transgenic seed set on ear of R_0 plant (h). *Gus* (i) and *bar* (j) transgene expression in segregating R_1 progeny. The leaf segment on the left was isolated from a GUS-expressing plant (positive), whereas that on right was from nonexpressing (negative) plant (i). The *bar*-expressing plantlet on the left (resistant) survived the glufosinate herbicide spray, whereas the nonexpressing plant on the right (sensitive) died.

event recovery. Whereas the average transformation efficiency at 20°C (6.2%) was somewhat higher than at 23°C (5.3%) in embryos cocultivated on 400 mg L^{-1} Cys, few stable events were recovered at either cocultivation temperature when embryos were cocultivated in the absence of Cys (Table II).

To test whether Cys concentrations lower than 400 mg L^{-1} in cocultivation medium would lead to an improvement in callus formation from treated embryos and a higher stable transformation rate, we conducted a preliminary experiment in which embryos infected with pTF102 were cocultivated on medium containing 0, 100, 200, 300, or 400 mg L^{-1} Cys. The highest rate of stable event recovery in these experiments was achieved from embryos cocultivated on medium containing 100 mg L^{-1} Cys (4.8%). Stable transformation efficiency for the 400 mg L^{-1} Cys treatment was 3.3%, and for the non-Cys control was 0.0% (Table III). The proportion of embryos responding after cocultivation decreased as Cys concentration increased. In contrast, transient GUS expression levels increased as Cys concentration increased, and showed a gradual shift from the embryo axis side and apical end of embryos (0 mg L^{-1} Cys) to the scutellar margins of embryos (100 and 200 mg L^{-1} Cys), to expression in all regions of the scutellar side of embryos, including the embryogenic competent basal scutellar cells at Cys levels of 300 and 400 mg L^{-1} (data not shown).

Plant Regeneration and Fertility

Bialaphos-resistant callus events were regenerated on medium containing 3 mg L^{-1} bialaphos as described in "Materials and Methods." To date, over 60 pTF102-derived transgenic events have been regenerated to plants and are growing in the greenhouse (Fig. 3g). Seed harvested from outcrosses of 97 plants representing 37 of these events averaged 51 kernels per ear (transgenic plant as female, Fig. 3h) and 60 kernels per ear (transgenic plant as male), indicating that these events are fertile. Thirty-five of these events were recovered from Cys-containing cocultivation medium, whereas the remaining two derive from the non-Cys cocultivation treatment.

Analysis of Stable Events

Histochemical GUS assays were carried out on all bialaphos-resistant callus events to determine whether those expressing the *bar* gene also expressed the *gus* reporter gene. Of 65 events analyzed, 51 (or 78%) were GUS positive (Table I). Because the *gus* gene in this construct contains an intron, blue staining was indicative of plant rather than *A. tumefaciens* expression of the transgene. Positive or negative GUS expression in leaf tissue correlated with callus expression of the reporter gene in all events.

Table I. *Efficiency of A. tumefaciens-mediated maize transformation*[a]

NA, Not applicable; NT, not tested.

Experiment Date	No. of Embryos Infected	No. of Bialaphos-Resistant Events Recovered	No. of Callus Events Expressing *gus* Gene	No. of Events Regenerated to Plants/No. of Events Attempted	Transformation Efficiency
					%
09/15/00	21	2	1	2/2	9.5
11/22/00	82	2	1	2/2	2.4
12/08/00	20	0	0	NA	0.0
01/29/01	27	6	4	5/5	22.2
02/05/01	63	2	1	2/2	3.2
02/12/01	121	2	2	2/2	1.7
02/19/01	17	1	1	NT	5.9
02/26/01	26	1	0	1/1	3.8
03/21/01	246	19	15	17/18	7.7
03/26/01	58	4	4	4/4	6.9
04/02/01	95	1	1	1/1	1.1
04/11/01	76	5	5	5/5	6.6
04/27/01	35	1	1	1/1	2.9
04/30/01	122	8	7	6/6	6.6
05/22/01	175	11	8	2/2	6.3
Total	1184	65	51	50/51	5.5

[a] All experiments used 400 mg L^{-1} cysteine in cocultivation medium. [b] Transformation efficiency = (no. of bialaphos-resistant events recovered/no. of embryos infected) × 100.

Southern-blot analysis was carried out to assess stable integration of the *bar* and *gus* transgenes in the R_0, R_1, or R_2 generations of numerous independent pTF102 events. Total genomic DNA was extracted from leaf tissue, digested with the restriction enzyme *Hin*dIII, and DNA blots prepared and hybridized with the *gus* or *bar* probes. As illustrated in Figure 1, *Hin*dIII restriction digestion of genomic DNA derived from pTF102 transgenic material would not only liberate a 3-kb fragment containing the *gus* gene cassette but would also yield various band sizes (>2.5 kb) that hybridize to the *bar* probe. The number of bands hybridizing with the *bar* probe would, in turn, reflect the different sites of transgene integration and represent an estimate of transgene copy number in the maize genome.

Samples of genomic DNA from 34 independent events were hybridized with the *gus* probe. Thirty-one of these events (91%) showed the expected 3-kb dropout band, suggesting the intactness of the *gus* gene cassette in the maize genome. A subset of 20 events is shown in Figure 4A. Some events contain multiple *gus*-hybridizing bands in addition to the predicted 3-kb dropout (Events 1, 13, 16, 40, 46, and 48, Fig. 4A). The 3-kb *gus*-hybridizing band is visible in all but Events 2, 6, and 23. Hybridizing bands bigger or smaller than 3 kb in these three events suggest that the *gus* gene cassette was rearranged (Fig. 4A) and may account for the lack of GUS expression in callus or plants of Events 6 and 23 (Table IV). However, Event 2 also carries a rearranged copy of the *gus* gene (Fig. 4A), but does express GUS in callus and plants. Conversely, Event 4 shows the expected 3-kb band (Fig. 4A), but does not express the *gus* gene in either callus or plants.

Duplicate DNA blots of 27 events hybridized with the *gus* probe were also hybridized with the *bar* probe and results for 20 of these events are shown in Figure 4B. Ninety-two percent of the events analyzed had various hybridizing bands larger than 2.5 kb, confirming the presence of the *bar* gene cassette and the T-DNA left border region in the maize genome. Events 2 and 23 contained a small *bar*-hybridizing band (<2.5 kb), suggesting that some gene rearrangement or truncation occurred between the *gus* and the *bar* genes in these events. In our analyses to date, 90% of events have shown three or fewer hybridizing

Table II. *Effect of cysteine on stable transformation efficiency*[a]

Cocultivation Temperature	Cysteine Concentration	No. of Experiments	No. of Embryos Infected	No. of Bialaphos-Resistant Events	Transformation Efficiency
°C	*mg L^{-1}*				*(%)*
23	0	8	497	1	0.2
	400	8	400	21	5.3
20	0	5	440	1	0.2
	400	5	487	30	6.2

[a] Transformation efficiency = (no. of bialaphos-resistant events recovered/no. of embryos infected) × 100.

Table III. *Effect of cysteine concentration on stable transformation efficiency*[a]

	Cysteine in Cocultivation Medium (mg L^{-1})				
	0	100	200	300	400
No. of embryos infected	52	42	134	132	153
No. of bialaphos resistant events	0	2	2	6	5
Transformation efficiency (%)	0.0	4.8	1.5	4.5	3.3

[a] Transformation efficiency = (no. of bialaphos-resistant events recovered/no. of embryos infected) × 100.

bands and no event has yielded more than five bands when hybridized with the *bar* probe.

Progeny screening of R_1 and R_2 generation seedlings was carried out on a subset of transgenic events to assess whether the *gus* and *bar* transgenes were inherited in a normal Mendelian fashion. Seed for these tests was derived from female transgenic plants that had been pollinated (outcrossed) with non-transgenic pollen. As such, the expected segregation ratio for inheritance of the transgene as a single locus was 1:1. Leaf samples from progeny plants of 17 independent events were assayed for histochemical GUS expression (Fig. 3i) and plantlets were then sprayed with glufosinate to test for expression of the *bar* gene (Fig. 3j). Three events (Events 4, 6, and 23) did not express the *gus* gene in callus (data not shown) or progeny plants (Table IV). For the majority of GUS-expressing events (11 of 14 events tested), the observed segregation ratio for the *gus* gene was as expected. Likewise, segregation ratios for *bar* gene expression confirmed normal Mendelian inheritance of the transgene in 14 of 17 events (Table IV). Whereas Events 16 and 45 showed abnormal segregation for both transgenes, Event 48 showed a 3:1 segregation ratio for the *gus* and *bar* genes, suggesting that each of the transgenes is segregating at two rather than one loci in this event.

R_2 generation progeny plants of Events 1 through 5 were also screened for expression of the *gus* and *bar* transgenes. Expected segregation ratios for both transgenes were observed except for Event 1 (*gus* gene) and Event 2 (*bar* gene, Table IV).

Although Southern blots of Events 2 and 23 indicate some rearrangement and truncation in the *bar* gene, both segregated normally for *bar* gene expression in the R_1 generation. However, segregation of R_2 progeny in Event 2 was abnormal (Table IV). Detailed sequence analysis will be required to determine the molecular nature of these events.

DISCUSSION

We have produced numerous transgenic events from Hi II immature zygotic embryos transformed using an *A. tumefaciens* standard binary vector system. Southern-blot analyses confirm integration of the transgenes in the maize genome. Transgenic plants from these experiments are fertile and we have observed normal inheritance and stable expression of the *bar* and *gus* transgenes in R_1 and R_2 progeny in

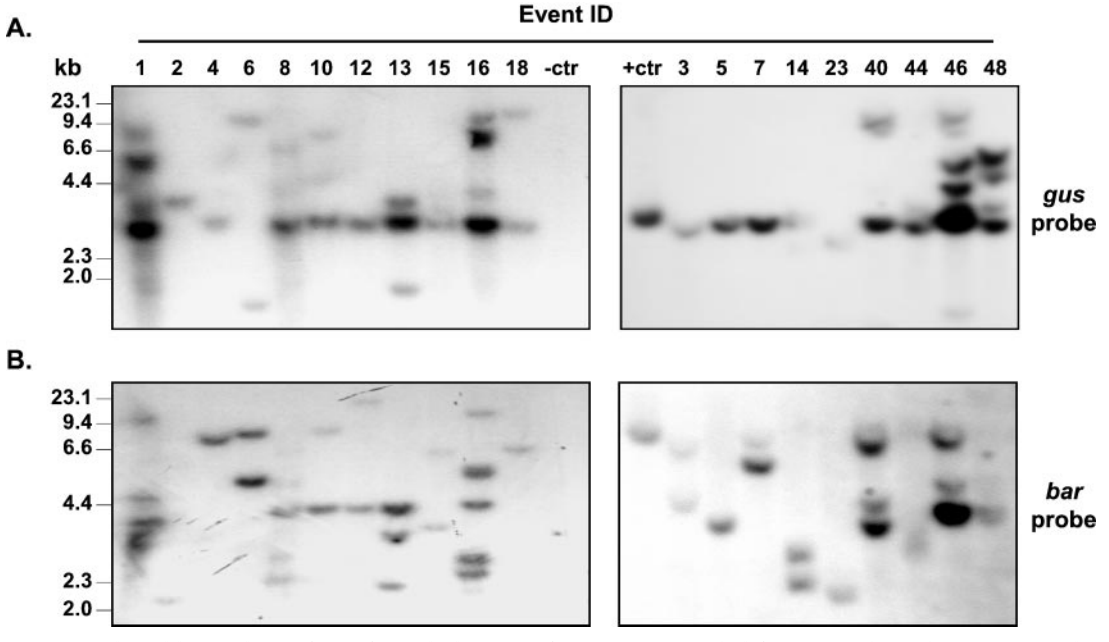

Figure 4. Southern-blot analysis of transformed plants. Leaf tissue was sampled from 20 independent events in the R_0 generation (6, 8, 10, 12, 13, 15, 16, and 18), the R_1 generation (1, 2, 4, 7, 14, 23, 40, 44, 46, and 48), or the R_2 generation (3, 5). No parent-progeny relationships are depicted. Ten micrograms of genomic DNAs was digested with *Hin*dIII and hybridized with the *gus* (A) or *bar* probe (B). −ctr, Negative control (genomic DNA from non-transformed maize inbred line B73); +ctr, positive control (non-transgenic maize genomic DNA spiked with 80 pg of pTF102 plasmid DNA digested with *Hin*dIII).

Table IV. *Segregation analysis for gus and bar gene expression in R_1 and R_2 generation progeny plants*[a]

NE, Non-expresser.

Event ID	Segregation Ratio					
	GUS		X^{2f}	Herbicide		X^{2f}
	Pos[b]	Neg[c]		Res[d]	Sen[e]	
R_1						
1	32	34	0.1	63	38	6.2
2	21	32	2.3	23	32	1.47
3	58	55	0.1	59	51	0.58
4	0	37	NE	18	18	0.0
5	34	43	1.1	36	42	0.23
6	0	39	NE	21	17	0.4
7	4	7	0.8	4	7	0.8
13	22	12	2.8	19	15	0.5
14	14	10	0.7	16	13	0.3
16	4	14	5.5	5	13	3.5
18	8	7	0.1	7	7	0.0
23	0	12	NE	11	11	0.0
40	6	7	0.1	9	6	0.6
44	7	8	0.1	7	8	0.1
45	4	13	4.8	2	16	10.9
46	4	6	0.4	4	7	0.8
48	17	3	9.8	15	5	5.0
R_2						
1	18	5	7.3	9	13	0.7
2	21	18	0.2	6	31	16.9
3	18	18	0.0	16	19	0.3
4	0	25	NE	11	15	0.6
5	16	15	0.0	15	18	0.3

[a] Transgenic plants were crossed as the female parent with pollen from non-transformed B73 plants. [b] Pos, GUS assay positive (*gus*-expresser). [c] Neg, GUS assay negative (*gus* non-expresser). [d] Res, resistant to glufosinate spray (*bar*-expresser). [e] Sen, Sensitive to glufosinate spray (*bar* non-expresser). [f] $\chi^2 = 3.8$ (0.05, 1 df).

the majority of events. In this study, we have demonstrated the reproducibility of this method using the pTF102 vector system. In separate studies, we have also used this protocol to introduce a number of other genes of interest into maize using the same vector backbone (B. Frame, unpublished data).

In all pTF102 events analyzed, estimated transgene copy number ranged from one to five copies per genome. A similar range in copy number estimate was reported by Zhao et al. (1998) in transgenic events derived from *A. tumefaciens* super binary vector transformation of maize Hi II immature zygotic embryos. In contrast, copy number estimates for biolistic gun-derived transgenic events of maize can range as high as 20 (H. Shou and R. Chikwamba, unpublished data).

The level of stable transformation achieved in this study is attributed to supplementation of cocultivation medium with 400 mg L^{-1} Cys. This antioxidant treatment also increased T-DNA delivery to embryogenic-competent scutellum cells of infected embryos. A similar increase in transient *gus* gene expression, followed by an increase in stable transformation efficiency, was reported in soybean cotyledonary node explants infected with *A. tumefaciens* and cocultivated on medium supplemented with Cys (Olhoft and Somers, 2001). Komari and Kubo (1999) suggested that the main hurdle in *A. tumefaciens*-mediated maize transformation may not be the infection step but may be the recovery of cells that have integrated the T-DNA into their chromosomes. Cys in cocultivation medium may be acting to minimize cell death caused by the hypersensitive response of maize scutellum cells to *A. tumefaciens* infection. This would favor postinfection survival of embryogenic-competent cells, thereby increasing stable transformation efficiency. Notably, we have observed an interaction between the Cys cocultivation treatment described in this study and treatments aimed instead at increasing *A. tumefaciens* virulence prior to infection. For example, in separate comparisons, reducing the 3-d incubation temperature for *A. tumefaciens* cultures from 28°C to 19°C and pre-inducing *A. tumefaciens* in plant infection medium supplemented with acetosyringone (AS) both lead to an increase in stable transformation, but only if infected embryos were subsequently cocultivated on 400 mg L^{-1} Cys (B. Frame, unpublished data).

Contrary to our expectation, the increase in stable transformation efficiency observed with the 400 mg L^{-1} Cys treatment was associated with a decrease in the proportion of embryos giving rise to embryogenic callus compared with the 0 mg L^{-1} Cys treatment. We know that this reduction in embryo response is not related to the plant-pathogen interaction per se because noninfected embryos also exhibited reduced response on 400 mg L^{-1} Cys. It is likely that Cys concentrations as high as 400 mg L^{-1} are toxic to maize cells. A similar negative impact of 80 mg L^{-1} Cys on embryogenesis in Japonica rice explants was reported by Enriquez-Obregon et al. (1999). We have achieved comparable stable transformation rates using Cys concentrations as low as 100 mg L^{-1}, and this treatment was associated with better embryo recovery after cocultivation than that observed using the 400 mg L^{-1} Cys treatment. Further studies are under way in our laboratory to define the optimum Cys concentration for stable transformation.

We have demonstrated that *A. tumefaciens*-mediated maize transformation using a standard binary vector system is reproducible although variability in experimental efficiency persists. In our experience, using cocultivation medium within 7 d of preparation minimizes this variability. Although our average transformation efficiency of 5.5% is low compared with that reported for Hi II immature zygotic embryos transformed using the super binary vector system (33%–51%, Zhao et al., 1998), further improvements in transformation efficiency using this procedure are likely to result from optimization of the Cys concentration used in cocultivation medium, or by examining the effect of using other antioxidant

compounds that are less toxic to maize. Consideration of interactions between Cys and experimental factors such as cocultivation duration and timing to selection, or parameters involved in *A. tumefaciens* virulence induction, may also lead to further improvements in stable transformation efficiency using *A. tumefaciens* standard binary vectors to transform maize.

MATERIALS AND METHODS

Agrobacterium tumefaciens Vector and Strain

A. tumefaciens strain EHA101 (Hood et al., 1986) containing the standard binary vector pTF102 (12.1 kb) was used in all experiments. The 5.9-kb T-DNA region of this construct is shown in Figure 1. The vector is a derivative of the pPZP binary vector (Hajdukiewicz et al., 1994) that contains the right and left T-DNA border fragments from a nopaline strain of *A. tumefaciens*, a broad host origin of replication (pVS1) and a spectinomycin-resistant marker gene (*aad*A) for bacterial selection. The CaMV 35S promoter (P35S) was used to drive both the *bar* selectable marker gene and the *gus* reporter gene. A tobacco etch virus translational enhancer (Carrington and Freed, 1990) was included in the 5′ end of the *bar* gene. The soybean (*Glycine max* L. Merrill) vegetative storage protein terminator (Mason et al., 1993) was cloned to the 3′ end of the *bar* gene. The *gus* gene contained a portable intron in its codon region (Vancanneyt et al., 1990) to prevent GUS activity in *A. tumefaciens* cells. This vector system, pTF102 in EHA101, was maintained on yeast extract peptone (YEP) medium (An et al., 1988) containing 100 mg L^{-1} spectinomycin (for pTF102) and 50 mg L^{-1} kanamycin (for EHA101). Bacteria cultures for weekly experiments were initiated from stock plates that were stored for up to 1 month at 4°C before being refreshed from long-term, −80°C glycerol stocks. In all experiments, bacteria cell densities were adjusted to an optical density (OD_{550}) between 0.35 to 0.45 using a spectrophotometer immediately before embryo infection.

Plant Material

F_2 immature zygotic embryos (1.5–2.0 mm) of the maize (*Zea mays*) Hi II hybrid genotype (Armstrong et al., 1991) were aseptically dissected from greenhouse-grown ears harvested 10 to 13 d post pollination. Ears were stored up to 3 d at 4°C before dissection.

Media

Infection, cocultivation, resting, and selection media were after Zhao et al. (1999) except that cocultivation medium was modified to contain Cys. All these media contained N6 salts and vitamins (Chu et al., 1975), 1.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid, and 0.7 g L^{-1} L-Pro in addition to the following ingredients: infection medium contained 68.4 g L^{-1} Suc and 36 g L^{-1} Glc (pH 5.2) and was supplemented with 100 μM AS (Sigma, St. Louis) before use; cocultivation medium contained 30 g L^{-1} Suc, 0.85 mg L^{-1} silver nitrate, 100 μM AS, and 3 g L^{-1} gelrite (pH 5.8); resting medium contained 30 g L^{-1} Suc, 0.5 g L^{-1} MES, 0.85 mg L^{-1} silver nitrate, 250 mg L^{-1} cefotaxime, and 8 g L^{-1} purified agar (pH 5.8). Selection medium was identical to resting medium with the addition of 1.5 or 3 mg L^{-1} bialaphos (Shinyo Sanyo, Tokyo). Infection medium was filter sterilized, whereas all other media were autoclaved. AS stock solutions (100 mM) were prepared by dissolving AS in 100% (v/v) dimethyl sulfoxide (DMSO) to make a 200 mM stock which was then diluted (1:1 [v/v]) with sterile water and stored in small aliquots at −20°C. Cys was added to cocultivation medium after autoclaving from freshly prepared, filter-sterilized stocks (100 mg mL^{-1}) and cocultivation medium was used within 2 to 5 d of preparation. Regeneration I medium contained Murashige and Skoog salts and vitamins (Murashige and Skoog, 1962), 60 g L^{-1} Suc, 100 mg L^{-1} myo-inositol, no hormones, and 3 g L^{-1} gelrite (pH 5.8) after Armstrong and Green (1985). Cefotaxime (250 mg L^{-1}) and bialaphos (3 mg L^{-1}) were added to this medium after autoclaving. Regeneration II medium differed from medium I in that it contained 30 g L^{-1} Suc and no bialaphos. All media was poured to 100- × 25-mL plates.

Infection and Cocultivation

A. tumefaciens cultures were grown for 3 d at 19°C on YEP medium amended with 100 mg L^{-1} spectinomycin and 50 mg L^{-1} kanamycin. One full loop (3 mm) of bacteria culture was scraped from the 3-d-old plate and suspended in 5 mL of liquid infection medium (Inf) supplemented with 100 μM AS (Inf + AS) in a 50-mL falcon tube. The tube was fixed horizontally to a bench-top shaker or a Vortex Genie platform head and shaken on low speed (approximately 75 rpm) for 4 to 5 h at room temperature. This pre-induction step was carried out for all experiments. For infection, immature zygotic embryos (1.5–2.0 mm) were dissected to bacteria-free Inf + AS medium (1.8 mL) in 2-mL eppendorf tubes (20–100 embryos per tube) and washed twice with this medium. The final wash was removed and 1 to 1.5 mL of *A. tumefaciens* suspension was added to the embryos. Embryo infection was accomplished by gently inverting the tube 20 times before resting it upright for 5 min with embryos submerged. Embryos were not vortexed at any time during this procedure. After infection, embryos were transferred to the surface of cocultivation medium and excess *A. tumefaciens* suspension was pipetted off the medium surface. Cocultivation medium contained 400 mg L^{-1} Cys unless stated otherwise. In experiments in which cocultivation medium treatments were compared, embryos were washed and infected in the same tube before being distributed between media treatments. Embryos were oriented with the embryo-axis side in contact with the medium (scutellum side up). Plates were wrapped with vent tape (Vallen Safety Supply, Irving, TX) and incubated in the dark at 20°C or 23°C for 3 d, after which embryos were transferred to 28°C on resting medium.

Embryo response (%) was measured as the number of cocultivated immature zygotic embryos that had initiated

embryogenic Type II callus formation at their scutellum base after 4 to 7 d on resting medium, compared with the total number plated. All embryos, responding or not, were transferred to selection medium.

Selection and Regeneration

After 4 to 7 d on resting medium (28°C, dark), embryos were transferred to selection medium (30 per plate) containing 1.5 mg L^{-1} bialaphos. Selection was increased to 3 mg L^{-1} bialaphos 2 weeks later. Putatively transformed events were identified as early as 5 weeks after infection. Regeneration of R_0 transgenic plants from Type II embryogenic callus was accomplished by a 2- to 3-week maturation step on Regeneration Medium I followed by germination in the light on Regeneration Medium II as described by Frame et al. (2000). Stable transformation efficiency (%) was calculated as the number of bialaphos-resistant callus events recovered per 100 embryos infected.

Acclimatization and Greenhouse Care of Transgenic Plants

Transplant and acclimatization of regenerated R_0 plants was accomplished as described previously (Frame et al., 2000). Transgenic plants were grown to maturity in the greenhouse.

Statistical Analysis

Data from eight independent experiments were used to compare stable transformation efficiency from pairs of plates treated alike aside from Cys exposure during cocultivation. A sign test was used to determine whether the benefit in transgenic event recovery rate observed for the 400 mg L^{-1} Cys treatment was significantly higher than that for the 0 mg L^{-1} Cys treatment.

A Chi square test was used to determine whether the segregation ratios we observed for *gus* and *bar* gene expressing progeny plants fit the expected 1:1 ratio.

Histochemical Analysis of Transient and Stable *gus* Expression

Histochemical GUS assays (Jefferson, 1987) were used to assess transient expression of the *gus* gene in immature zygotic embryos 1 or 2 d after the 3-d cocultivation (4 or 5 d after infection). Level of transient *gus* expression was assessed on a per embryo basis by estimating the number of blue foci visible on the scutellum side of each embryo. Embryos displaying blue foci only on the embryo-axis side of the explant were scored as non-expressors. The embryo was then categorized as follows: nonexpresser (no blue foci), low expresser (one–25), moderate expresser (26–100), or high expresser (>101). The number of embryos in each of these four groups was compared with the total number of embryos assessed to determine percent of total embryos in each of the expression categories. Histochemical GUS assays were also used to assess stable expression of the *gus* gene in bialaphos-resistant callus samples and in leaf tissue of transgenic plants in the R_1 and R_2 generations. Leaf segments (0.5 cm) were submerged in the substrate, vacuum infiltrated (20 inch Hg) for 10 min, and incubated at 37°C overnight. Blue staining cells were visualized by soaking leaf tissue in 75% followed by 95% (v/v) ethanol to remove chlorophyll and leaf pieces scored as positive or negative for GUS expression.

Southern-Blot Analysis

Leaf genomic DNA was prepared from 2 to 3 g of fresh leaf tissue from putative transgenic maize plants using the cetyltrimethylammonium bromide (CTAB) method, as described by Murray and Thompson (1980). Ten micrograms of genomic DNA per sample was digested with the *Hin*dIII restriction enzyme at 37°C overnight and separated on a 0.8% (w/v) agarose gel. DNA gel-blot analyses (Sambrook et al., 1989) were conducted on DNA samples using the ^{32}P-labeled *bar* or *gus* fragments as shown in Figure 1.

Progeny Segregation Analysis for *bar* Gene Expression

A glufosinate leaf-spray test (Brettschneider et al., 1997) was used to establish segregation ratios for expression of the *bar* gene in progeny. The herbicide Liberty (Aventis, Strasbourg, France) was dissolved in water (1.25 mL L^{-1}) along with 0.1% (v/v) Tween 20 for a final glufosinate concentration of 250 mg L^{-1}. Beginning 9 d after planting, seedlings were sprayed three times at 1- to 2-d intervals with a freshly prepared glufosinate solution and then scored for herbicide resistance (alive) or herbicide sensitivity (dead).

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for non-commercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permission will be the responsibility of the requestor.

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