

Improvement of *Agrobacterium*-mediated transformation in Hi-II maize (*Zea mays*) using standard binary vectors

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Received: 30 July 2007 / Revised: 26 September 2007 / Accepted: 30 September 2007 / Published online: 16 October 2007
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Abstract High-frequency transformation of maize (*Zea mays* L.) using standard binary vectors is advantageous for functional genomics and other genetic engineering studies. Recent advances in *Agrobacterium tumefaciens*-mediated transformation of maize have made it possible for the public to transform maize using standard binary vectors without a need of the superbinary vector. While maize Hi-II has been a preferred maize genotype to use in various maize transformation efforts, there is still potential and need in further improving its transformation frequency. Here we report the enhanced *Agrobacterium*-mediated transformation of immature zygotic embryos of maize Hi-II using standard binary vectors. This improved transformation process employs low-salt media in combined use with antioxidant L-cysteine alone or L-cysteine and dithiothreitol (DTT) during the *Agrobacterium* infection stage. Three levels of N6 medium salts, 10, 50, and 100%, were tested. Both 10 and 50% salts were found to enhance the T-DNA transfer in Hi-II. Addition of DTT to the cocultivation medium also improves the T-DNA transformation. About 12% overall and the highest average of 18%

transformation frequencies were achieved from a large number of experiments using immature embryos grown in various seasons. The enhanced transformation protocol established here will be advantageous for maize genetic engineering studies including transformation-based functional genomics.

Keywords *Agrobacterium tumefaciens* · Binary vector · Hi-II maize · *Zea mays* · Transformation

Introduction

Maize (*Zea mays*) is a major world crop and an important model monocot plant for studying genetics, genomics, and molecular biology. Recent advances in maize genome research have generated a wealth of new genetic information (<http://www.plantgdb.org>) (for website use, see Dong et al. 2005). These valuable genetic resources will be better utilized if a high-quality and high-frequency maize transformation system is developed. One immediate application of these resources will be to verify the functions of ESTs through genetic transformation in a high-throughput manner.

Agrobacterium tumefaciens has become a preferred transgene delivery vehicle in maize transformation in public laboratories. This preference is largely due to the advantages that the T-DNA transfer process has over other gene delivery systems, i.e., the high proportion of simple insertion events with intact transgenes and stable transgene expression and inheritance (Dai et al. 2001; Hu et al. 2003; Shou et al. 2004; Travella et al. 2005). Functional genomic studies of crop plants such as maize necessitate a cost-effective functional testing of a huge number of candidate genes or other nucleotide sequences and require high-throughput transformation systems.

Communicated by P. Ozias-Akins.

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Of the several target maize genotypes, maize hybrid line Hi-II has been a predominant genotype used for genetic transformation in public laboratories. The primary advantage of using Hi-II maize is its high-frequency of type II somatic embryogenic callus induction, a type of callus that is friable, fast growing, and highly embryogenic (Armstrong and Green 1985, 1992; Armstrong 1999). These qualities have allowed development of *Agrobacterium*-mediated T-DNA transfer in Hi-II maize that is either high-throughput (Zhao et al. 1999, 2002) or adequately efficient (Frame et al. 2002).

Two additional factors have contributed to the increase in transformation frequencies: the superbinary vector and the use of antioxidant L-cysteine during the *Agrobacterium* infection stage. While the superbinary vector has enabled very high-frequency transformation of maize in the industrial sector (Ishida et al. 1996; Zhao et al. 1999, 2002), the use of L-cysteine has significantly enhanced Hi-II maize transformation employing standard binary vectors in public laboratories (Frame et al. 2002). More recently, the use of L-cysteine in combination with modified medium salts has improved *Agrobacterium*-mediated transformation of three maize inbred lines (Frame et al. 2006).

Several antioxidants have been found to improve T-DNA transformation of recalcitrant crop species. For example, dithiothreitol (DTT) coupled with polyvinylpyrrolidone (PVP) in grape (*Vitis vinifera* L.) (Perl et al. 1996) or DTT in combination with L-cysteine in soybean (Olhoft and Somers 2001; Olhoft et al. 2001, 2003; Paz et al. 2004) have been shown to enhance T-DNA transfer. However, the potential role of DTT in promoting maize transformation remains to be explored.

Use of low-salt media during the *Agrobacterium* infection stage of transformation represents an additional strategy to improve T-DNA transfer. Although the precise mechanism of transformation enhancement is not understood, low salt-medium is now commonly used to improve T-DNA transformation in a few major crop species, except for maize (Fry et al. 1987; Cheng et al. 1997; Zhang et al. 1999, 2003; Zeng et al. 2004; Paz et al. 2004).

Here we report the improvement of *Agrobacterium*-mediated transformation in maize Hi-II using standard binary vectors. We show that the use of low salt media and the addition of DTT along with L-cysteine in the cocultivation medium improved *Agrobacterium*-mediated stable transformation frequencies from an average of 5.5 to 12% in Hi-II.

Materials and methods

Plant materials

Maize genotypes Hi-II A and Hi-II B were acquired from the Maize Genetic Cooperation Stock Center (United

States Department of Agriculture-Agriculture Research Service) at the University of Illinois at Urbana-Champaign, USA. They were grown and crossed to generate Hi-IIA × B (hybrid cross) seeds (F1) under either greenhouse or field conditions. These seeds were planted and grown in 9.1-l pots filled with Pro-mix soil in the Sears Plant Growth Facility at the University of Missouri-Columbia. The greenhouse is located at latitude 38°57'8"N and longitude 92°19'48"W. The greenhouse conditions included supplemental lighting with high-sodium pressure lights (intensity 600–1,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and a 16:8 h photoperiod from late May to early November and a 18:6 h photoperiod during the remaining months. The average temperatures were set to 22°C during the night and 28°C during the day throughout the growing seasons. Plants were fertilized once with Osmocot 18-6-12 time-release fertilizer about a month after planting or transplanting and watered as required. F1 plants were self-pollinated and immature embryos (F2) were isolated from kernels 9–13 days after self-pollination and used for *Agrobacterium* infection. Unless otherwise specified, all materials and supplies for maize growth in the greenhouse were purchased from Hummert International, Earth City, IA, USA.

Agrobacterium strains and vectors

We used *Agrobacterium tumefaciens* strains LBA4404 (Hoekema et al. 1983) and EHA101 (Hood et al. 1986) to harbor the transformation constructs. The first construct was a standard binary vector pCAMBIA3301 (CAMBIA, Australia), which is publicly available. The second standard binary vector, pZY101 (or its derivatives) was used to subclone a GUS reporter gene or other genes of interest into the multiple cloning sites of the vector. The vector pZY101 was derived from pZY101.1 (also called PTF101.1) (Frame et al. 2002) by replacing the double CaMV35S promoter with a single CaMV35S promoter. Figure 1 shows these two vectors. The construct carrying GUS Intron reporter gene cassette is pZY102 and has been reported previously (Zeng et al. 2004). All constructs were mobilized into *Agrobacterium* by direct DNA transfer (An et al. 1988) and their integrity within the *Agrobacterium* cells was confirmed through restriction digest analysis.

Medium formulations

The types of media and composition of maize cultures used at various stages were derived from Zhao et al. (1999) and are listed in Table 1. Critical modifications in our established standard protocol included the use of 50% N6 basal salt mixture and full-strength vitamins (Chu et al. 1975) in

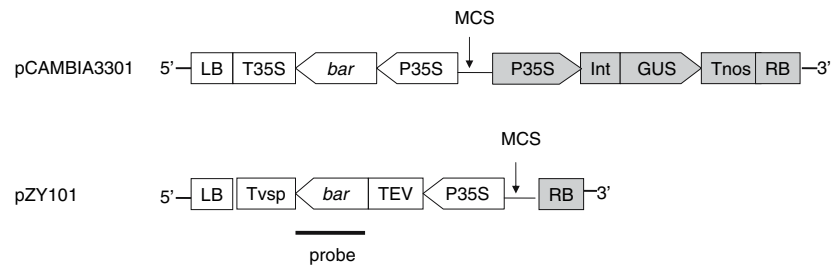


Fig. 1 Diagram of the two binary transformation vectors used in the study. Shown are only T-DNA regions of standard binary vectors pCAMBIA3301 (*top*) and pZY101 (*bottom*). LB and RB, T-DNA *left* and *right* borders, respectively; P35S and T35S, CaMV35S promoter and terminator, respectively; *bar* bialaphos resistance gene for

selecting transformants; *Int-GUS* intron-containing β -glucuronidase (*GUS*) gene; *Tnos* nopaline synthase gene terminator; *Tvsp* soybean seed storage protein gene terminator; *TEV* tobacco etch virus translational enhancer; *MCS* multiple cloning sites

both inoculation and cocultivation media and the addition of L-cysteine and DTT to the cocultivation medium. Basal salt medium and sucrose were autoclaved while additives including vitamins, plant growth regulators, antioxidants, selective agent and antibiotics were filter-sterilized into the medium. Minor modifications included the use of 8 g l^{-1} washed agar (Cat: A8678, Sigma-Aldrich, USA) to replace gelrite in the cocultivation medium. This cocultivation medium was overlaid with a piece of sterile filter paper in

our earlier experiments which tested the impact of three levels of N6 salt mixture.

Transformation protocol

Described below is our established standard protocol, which is modified from the previous ones (Zhao et al. 1999; Frame et al. 2002). The variations of this protocol used for

Table 1 Medium formulation

Medium compositions	Unit per liter	Concentrations							
		A	B	C	D1	D2	E	F	
N6 salt ^a	g	2.0	2.0	4.0	4.0	4.0	–	–	
MS salt	g	–	–	–	–	–	4.3	2.9	
Sucrose	g	68.5	30	30	30	30	60	30	
Glucose	g	36.0	–	–	–	–	–	–	
L-proline	g	0.7	0.7	0.7	0.7	0.7	–	–	
MES	g	0.5	0.5	0.5	0.5	0.5	–	–	
2,4-D	mg	1.5	1.5	1.5	1.5	1.5	–	–	
pH		5.2	5.8	5.8	5.8	5.8	5.6	5.6	
Agar (washed)	g	–	8.0	–	–	–	–	–	
Gelrite	g	–	–	3.0	3.0	3.0	3.0	3.0	
N6 vitamins, 1,000×	ml	1.0	1.0	1.0	1.0	1.0	–	–	
MS vitamins, 1,000×	ml	–	–	–	–	–	1.0	1.0	
Glycine	mg	–	–	–	–	–	2.0	2.0	
Silver nitrate	mg	0.85	0.85	0.85	–	–	–	–	
L-cysteine	g	–	0.4	–	–	–	–	–	
DTT ^a	g	–	0.15	–	–	–	–	–	
Acetosyringone	μM	–	100	–	–	–	–	–	
Cefotaxime	g	–	–	0.25	0.25	0.25	0.25	–	
Bialaphos	mg	–	–	–	1.5	3.0	3.0	–	

The N6 and MS salts are pre-mixed basal salts mixtures without vitamins, respectively. Treatments (salt levels) of N6 salt mixture in inoculation (A) and cocultivation (B) media were 10, 50, and 100%, while remaining media and their compositions for all treatments were identical. Salt and vitamin compositions were the same as described for MS (Murashige and Skoog 1962) and N6 (Chu et al. 1975). Sigma-Aldrich Inc., USA was supplier of all reagents except for MES (Fisher Scientific, USA), DTT (Invitrogen, USA), and bialaphos (Shinyo Sangyo Co. Ltd, Japan)

MES, 2-(4-morpholino)-ethane sulfonic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; *acetosyringone*, 3',5'-dimethoxy-4'-hydroxyacetophenone

^a Only 50% salts and 0.15 g l^{-1} (1 mM) DTT are listed as our established, standard protocol

testing the effect of different treatment conditions are also described in detail in the Results section.

Agrobacterium culture initiation

Agrobacterium tumefaciens strain LBA4404 (harboring construct pCAMBIA3301) or EHA101 (harboring pZY101 derivatives) was streaked out from a -80°C glycerol stock onto an AB minimal medium (Chilton et al. 1974) plate containing appropriate antibiotics. LBA4404 (carrying pCAMBIA3301) required 100 mg l^{-1} streptomycin and 50 mg l^{-1} kanamycin; whereas EHA101 (carrying pZY101 derivatives) required 25 mg l^{-1} chloramphenicol, 50 mg l^{-1} kanamycin, 100 mg l^{-1} each of spectinomycin and streptomycin. The plate was incubated at 28°C for 3 days until single colonies developed. This master plate was used on a weekly basis for up to a month. A single colony was streaked out onto YEP (5 g l^{-1} yeast extract, 10 g l^{-1} peptone, 5 g l^{-1} NaCl, and pH 7.0) containing the same antibiotics as the AB plate. The YEP plate was then incubated at 20°C for 2–3 days until bacterial colonies developed fully.

Inoculation and cocultivation

Agrobacterium colonies were taken from the YEP plate, suspended in 5 ml of A medium (in a 15 ml tube) with cell density of $\text{OD}_{550} = 0.3\text{--}0.4$. The culture tube was then shaken horizontally on a platform at 160 rpm at room temperature (23°C) for 4 h, i.e., pre-incubation (Frame et al. 2002) before being used for embryo infection. Before embryo isolation, ears were sterilized in 50% commercial bleach containing 5.25% sodium hypochlorite for 20 min and washed three times with sterile water. These ears were either freshly harvested or stored in 4°C refrigerator for up to 2 days (ears with husk were wrapped in a plastic bag). Immature zygotic embryos, around 1.5 mm (1.4–1.8 mm) in size, were isolated from maize kernels and placed in 1 ml of the medium A (about 100 embryos in a 1.5 ml Eppendorf tube), followed by 3–4 washes with the same medium. Then 1 ml of *Agrobacterium* suspension was added to the 1.5 ml tube, and inoculation was carried out for 10 min before embryos were transferred onto the medium B plate, with embryo scutellum facing up. To test the effect of the salt concentrations in media A and B on stable transformation, three levels of the N6 basal salt mixture, i.e., 10, 50, and 100%, were examined. In this test, 0.4 g l^{-1} cysteine was added to the cocultivation medium for all three levels of salt mixture treatments and no pre-inoculation induction of *Agrobacterium* cells was employed. The cocultivation lasted 3 days at 25°C . To test the impact of DTT on stable transformation, we conducted

a side-by-side comparison between no and addition of 0.15 g l^{-1} (1 mM) DTT in the cocultivation medium using the most advanced public procedure for maize Hi-II (Frame et al. 2002) as a basic protocol except that 50% N6 salt mixture was used in both treatments. In this test, a 4-h pre-inoculation induction of *Agrobacterium* cells was employed and cocultivation lasted 3 days at 20°C . In all cases, the cocultivation medium B plate was wrapped with parafilm and placed in darkness.

Culture selection and maturation

After cocultivation, embryos were then transferred to the medium C plate for 5–8 days without herbicide selection (the resting stage). Embryos were then subcultured on medium D1 containing 1.5 mg l^{-1} bialaphos for 2 weeks for initial herbicide selection. Subsequent cultures used 3 mg l^{-1} bialaphos in the medium D2; embryogenic, bialaphos-resistant calluses were transferred to fresh medium D2 biweekly until somatic embryos turned opaque. During the subculture on medium D2 the growing embryogenic calluses (over 1 cm in size) were divided into smaller pieces and only highly-embryogenic calluses (i.e., dry, friable, and fast growing tissues) were maintained. All divided calluses from the same embryo that later developed into fertile transgenic plants were treated as one event. The total culture period on the medium D2 lasted about 3–4 months. Maturation took place when embryos showing opaque color were transferred to the medium E for 3–4 weeks. All of the above culture plates were wrapped with parafilm and maintained in darkness at 28°C , except for maturation plates, which were wrapped with venting tape and placed in darkness at 25°C .

Regeneration, acclimatization and greenhouse care

The maturation stage ended when embryos turned ivory in color; embryos were subsequently transferred to medium F plates (wrapped with venting tape) for regeneration using a 18:6 h photoperiod with light intensity of $100\text{--}150\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ at 24°C . Shortly thereafter, both shoots and roots developed from each mature embryo. Each small plantlet was subsequently transferred to a $150 \times 25\text{ mm}$ tube containing the medium F for further development. Plantlets with fully grown shoots and roots were then transferred to Deep Traditional Inserts ($3\frac{1}{2}'' \times 3\frac{1}{2}''$, Hummert International) containing Pro-mix soil and allowed to acclimatize for 2–3 weeks in growth chamber conditions (a 18:6 h photoperiod and mixed cool white florescence and high-sodium pressure lights with light intensity of $300\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ at 24°C) before being moved to 9-l pots in greenhouse conditions. We carried 2–5 clones per event to the greenhouse

to secure the fertility. Subsequent greenhouse plant care followed the procedures described in the Plant materials section.

Analysis of transgenes

The histochemical β -glucuronidase (GUS) assay (Jefferson et al. 1987) was used to evaluate early events of T-DNA transfer to maize cells located in the embryo scutellum area at the end of the resting stage. The number of GUS-positive sectors and their distribution in the scutellum region were scored. Thus, the status of T-DNA transfer was estimated by percent areas infected on scutellum as indicated by GUS staining. Six infection ranges were used to estimate the magnitude of infection: 0, <25, 25–50, 50–75, >75, and 100% of scutellum areas showing GUS staining. The infection status was also evaluated using the “infection frequency (%)” that is defined as the number of embryos infected over the total number of embryos cocultivated.

We used a leaf-painting assay (Zhang et al. 1999) for preliminary confirmation of T-DNA insertion and progeny segregation to verify the functional expression of the *bar* gene. Leaf-painting was conducted 2–3 times on each putative transgenic event and progeny plant using 0.5% Liberty[®] (Aventis CropScience, USA). Southern blot analysis (Southern 1975) was used to confirm the integration and stable inheritance of transgene inserts. For Southern blot analysis, genomic DNA was extracted from Hi-II maize leaf tissues following a modified CTAB-based protocol (Dellaporta et al. 1983). Thirty μ g of DNA were digested with *Swa*I restriction enzyme that cut only once within the T-DNA region. The digested genomic DNA was fractionated on a 1.0% agarose gel prior to transferring to Zeta-Probe[®] GT nylon membrane (Bio-Rad, CA, USA). DNA was fixed to the membrane by UV cross-linking. Hybridization and washing conditions for Southern blot analysis followed the Zeta-Probe[®] GT manufacturer's instructions. The *bar* ORF from vector pZY101 was used to generate a ³²P-labeled probe. The probe was prepared by random primer synthesis incorporating ³²P-dATP utilizing the Prime-it[®] II kit (Stratagene, USA).

For progeny analysis, ten random sets of progeny plants were analyzed first using the leaf-painting assay to examine segregation patterns. One random set of progeny was further analyzed by Southern blot analysis to verify transgene inheritance.

Experimental design and data analysis

Randomized complete block design (RCBD) was applied to all experiments in which all treatments were assigned to

each ear (block) to minimize the variation between the experimental units (embryos). Analysis of variance was conducted using the SAS GLM program (Der and Everitt 2001) and means were separated by Duncan's multiple range test at $\alpha = 0.05$. Progeny segregation was analyzed using the χ^2 -test for Goodness of fit. Transformation frequency (%) was calculated based on stable transformation and was defined as the number of independent fertile transgenic maize events per explant, excluding contaminated explants when treatment effects were compared, or including contaminated explants when large scale stable transformation experiments were conducted. Each independent event was defined as a bialaphos-resistant plant derived from a single inoculated embryo that also transmitted the transgene to progeny. Random samples of some of these bialaphos-resistant plants were then tested for confirmation of different transgene integration events by Southern blot analysis and for transgene transmission by progeny segregation analysis.

Results

Impact of low-salt media during the cocultivation on transformation

The binary vector pCAMBIA3301 (Fig. 1) harbored by *Agrobacterium* strain LBA4404 was used to test the effect of three different salt strengths of N6 basal medium, 10, 50, and 100% on enhancing stable transformation. In these experiments, no pre-inoculation induction of *Agrobacterium* cells was employed and cocultivation lasted 3 days at 25°C. Our results demonstrate that as the salt concentration decreased in the inoculation (A) and cocultivation (B) media, the transformation frequency increased to as high as 17.5% at 10% salt concentration (Table 2). This was three times higher than the 5.5% transformation frequency previously reported by Frame et al. (2002). Since 10% salt in some cases decreased the quality of the embryogenic response, 50% was used for further experiments.

Impact of addition of DTT in cocultivation medium on T-DNA transfer

Previous studies showed enhanced transformation of Hi-II by employing L-cysteine during cocultivation (Frame et al. 2002). To determine if the addition of DTT in the cocultivation medium could further enhance transformation, we conducted side-by-side experiments to compare this modification with the Frame et al. (2002) method. Since 50% salts was proven to be superior to 100% salts in inoculation (A) and cocultivation (B) media for the T-DNA transfer in

Table 2 Impact of medium salt concentrations on stable transformation of Hi-II maize

Medium salt strength (%)	Number of embryos	Number of transgenic events recovered	Transformation frequency (%)
10	200	35	17.5a
50	200	8	4.0b
100	200	0	0.0c

Data were from three independent experiments using immature embryos from Hi-IIA × B:F1 maize plants. L-cysteine at 0.4 g l^{-1} (3.3 mM) as a sole antioxidant was used in combination with three different salt concentrations in the cocultivation medium (B) to test the effect of medium salt levels on stable transformation. Transformation frequencies (%) followed by the same letter are not statistically different from one another as detected by Duncan's multiple range test at $\alpha = 0.05$ level

our preliminary experiments, we used 50% salt media across the two treatments contrasting L-cysteine alone and L-cysteine + DTT in cocultivation medium. The *Agrobacterium* strain EHA101 carrying the binary vector pZY102 (PTF102) was used to transform the embryos after a 4-h pre-incubation (see also Materials and methods section) and the cocultivation lasted 3 days at 20°C.

We first conducted preliminary experiments to examine the effect of adding DTT to the cocultivation medium on T-DNA transfer by using a GUS assay at an early culture stage, i.e., right after the resting stage. The percent infected area as indicated by GUS-positive sectors was estimated in scutellum areas, since only scutellum areas are responsible for somatic embryo formation and T-DNA transfer to this area is more relevant. Data from two independent experiments with a total of 64 embryos per treatment indicated that addition of DTT plus L-cysteine was superior to L-cysteine alone. The former treatment led to 63.7% of embryos exhibiting GUS staining on scutellum areas as compared with 17.2% of embryos in the latter treatment (L-cysteine alone) (Fig. 2). Furthermore, 12.5% of embryos in DTT plus L-cysteine treatment exhibited large (25–75%) scutellum areas infected as compared with no scutellum displaying this infection range in L-cysteine alone.

We then conducted four independent, side-by-side stable transformation experiments comparing the two treatments (L-cysteine alone and L-cysteine + DTT) using the same experimental conditions. The L-cysteine alone and L-cysteine + DTT treatments included a total of 192 and 190 embryos, respectively. Our results showed that addition of DTT in cocultivation medium increased the transformation frequency fourfold over that using L-cysteine alone. This enhanced transformation was also statistically significant according to Duncan's multiple range test at $\alpha = 0.05$ level.

To further confirm the reproducibility of our improved transformation process, we then conducted multiple independent stable transformation experiments using the

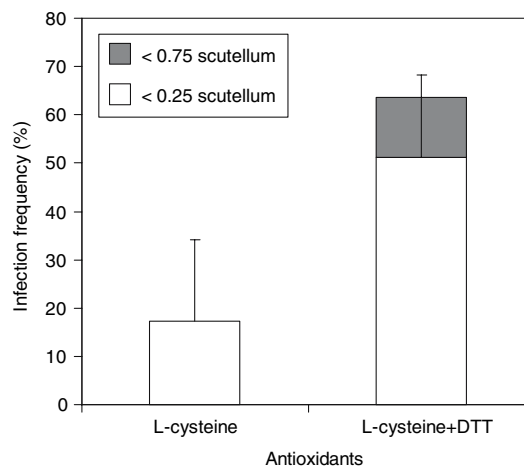


Fig. 2 Impact of addition of dithiothreitol (DTT) to the cocultivation medium on early events of T-DNA transfer. Results were evaluated based on GUS assays of infected embryos right after resting stage. The status of T-DNA transfer was estimated by percent areas infected on scutellum as indicated by GUS staining. Six infection ranges were used for estimation: 0, <25, 25–50, 50–75, >75, and 100% of scutellum areas. Data were from two independent experiments with a total of 64 embryos per treatment. Each error bar stands for a standard error of a mean for each treatment

improved infection conditions (50% salts and L-cysteine + DTT) with a large number of immature embryos from various growing seasons. Table 3 lists the transformation results obtained from the experiments using *Agrobacterium* strain EHA101 carrying 12 different constructs (containing different genes of interest) derived from pZY101 (PTF101.1). The overall transformation frequency across these constructs was 12.2%, and the highest average frequency was 18%. The overall recovery was consistent, although embryo vigor influenced transformation frequency (data not shown). The time frame of this entire transformation process from embryo isolation to harvesting transgenic T₁ seeds was 7–9 months.

Confirmation of transgene inheritance and integration patterns

To test the transgene inheritance and integration patterns, we randomly chose ten transgenic Hi-II maize events (T₀) expressing the HcPro construct (carrying a virus helper component for gene silencing study). Progeny plants of these events were first screened by herbicide leaf-painting to verify transgene segregation. Because all T₀ lines were crossed with wild type maize plants, the expected segregation ratio was 1:1 (resistant versus susceptible). The results showed that six out of ten events followed the expected 1:1 segregation ratio for a single locus ($P \geq 0.059$) and four events deviated from this ratio ($P \leq 0.018$) (Table 4). The deviation in these four events,

Table 3 Stable transformation of Hi-II maize using the improved protocol

Binary vectors	Number of embryos	Number of transgenic events recovered	Transformation frequency (%)
WY76	746	85	11.4
WY86	377	65	17.2
HcPro	1,013	84	8.3
MT101	275	37	13.5
MT102	672	70	10.4
MT103	273	29	10.6
HK191	378	68	18.0
HK245	380	58	15.3
MIPT	476	84	17.6
MOY	530	40	7.5
MYFP	536	51	9.5
MMRP	469	79	16.8
Total	6,125	750	12.2

Data were from at least three independent experiments for each construct using immature embryos from Hi-IIA × B: F1 maize plants grown in various seasons. The transformation frequency was calculated by the number of independent fertile transgenic events over the total number of embryos to start with (see also Materials and methods for event definition)

which was always a deficiency of resistant offspring, could be attributable to the small number of progeny plants examined or *bar* gene silencing. To examine the transgene integration patterns, we analyzed the genomic DNA samples of ten randomly selected maize events derived from construct HcPro using Southern blot analysis with the *bar*

Table 4 Progeny segregation analysis of primary transgenic Hi-II maize events

Events	Total progeny	Progeny		Segregation: R:S ratio	χ^2 -value	<i>P</i> -value
		R	S			
JB13-1	18	2	16	1:1	10.89	0.001
JB13-2	16	7	9	1:1	0.25	0.617
JB13-3	15	8	7	1:1	0.67	0.796
JB13-4	18	12	6	1:1	2.00	0.157
JB13-7	18	13	5	1:1	3.56	0.059
JB13-8	18	4	14	1:1	5.56	0.018
JB13-9	16	5	11	1:1	2.25	0.134
JB13-10	18	2	16	1:1	10.89	0.001
JB13-12	18	9	9	1:1	0.00	1.000
JB13-14	14	2	12	1:1	7.14	0.007

Progeny R and S columns indicate the number of progeny plants showing herbicide resistance and susceptible, respectively. Resistant progeny plants were scored as R and susceptible ones as S. χ^2 -test was used to test Goodness of Fit of observed progeny plants against theoretical number of plants showing R and S, respectively. A single degree of freedom was used to obtain *P*-values

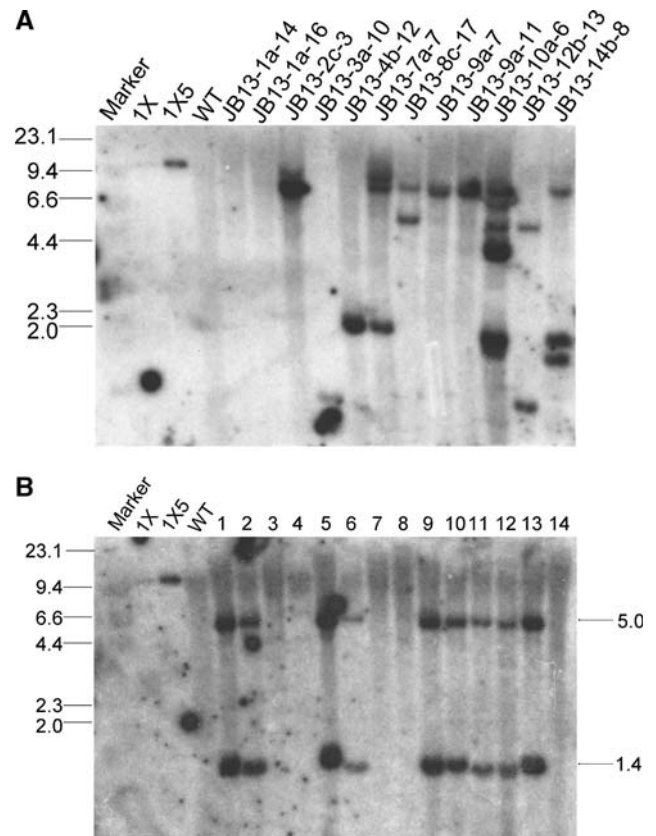


Fig. 3 Southern blot analysis of transgenic maize events and a set of representative progeny. **a** Analysis of transgene integration of ten putative events derived from vector HcPro. **b** Analysis of progeny plants 1–15 from event JB13–12b, showing segregation of transgene; two distinct bands, one at 5 kb and the other at 1.4 kb are indicated. A λ HindIII ladder was used as molecular marker and sizes are labeled as kb; WT wild-type control, 1X and 5X 1X and 5 X genome equivalent copy number controls using 24 and 120 μ g of vector HcPro, respectively. A total of 30 μ g DNA per sample was digested with *Swa*I, which cuts once within the T-DNA region of vector HcPro. Membranes were probed with *bar* ORF

gene ORF as a probe. We used restriction enzyme *Swa*I, which cuts once within the T-DNA region of the HcPro. Our results confirmed that at least nine of the ten putative events carrying the transgene were independent, as evidenced by different banding patterns (Fig. 3a). Plants JB13-9a-7 and JB13-9a-11 are apparently from the same event, as expected (bearing the same core code JB13-9a), because of the same banding pattern. Furthermore, five of nine events were simple insertions, with 1–2 transgene loci per genome; only one event exhibited a complex integration pattern (with seven transgene loci). It is possible that event JB13-1a may still carry a transgene but failed to exhibit insertion signal due to an insufficient amount of genomic DNA sample loaded, as is the case with the single copy control using vector HcPro that is undetectable on the Southern blot. Moreover, we analyzed genomic DNA samples from a random set of progeny (event JB13-12b

showing 1:1 segregation) using Southern blot analysis with the *bar* probe. The result indicates that the transgene was indeed transmitted to the progeny plants; thus the presence of a Southern band corroborated the observed herbicide resistance (Table 4, Fig. 3b).

Discussion

We have improved *Agrobacterium*-mediated gene delivery in maize Hi-II using standard binary vectors. These transformation results are representative because the immature embryos we used were from various growing seasons including winters when embryos exhibit relatively lower embryogenic responses (anecdotal observations). Southern blot and progeny segregation analyses confirmed stable transgene integration and inheritance with a high proportion of low-copy inserts per genome. Because our improved protocol utilizes standard binary constructs and thus does not require a superbinary vector, such an improved gene delivery system will be instrumental for public laboratories to study both basic and applied plant biology using transgenic maize. Indeed, one immediate application for this transformation protocol is telomere-mediated chromosomal truncation in maize (Table 3, pWY76 and pWY86) (Yu et al. 2006). This telomere-mediated chromosomal truncation requires high-frequency transformation because such truncation often has a deleterious effect on transformed maize cells, which could be selectively eliminated during the *in vitro* tissue culture stage. Consequently, the maize events recovered in this study may represent a fraction of transgenic cells that survived such lethality. Conceivably, other important maize engineering efforts, such as functional genome studies, will also benefit from this high-frequency transformation process.

The role of low salt mixture and DTT in promoting T-DNA transfer during the *Agrobacterium*-embryo cocultivation stage is illustrated by our study. The mechanisms that promote the observed enhancement of T-DNA transfer by the low salts and the antioxidants, however, remain largely uncharacterized. The dramatic effect of 10% salt media on T-DNA transfer has also been reported to optimize other cereal (wheat) transformation, but only in an assay that was based on transient expression (Ke et al. 2002). Our anecdotal observations indicated that an important condition in validating the use of 10% salt mixture during the infection stage for dramatic enhancement of T-DNA transformation in maize Hi-II is related to the vigor of embryos for infection. However, 50% salt in combination with DTT (plus L-cysteine) enabled the use of embryos of a wider vigor range. A more thorough systematic comparison would be necessary to validate

the above observations including the correlations between the embryo sizes and vigor and their transformation frequencies.

Acknowledgments We thank Regina Wamsley (from Zhanyuan J. Zhang's laboratory) for her excellent technical assistance; CAMBIA (Australia) for providing pCAMBIA3301; Aventis CropScience (Research Triangle Park, NC, USA) for herbicide Liberty[®], and James A. Birchler and Seth D. Findley (University of Missouri-Columbia) for a critical review of this manuscript. University of Missouri-Columbia Life Science Mission Enhancement program supported Angela R. Kennon and, in part, Xinlu Chen (from Zhanyuan J. Zhang's lab). All transformation experiments were conducted in the Plant Transformation Core Facility at the University of Missouri-Columbia.

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