

## PRODUCTION OF TRANSGENIC MAIZE FROM BOMBARDED TYPE II CALLUS: EFFECT OF GOLD PARTICLE SIZE AND CALLUS MORPHOLOGY ON TRANSFORMATION EFFICIENCY

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### SUMMARY

Here we present a routine and efficient protocol for year-round production of fertile transgenic maize plants. Type II callus derived from maize Hi II immature zygotic embryos was transformed using the PDS 1000/He biolistic gun and selected on bialaphos. In an effort to improve the transformation protocol, the effects of gold particle size and callus morphology on transformation efficiency were investigated. Reducing gold particle size from 1.0  $\mu\text{m}$  or 0.6  $\mu\text{m}$  resulted in a significant increase in the rate of recovery of bialaphos-resistant clones from Type II callus. The average transformation efficiency of pre-embryogenic, early embryogenic and late embryogenic callus did not vary significantly. Rates of transformation, regeneration and fertility achieved for Type II callus are summarized and compared to those achieved for greenhouse- and field-derived immature zygotic embryos.

*Key words:* zygotic embryo; regeneration; biolistic; *Zea mays*; Hi II callus; osmotic.

### INTRODUCTION

Biolistic transformation of maize has become routine since the recovery of fertile transgenic plants by this method was first reported by Gordon-Kamm et al. (1990). This technology has been used to transform various maize target tissues (Gordon-Kamm et al., 1999), including immature zygotic embryos from inbred lines (Koziel et al., 1993; Dunder et al., 1995; Brettschneider et al., 1997) and Hi II germplasm (Songstad et al., 1996; Pareddy et al., 1997). Immature zygotic embryos provide an excellent transformation target because they minimize both time in tissue culture and the expertise needed for callus induction and maintenance (Brettschneider et al., 1997; Songstad et al., 1996). However, in circumstances where inadequate greenhouse facilities may limit the quantity or quality of zygotic embryos available, and where continuous construct testing *in planta* is desirable, biolistic transformation experiments can instead be carried out on embryogenic callus derived from these or other explants. Biolistic transformation of both slow-growing, compact Type I embryogenic maize callus and fast-growing, friable Type II maize callus (Armstrong and Green, 1985) has been reported (Wan et al., 1995; Fromm et al., 1990; Walters et al., 1992; Dennehey et al., 1994; Armstrong et al., 1995; Zhang et al., 1996; Pareddy et al., 1997).

Type I callus can be induced to develop from immature zygotic embryos of several maize inbred genotypes (Wan et al., 1995;

Brettschneider et al., 1997), thereby facilitating the transformation of some elite germplasms using this target tissue. However, the slow growth of Type I callus limits the rate at which it can be increased through subculture unless a constant supply of immature embryo starting material is available. Furthermore, the compact growth habit of Type I callus, along with its high degree of differentiation, demands intensive subculturing during selection (Pareddy et al., 1997). Even then, a second tier of screening at the plant level may be required to eliminate chimeric events or nontransformed, regenerated plants that have survived selection (Wan et al., 1995; Brettschneider et al., 1997).

In contrast, Type II maize callus can be rapidly increased through subculture to meet flexible demand. The friability and lack of differentiation characteristic of Type II callus also facilitates effective *in vitro* selection thereby minimizing the chance of escapes (Fromm, 1994). While not readily induced in most inbred germplasms, Type II callus can be efficiently produced from immature zygotic embryos of the Hi II germplasm derived from A188×B73 (Armstrong et al., 1991). In addition, this material was reported to form excellent Type II callus in F1 combinations with other more recalcitrant genotypes (Armstrong et al., 1991). These features of regenerable Type II callus make it a favorable tissue for biolistic transformation, particularly if the goal of the research is to produce large numbers of fertile transgenic plants for construct testing in noninbred germplasm. Existing protocols for Type II callus development (Sellmer et al., 1994), biolistic transformation (Fromm, 1994), and regeneration (Armstrong, 1994) attest to its effectiveness as a target tissue for transformation using micro-projectile bombardment.

Our goal was to establish a routine and efficient protocol for

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year-round production of transgenic maize plants. Using Type II callus, we implemented a transformation protocol using the biolistic PDS 1000/He delivery system and then set out to improve upon its efficiency by examining the effect of callus morphology, osmotic treatment and gold particle size on transgenic clone yield.

Type II callus morphology can vary considerably while maintaining characteristic attributes such as friability and rapid growth. For example, Welter et al. (1995) described the histology of three distinct morphologies of embryogenic Type II maize callus (pre-embryogenic, early embryogenic and late embryogenic) and proposed that pre-embryogenic callus may be particularly amenable to biolistic transformation due to the 1–2-layer thick mantle of embryogenic units that characterize its surface morphology. The predominance of cells at this early developmental stage in the surface layers known to be targeted at bombardment (Kausch et al., 1995) does suggest that this callus may be a preferred target. To test whether differences in callus morphology could be exploited to achieve higher transformation efficiencies, we bombarded pre-embryogenic, early embryogenic and late embryogenic Type II callus and compared transgenic clone yield, regeneration and fertility for these three distinct callus morphologies.

Efforts to improve the efficiency of biolistic maize transformation have focused not only on identifying a suitable target tissue, but, among other factors, on osmotic treatments (Vain et al., 1993), and biolistic parameters (Klein et al., 1988; Sanford et al., 1993; Kausch et al., 1995). For example, sorbitol and/or mannitol osmotic treatments have been shown to increase stable transformation rates in maize cell suspensions (Vain et al., 1993) and a 12% sucrose osmotic treatment was reported effective in obtaining stable events from bombarded immature zygotic embryos (Dunder et al., 1995). The beneficial effect of osmotic treatment in biolistic transformation is attributed to its role in plasmolyzing the cells prior to bombardment (Vain et al., 1993). In this state of reduced turgor, the bombarded cells are thought to be less likely to burst upon impact. Transient *gus* expression in Type II callus treated at the time of bombardment on either of these two osmotic media is comparable (Pareddy et al., 1997). In our study, we compare stable clone recovery from bombarded Type II callus treated for 4 h pre- and 1 h post-bombardment on these two osmotic media.

Biolistic parameters such as particle size also affect stable transformation efficiency in maize. Kausch et al. (1995) surmised that excessive wounding of targeted cell layers by particles (microprojectiles) during biolistic transformation could compromise transformation success. Randolph-Anderson et al. (1995) measured a 5–7-fold increase in anthocyanin-expressing stable sectors of maize Type I callus bombarded with 0.6  $\mu\text{m}$  gold particles rather than 1.0  $\mu\text{m}$  gold particles. The authors suggested that this increase in stable sector recovery was due to the increased survival rate of cells bombarded with the smaller gold particles. We compare the effect of 0.6  $\mu\text{m}$  and 1.0  $\mu\text{m}$  gold particles on the efficiency of stable clone recovery from bombarded Type II callus.

Finally, we summarize the rates of co-transformation, regeneration and fertility of transgenic events obtained from our Type II callus protocol and compare the efficiency with that achieved by bombarding Hi II immature zygotic embryos derived from field- or greenhouse-grown plants.

## MATERIALS AND METHODS

**Plant material.** Seeds of parent A and parent B of the Hi II maize germplasm (Armstrong et al., 1991), were generously provided by Mark Galatowitsch (University of Minnesota, Minneapolis/St. Paul, MN) and the Maize Genetics Cooperation – Stock Center (Stock Number I.D. T940A and T940B). Seed stocks were propagated in the genetic nursery of Patrick Schnable and the source field of the Plant Transformation Facility at Iowa State University (Ames, Iowa). Zygotic embryo source plants were grown in the field (1996–1998) or the greenhouse (16-h photoperiod, 28°C day/20°C night temperature, 1–400 W high-pressure sodium lamp/22 ft<sup>2</sup>) in Ames, Iowa.

**Callus culture initiation and maintenance.** Ears were harvested 9–12 d after pollination and sterilized for 20 min in 50% (v/v) commercial bleach (Clorox™) followed by three 5-min rinses with sterile water. Field ears were often pre-washed and rotting sections removed prior to sterilization to help alleviate fungal and bacterial contamination, which developed more often from field- than greenhouse-derived material. Calluses were initiated from F2 embryos and, in fewer cases, from F1 embryos (parent A × parent B) of Hi II germplasm unless otherwise stated. For callus initiation, immature zygotic embryos (1.5–2.0 mm) were dissected and cultured embryo-axis-side down on N6-based media (Chu et al., 1975) supplemented with 25 mM proline, 100 mg l<sup>-1</sup> casein hydrolysate, 50  $\mu\text{M}$  silver nitrate (Armstrong and Green, 1985; Songstad et al., 1991), 2 mg l<sup>-1</sup> 2,4-dichlorophenoxyacetic acid (2,4-D) (9  $\mu\text{M}$ ), 3% sucrose and 0.25% Gelrite (Sigma Chemical Co., St. Louis, MO). All media were made with Millipore™ (Bedford, MA) filtered, distilled water and were autoclaved at 121°C, 125 kPa, for 18–23 min (depending on the volume). At all times, filter-sterilized silver nitrate was added to medium after autoclaving. Culture plates were wrapped with micropore tape (Vallen Safety Supply Co., Irving, TX) and incubated for 2 wk at 28°C in the dark. Thereafter, developing calluses were subcultured weekly to the same medium and wrapped with Parafilm (Fisher, Itasca, IL). Transformable callus was visually identified using an Olympus SZ-4045 dissecting scope (Leeds Precision Instruments, Minneapolis, MN).

Callus lines were maintained based on individual zygotic embryo origin, an approach adopted by Pescitelli and Sukhapingda (1995). This allowed careful tracking of age, origin and distinct morphological characteristics of each plate of bombarded callus.

**Bombardment.** Callus lines used in bombardment ranged in age from 2 to 6 mo from the date of initiation. The maximum duration of bombardments for any single callus line was 6 wk. Thirty callus pieces (2–3 mm diameter) from a callus line 4–7 d after subculture were evenly distributed within a 3.5-cm diameter target area in the middle of a plate of osmotic medium 4 h prior to bombardment. Osmotic medium was identical to the callus initiation medium but contained either 12% sucrose (Dunder et al., 1995) or 0.2 M sorbitol and 0.2 M mannitol (Vain et al., 1993), and reduced proline (6 mM). Transformation was carried out with the PDS 1000/He biolistic gun (Bio-Rad, Hercules, CA) using the following parameters: 650 psi rupture disk pressure; 6 cm target distance (from middle of launch assembly to target plate); 6 mm gap; 1.2 cm from macro-carrier to stopping plate; and 28 Torr vacuum at rupture. In most bombardment experiments, a 150  $\mu\text{m}$  screen (McMaster-Carr, Elmhurst, IL) was inserted between the target tissue and the launch assembly (Gordon-Kamm et al., 1990; Wilson et al., 1995). All hardware and disposables for the biolistic gun were obtained from Bio-Rad. Callus pieces were transferred off the osmotic medium to callus initiation medium 1 h after bombardment. Culture plates were wrapped with micropore tape, and maintained at 28°C in the dark.

**Gold particle preparation and coating with DNA.** Gold particles were washed and coated according to Klein et al. (1988) and Sanford et al. (1993) with some variations, as follows:

- **Gold washing.** Fifteen milligrams of gold particles (1.0  $\mu\text{m}$  or 0.6  $\mu\text{m}$ ) was washed with 500  $\mu\text{l}$  cold, 100% ethanol in a 1.5 ml siliconized Treff tube (Tekmar Co., Cincinnati, OH) by sonicating for 15 s in an 18 oz ultrasonic cleaner water bath (Fisher). The particles were allowed to settle for up to 30 min and then centrifuged for 60 s at 3000 rpm. The supernatant was discarded. The pellet was washed three times with 1 ml cold, sterile distilled water, each time disturbing the pellet only slightly by finger-vortexing the tube, allowing the gold to settle, and centrifuging at 3000 rpm for 60 s. After removing the final rinse, 250  $\mu\text{l}$  of sterile water was added to the gold pellet, and the gold suspension was ultra-sonicated for 15 s. Fifty microliters of the gold suspension was aliquoted to each of five sterile Treff™ tubes. These

samples, each designated as a 1X gold aliquot (3 mg gold particles in 50  $\mu$ l water), were frozen ( $-20^{\circ}\text{C}$ ) and used within 2 mo.

• *Coating the gold with DNA.* One hour prior to bombardment, a 1X aliquot of sterile gold particles (3 mg) was thawed and sonicated for 15 s. Between 0.2–2.0  $\mu$ g of the selectable-marker construct, pBARI84(-), and gene of interest (GOI) construct were added per 1X gold tube such that the final concentration of the GOI construct was 2–3-fold higher than that of the selectable-marker construct. After finger-vortexing, the sample was continuously mixed on the vortexer while 50  $\mu$ l of  $\text{CaCl}_2$  (2.5 M) was added followed by 20  $\mu$ l of spermidine (0.1 M). After 5–10 min of continuous mixing, the gold was allowed to settle and the sample was centrifuged at 5000 rpm for 15 s. The supernatant was discarded, and the pellet was completely re-suspended in 250  $\mu$ l of cold, 100% ethanol by gently rocking the tube. The gold was again allowed to settle before the sample was centrifuged (5000 rpm, 15 s). After the supernatant was discarded, the pellet was re-suspended in 120  $\mu$ l cold, 100% ethanol. Ten microliters of the DNA-coated gold was pipetted onto each macro-carrier while the suspension was continuously shaken. Prepared macro-carriers were placed in sterile petri plates over a bed of indicator Drierite (Fisher) to maintain maximum dryness prior to bombardment. Up to nine plates were bombarded from each 1X tube of DNA-coated gold particles. Each plate of callus was bombarded once.

*Selection of transformed callus.* Selection on 2 mg  $\text{l}^{-1}$  bialaphos (Shinyo Sangyo Co., Tokyo, Japan), as described by Spencer et al. (1990), was begun 7–10 d after bombardment when callus pieces had at least tripled in diameter. Selection medium was similar to that used for callus initiation but without proline and casein hydrolysate and with 10  $\mu\text{M}$   $\text{AgNO}_3$ . Filter-sterilized bialaphos was added to the medium after autoclaving. Callus pieces were transferred every 2 wk to fresh selection media. After three or four subcultures, putative transformation events (bialaphos-resistant clones) were distinguishable as clusters of white, rapidly growing callus emerging from otherwise nongrowing, slightly brown callus. After an individual clone was identified, it was transferred to fresh selection media and the callus clump from which it originated was crossed out on the plate bottom to ensure that a single transformation event was picked only once. No effort was made to recover more than one transformation event per bombarded callus piece. Individual clones were subcultured two more times on selection media containing 2 mg  $\text{l}^{-1}$  bialaphos before sampling for molecular analysis using polymerase chain reaction (PCR).

Transformation efficiency (%) was measured as the number of independent, bialaphos-resistant calluses recovered after 10 wk of selection on 2 mg  $\text{l}^{-1}$  bialaphos per 100 pieces of callus bombarded.

*Zygotic embryo transformation.* Hi II immature zygotic embryos were dissected embryo-axis-side down onto filter paper (Whatman No. 4, 5.5 cm) overlaying callus initiation media in a 3.5 cm grid (36–42 embryos per plate). After 4 d (Songstad et al., 1996), the embryos and filter paper were transferred to 0.2 M sorbitol + 0.2 M mannitol osmotic media (described above) and bombarded 4 h later at 6 mm gap, and 900 psi / 10 cm target or 650 psi / 6 cm target. Sixteen hours later, embryos were transferred off the filter paper, embryo-axis-side down, to callus initiation medium. Selection was begun on 2 or 3 mg  $\text{l}^{-1}$  bialaphos 7 d after bombardment, or when Type II callus formation at the base of the scutellum was visible. All media components and regeneration protocols were as described for callus. One putative clone was picked per bombarded embryo, and transformation efficiency (%) was measured as the number of independent, bialaphos-resistant calluses growing actively on 2 or 3 mg  $\text{l}^{-1}$  bialaphos, recovered per 100 immature zygotic embryos selected.

*Plant regeneration.* Transformed clones carrying the GOI were regenerated according to Armstrong and Green (1985) and McCain et al. (1988). Small pieces of Type II callus (with clearly defined somatic embryos) were transferred to MS media (Murashige and Skoog, 1962) containing no hormones, 6% sucrose, 0–3 mg  $\text{l}^{-1}$  bialaphos, and 0.3% Gelrite. In some cases, when no somatic embryos were visible, callus was first transferred to MS media with 0.25 mg  $\text{l}^{-1}$  2,4-D and 1 mg  $\text{l}^{-1}$  bialaphos ( $25^{\circ}\text{C}$ , dark) for 2 wk. This reduction in auxin level stimulated embryo formation prior to subculturing to the high sucrose maturation medium. Armstrong (1994) describes a similar step-down regeneration pathway. Throughout regeneration, all material was cultured in 100  $\times$  25 mm petri plates wrapped with micropore tape and incubated at  $25^{\circ}\text{C}$  in the dark. In our experience, the increased ventilation provided by deep-dish petri plates and micropore tape increased the rate and quality of regeneration. After 2–3 wk on high sucrose

medium, mature somatic embryos were transferred to the light (80  $\mu\text{E m}^{-2} \text{s}^{-1}$ ,  $22^{\circ}\text{C}$ , 16-h photoperiod) on MS media containing 3% sucrose and no hormones where they germinated to form plantlets with fully formed roots and shoots. One to 2 wk later, plantlets were transferred to glass vials (25  $\times$  150 mm) containing 15 ml of 2/3 strength MS solid media for further elongation.

Regeneration (%) was measured as the number of independent transgenic events successfully regenerated to plants per 100 events for which regeneration was attempted.

*Acclimatization, greenhouse care, transgenic seed recovery.* When roots were well established, plantlets in vials were transplanted into pre-wetted Redi-earth (Consumer Supply, Storm Lake, IA) in small pots (2 in<sup>2</sup>) in the growth chamber (300  $\mu\text{E m}^{-2} \text{s}^{-1}$ ,  $22^{\circ}\text{C}$ , 16-h photoperiod). Roots were rinsed with water prior to transplant. Flats were initially covered with a transparent plastic lid, or Humi-dome™ (Hummert International, Earth City, MO) and fertilized with liquid Peter's (20-20-20) (Hummert International) 1 wk later. After one more week of hardening in the growth chamber, plants were moved to the greenhouse and transplanted 1 wk later to 2-gallon pots containing Universal Mix (Consumer Supply) and Sierra (17-6-12) granular fertilizer (Hummerts International). A second application of Sierra was added at the 5–7 leaf plant stage. At transplant, soil was drenched and then left for up to 10 d before watering was recommenced to coincide with renewed growth by the established plants. In our experience, thorough but infrequent watering during the early stages of plant development favored good root development in mature plants. Plants were grown to maturity in the greenhouse (~ one plant per 2 ft<sup>2</sup>). Seed was dried down on the plant and harvested 45 d after pollination.

Fertility (%) was measured as the number of transformation events from which more than 20 seeds were obtained (from the sum of transgenic plants comprising the event) per 100 events for which at least one  $\text{R}_0$  transgenic ear was pollinated. Each transgenic event was comprised of an average of eight plants. In general, reciprocal crosses using transgenic pollen were not done due to lack of space for recipient plants and limited male fertility in some clones.

*Selection construct.* A selection construct for use in routine co-transformation experiments was made as follows: a 2.9 kb fragment containing the ubiquitin promoter, first exon, and first intron, driving the *bar* gene with a *nos* terminator was isolated by partial digestion of pAHC25 with *EcoRI* (Christensen and Quail, 1996). This fragment was inserted into the *EcoRI* site within the chloramphenicol gene of pACYC184 (New England Biolabs, Beverly, MA) in the opposite orientation to the chloramphenicol gene to create the ubiquitin-*bar* selectable-marker plasmid, pBARI84(-). pACYC184 was chosen as the backbone of this vector because of its low homologous sequence with vectors such as pUC and pBluescript most commonly used for carrying the GOI. The plasmid was constructed by standard cloning procedures (Sambrook et al., 1989), and purified using a Qiagen Plasmid Maxi Kit (Valencia, CA).

GOI constructs co-transformed with pBARI84(-) ranged in size from 4.3 to 11.4 kb.

*Molecular analysis.* PCR analyses were performed using GOI-specific primers on DNA isolated from bialaphos-resistant callus clones. Co-transformation rate (%) was measured as the number of events in which a GOI-specific product was amplified for every 100 bialaphos-resistant clones analyzed.

*Statistical analysis.* Single factor analysis of variance (ANOVA) was carried out on experiments for which *P*-values are presented in results. Excel™ data analysis was used ( $\alpha = 0.05$ ).

## RESULTS

*Effect of gold particle size on transformation efficiency.* Target plates derived from 10 early embryogenic callus lines were bombarded with equal weights (300  $\mu\text{g}$  shot<sup>-1</sup>) of 0.6  $\mu\text{m}$  or 1.0  $\mu\text{m}$  gold particles in five separate experiments to determine the effect of gold particle size on transformation efficiency. Transformation efficiency was measured as the number of independent, bialaphos-resistant calluses recovered after 10 wk of selection on 2 mg  $\text{l}^{-1}$  bialaphos per 100 pieces of callus bombarded. The results show that the transformation efficiency

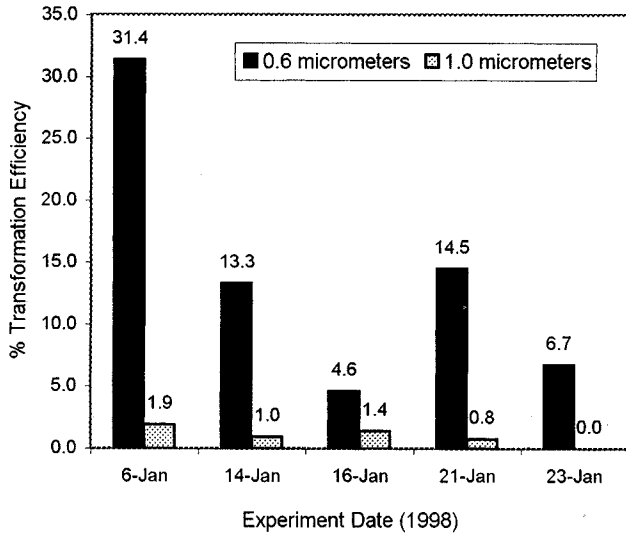


FIG. 1. Comparison of the effect of two gold particle sizes on the transformation efficiency of bombarded Type II callus. Six to 8 target plates prepared from the same callus line were bombarded with 0.6  $\mu\text{m}$  or 1.0  $\mu\text{m}$  gold particles in each of the five experiments.

using 0.6  $\mu\text{m}$  gold ranged from 4.6 to 31.4% (Fig. 1). When the same callus lines were bombarded on the same day with 1.0  $\mu\text{m}$  gold particles, transformation efficiencies ranged from 0.0 to 1.9% (Fig. 1). In one experiment, the 1.0  $\mu\text{m}$  gold particle treatment produced no transgenic clones, whereas the 0.6  $\mu\text{m}$  gold treatment produced transformants in every experiment performed. The mean transformation efficiency for experiments outlined in Fig. 1 was  $14.1 \pm 4.7\%$  for plates bombarded with 0.6  $\mu\text{m}$  and  $1.0 \pm 0.3\%$  for plates bombarded with 1.0  $\mu\text{m}$  gold particles. The mean transformation efficiency achieved with the smaller gold particle size was significantly higher ( $P = 0.02$ ) than that for the larger gold particle.

*Effect of callus morphology on transformation efficiency.* To determine the relationship between Type II callus morphology and biolistic transformation efficiency, we first categorized callus lines as having late, early, or pre-embryogenic morphology (Welter et al., 1995). Representative calluses from each group are shown in Fig. 2 (A-C) and a description of the predominant characteristics of callus lines comprising each morphology grouping is outlined in Table 1. While numerous early and late embryogenic callus lines were developed and bombarded in the course of this study, pre-embryogenic callus proved to be more difficult to develop and maintain. As a result, only two such callus lines were bombarded. These lines exhibited a high average transformation rate ( $27.7 \pm 7.7\%$ ) when compared to the transformation efficiency of

a subset of early embryogenic callus ( $9.5 \pm 5.5\%$ ) and late embryogenic callus ( $11.6 \pm 5.7\%$ ) in a replicated bombardment experiment (data not shown). However, variability was too high in this experiment to detect any significant difference between mean transformation efficiencies for the three callus morphologies ( $P = 0.20$ ). Furthermore, because so few pre-embryogenic callus lines were developed overall, we could not establish whether this transformation rate was representative of the pre-embryogenic callus morphology or simply an attribute of the two callus lines tested. To compare transformation efficiencies of two callus morphologies while controlling for variability inherent in callus lines, a single Type II callus line (initiated from one B73  $\times$  Hi II immature zygotic embryo) was serially subcultured to produce two sub-lines: one of late embryogenic callus, the other of pre-embryogenic callus. Within each of the five replicates, these sub-lines were bombarded using the same gold preparation. Results (Table 2) show that the average transformation rate of the pre-embryogenic callus sub-line was not significantly different from that of late embryogenic callus ( $P = 0.68$ ). This result also demonstrates that Type II callus from the Hi II cross to B73 can be developed and transformed at rates equivalent to those of the Hi II  $\times$  Hi II cross (Armstrong et al., 1991).

Nontransformed plants were regenerated from the three callus morphologies to test for the suitability of each as a transformation target. In agreement with Welter et al. (1995), we recovered regenerated plants from representative calluses of these three morphologies (data not shown). Early and late embryogenic callus were easily regenerated on high sucrose-containing regeneration medium. In pre-embryogenic callus, embryogenesis was first induced by lowering the 2,4-D concentration of the medium to  $0.25 \text{ mg l}^{-1}$  ( $1.1 \mu\text{M}$ ) before subculturing somatic embryos onto high sucrose-containing medium.

Transgenic callus clones were grouped by the morphology of the bombarded callus lines from which they were derived, and their rates of regeneration and fertility were compared to determine whether the morphology of bombarded callus influenced downstream efficiencies. Of 141 callus clones derived from the bombardment of 20 different late embryogenic callus lines, 78% produced plants. Likewise, 84% of 222 clones derived from the bombardment of 15 different early embryogenic callus lines produced plants (data not shown). Rates of fertility for a subset of these late and early embryogenic callus-derived clones were 75% and 83%, respectively (data not shown). Transgenic events were regenerated from only one pre-embryogenic callus line and are therefore not reported here.

*Effect of osmotic treatment on transformation efficiency.* Type II callus from the same callus lines was plated to 12% sucrose or 0.2 M mannitol + 0.2 M sorbitol (4 h pre-, 1 h post-bombardment)

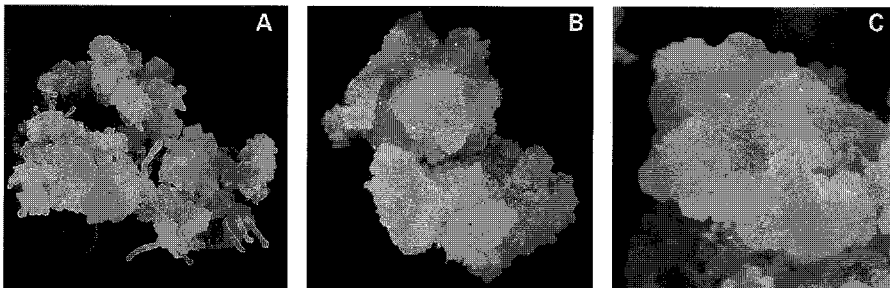


FIG. 2. Three Type II callus morphologies. Late embryogenic callus (A), early embryogenic callus (B), pre-embryogenic callus (C).

TABLE 1  
DESCRIPTION OF THREE MAIZE TYPE II CALLUS MORPHOLOGIES

Parameter	Late embryogenic	Early embryogenic	Pre-embryogenic
Color	White, light yellow	Light yellow, yellow	White, somewhat transparent
Growth rate	Fast	Fast	Very fast
Texture	Dry, very friable	Wet, grainy, friable	Dry smooth surfaces, friable, wet matrix
Overall characteristics	Heterogenous mixture of blocky, smooth-surfaced aggregates and late, Type II somatic embryos. Fluffy in appearance	Homogeneous mixture of early somatic embryos embedded in wet matrix	Homogeneous mixture of large, uniform aggregates consisting of a skin-like mantle overlaying wet matrix, no somatic embryos visible

in three separate experiments to determine which osmotic treatment resulted in the highest stable transformation efficiency. All plates in an experiment were bombarded from the same gold preparation. In two of the three experiments, callus was plated to callus initiation medium prior to bombardment as a non-osmotic control treatment. Twelve percent sucrose and 0.2 M mannitol + 0.2 M sorbitol osmotic treatments resulted in average transformation rates of  $10.4 \pm 0.9\%$  and  $12.2 \pm 0.8\%$ , respectively (Table 3). These treatments did not differ significantly ( $P = 0.57$ ). In contrast, from 10 plates bombarded with no osmotic pretreatment, one putative clone was obtained resulting in a 0.3% transformation efficiency. These results underscore the beneficial role of osmotic pretreatment in increasing stable clone recovery (Vain et al., 1993) and demonstrate that sucrose or sorbitol/mannitol osmotic medium can be effective. Based on these results, we routinely use the sorbitol/mannitol osmotic treatment in Type II callus bombardment experiments.

*Effect of target tissue and donor plant origin on average rates of transformation, regeneration and fertility.* To identify the best target tissue for our routine transformation experiments, we compared the average transformation efficiencies achieved (using 0.6  $\mu\text{m}$  gold particles) by bombarding Type II callus or immature zygotic embryo target tissues from Hi II germplasm. We further categorized results based on the origin of the plant material from which the bombarded target tissue was derived (greenhouse or field).

Type II callus initiated from greenhouse- or field-derived immature zygotic embryos transformed at rates of  $9.3 \pm 0.9\%$  and  $10.9 \pm 1.4\%$ , respectively (Table 4). In contrast, a marked

difference in efficiency was observed for bombarded immature zygotic embryos depending on whether they were harvested from field- or greenhouse-grown plants. The average transformation efficiency achieved with field-derived immature zygotic embryos was  $18.9 \pm 1.7\%$  while bombarded immature zygotic embryos harvested from the greenhouse exhibited an average transformation efficiency of  $4.0 \pm 0.9\%$ .

The regeneration rate of transgenic events derived from bombarded callus or embryos of field or greenhouse origin ranged between 78–85% (Table 4). By comparison, 20 of 23 (87%) non-transformed callus lines regenerated over the course of the study produced plants (data not shown).

Average seed set per transgenic event (comprised of an average of eight plants) was 500 kernels, or 60 kernels per transgenic ear. Clones originating from bombardments of greenhouse-derived embryos exhibited a 70% fertility rate while 83% of transgenic events derived from bombarded field embryos were fertile (Table 4). Under the greenhouse conditions described, we have observed consistently better plant vigor for transgenic events derived from field embryo bombardments than other target tissues. For example, close to 100% of transgenic events originating from field embryo bombardments shed pollen, whereas little or no pollen shed was common in events originating from callus or greenhouse embryo bombardments.

The fertility of transgenic events derived from bombarded callus did not differ based on the origin of the embryo explant from which the callus was initiated (Table 4). Furthermore, we could distinguish no clear relationship between fertility and the age of a callus line at bombardment. No single callus line was bombarded for longer than 6 wk; therefore a meaningful time line for individual callus lines was not established. Within the 6-mo age limit we imposed on bombarded callus lines, our observations were that clone infertility was more often associated with callus line than callus age.

TABLE 2

COMPARISON OF THE TRANSFORMATION EFFICIENCY OF PRE-EMBRYOGENIC AND LATE EMBRYOGENIC CALLUS SUB-LINES DEVELOPED FROM THE SAME IMMATURE ZYGOTIC EMBRYO

Callus morphology	Transformation efficiency (%) <sup>a</sup>					
	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Mean $\pm$ SE <sup>b</sup>
Pre-embryogenic	16.7	28.0	6.3	7.3	7.1	13.0 $\pm$ 4.4
Late embryogenic	5.4	26.0	14.0	3.2	3.6	10.5 $\pm$ 4.2

<sup>a</sup>All experiments with 0.6  $\mu\text{m}$  gold particles.

<sup>b</sup>Standard error of the mean transformation efficiency over five experiments. In each experiment, 2–4 plates of each callus morphology were bombarded from the same gold tube. Both callus sub-lines were derived from the same greenhouse-derived immature zygotic embryo of a B73  $\times$  Hi II cross.

## DISCUSSION

*Effect of gold particle size on transformation efficiency.* Our study shows that bombardment of Type II callus using 0.6  $\mu\text{m}$  gold particles results in a higher stable transformation efficiency than bombardment using 1.0  $\mu\text{m}$  gold particles. This is in agreement with Type I callus results reported by Randolph-Anderson et al. (1995). These authors measured an increase in the number of anthocyanin-expressing sectors in unselected Type I maize callus after bombardment with 0.6  $\mu\text{m}$  gold particles relative to bombardment with 1.0  $\mu\text{m}$  gold particles. Our results apply to

TABLE 3

## EFFECT OF OSMOTIC TREATMENT ON TRANSFORMATION EFFICIENCY OF BOMBARDED TYPE II CALLUS

Osmotic treatment	Total no. callus pieces bombarded	Transformation efficiency (%) <sup>a</sup>			
		Exp 1	Exp 2	Exp 3	Mean $\pm$ SE <sup>b</sup>
12% sucrose	499	11.6	8.6	11.0	10.4 $\pm$ 0.9
0.2 M sorbitol + 0.2 M mannitol	510	12.9	10.6	13.3	12.2 $\pm$ 0.8
None	332	NT	0.6	0.0	0.3 $\pm$ 0.3

<sup>a</sup>All experiments with 0.6  $\mu$ m gold particles.

<sup>b</sup>SE is standard error of the mean transformation efficiency of three experiments. In each experiment, 4–8 plates were bombarded for each osmotic treatment, three or four plates for the nonosmotic treatment.

NT = not tested.

TABLE 4

## EFFECT OF TARGET TISSUE AND DONOR PLANT ORIGIN ON TRANSFORMATION EFFICIENCY AND THE REGENERATION AND FERTILITY OF TRANSFORMATION EVENTS

Target tissue	Donor plant origin	No. embryos or callus pieces assessed	Mean transformation efficiency (%) $\pm$ SE <sup>a</sup>	No. events to regeneration	Regeneration (%)	No. events crossed as female	Female fertile events (%)
Embryo	Greenhouse	1020	4.0 $\pm$ 0.9	49	78.0	20	70.0
	Field	1568	18.9 $\pm$ 1.7	27	81.0	18	83.0
Callus	Greenhouse	11 540	9.3 $\pm$ 0.9	314	81.0	26	77.0
	Field	4022	10.9 $\pm$ 1.4	168	85.0	20	80.0

<sup>a</sup>All experiments used 0.6  $\mu$ m gold particles. Summary of results from experiments between December 1996 and September 1998 using Hi II germplasm and pBAR184(-) as selection construct. SE is standard error of the mean transformation efficiency. For greenhouse embryos,  $n = 9$  (19 ears); for field embryos,  $n = 2$  (33 ears); for greenhouse callus,  $n = 56$ ; and field callus,  $n = 21$ ; where  $n =$  the number of independent experiments performed.

Type II callus clones recovered from selection on bialaphos. Similarly, we have seen a favorable effect of reducing gold particle size from 1.0  $\mu$ m to 0.6  $\mu$ m on stable clone recovery from bombarded immature zygotic embryos (data not shown). This result has been observed by other research groups (Mark Galatowitsch, personal communication).

This beneficial effect of reduced particle size on transformation efficiency is likely attributable to less sustained damage being inflicted on bombarded cells by the smaller particles than the larger ones. Maize cells bombarded with 0.6  $\mu$ m gold showed less damage than those bombarded with 1.0  $\mu$ m gold particles (Randolph-Anderson et al., 1995) and resulted in an increase in the number of anthocyanin-expressing stable sectors observed. Importantly, this increase was observed regardless whether equal weights or equal particle numbers of the two gold particle sizes were bombarded. In our experiments, transient *gus* expression in four callus lines bombarded with equal weights of 0.6  $\mu$ m or 1.0  $\mu$ m gold particles was three-fold higher in cells bombarded with the smaller gold particles (data not shown). This might be expected given that a fixed weight of 0.6  $\mu$ m gold particles contains approximately five-fold more particles than an equivalent weight of 1.0  $\mu$ m gold particles (Randolph-Anderson et al., 1995). However, in experiments where we have increased the number of 1.0  $\mu$ m gold particles used per bombardment, we have seen a decrease rather than an increase in stable clone recovery (data not shown). Conversely, in preliminary experiments where we have decreased 0.6  $\mu$ m gold particle concentration per bombardment five-fold, we have either measured no decrease (immature zygotic embryos) in transformation efficiency compared to the undiluted control treatment or a one-fold

drop (Type II callus) in transformation efficiency (data not shown). Similarly, scutellar tissues of H99 immature zygotic embryos bombarded at different dilutions of the same (0.4–1.2  $\mu$ m) gold particle solution showed a significantly greater number of embryos undergoing somatic embryogenesis when fewer gold particles were used in bombardment (Brettschneider et al., 1997). In these experiments, treatments which favored the post-bombardment recovery of targeted tissue also led to higher stable transformation rates. These data support the argument that particle size and not increased particle number is primarily responsible for the increase in stable clone recovery measured here, and that reducing particle size serves to minimize damage to targeted cells.

The average callus transformation rate achieved using the improved protocol described here is three clones per bombarded plate, or 10 clones per 100 callus pieces bombarded (Fig. 3). This marks a 3.5-fold improvement in efficiency when compared to the highest average efficiency achieved in our laboratory using 1.0  $\mu$ m gold particles (data not shown). Furthermore, this increased efficiency has been achieved with no detrimental effect on the subsequent regenerability or fertility of recovered clones, as is the case, for example, when the biolistic transformation rate of a cell or callus line improves with age (Wilson et al., 1995; Pareddy et al., 1997).

*Effect of callus morphology on transformation efficiency.* We have demonstrated that late, early and pre-embryogenic Type II callus, as described by Welter et al. (1995) and outlined here, can be transformed using the PDS 1000/He biolistic gun. Furthermore, no significant effect of callus morphology on transformation efficiency could be demonstrated based on our results. Pareddy et al. (1997), using helium blasting for Type II callus transformation,

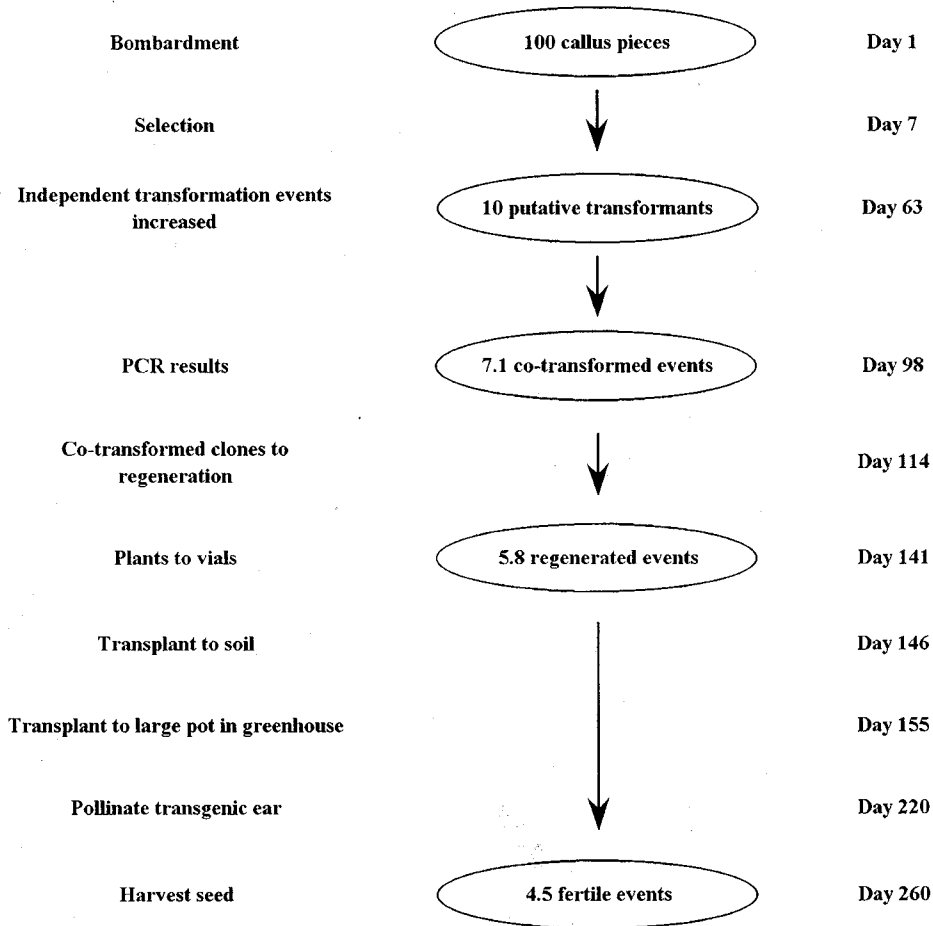


FIG. 3. Efficiency cascade and time elapsed (d) for Type II callus transformation using the PDS 1000/He gene gun and 0.6  $\mu\text{m}$  gold particles.

also claimed that no clear correlation between callus morphology and transformability could be established. In agreement with Welter et al. (1995), we found pre-embryogenic callus cultures more difficult to develop and maintain at optimum quality than either early or late embryogenic callus. These results and observations suggest that pre-embryogenic callus is not a preferred target for bombardment using this PDS 1000/He gene gun protocol. Furthermore, because we found that early and late embryogenic callus could be transformed with similar efficiency, we no longer differentiate between these two morphologies in developing callus lines for transformation. In our experience, if Type II callus was friable, light yellow or white and fast-growing, it transformed well. In contrast, Type II callus that was excessively dry, slow-growing, or lacked friability transformed poorly.

*Clone production cascade.* Type II callus transformation efficiency using this improved protocol was 10% in experiments in which 25 constructs were co-bombarded with pBAR184(-) over a 21-mo. period (Fig. 3, data not shown). As illustrated in Fig. 3, 71% of the bialaphos-resistant clones recovered from selection were co-transformed and of these, 82% were regenerable. Female fertility of regenerated events was 78%. Overall, for every 10 transgenic events recovered from selection, 4.5 set seed. The time elapsed from bombardment to the recovery of transgenic seed was approximately 260 d. These results expand upon the precedent literature for Type

II callus biolistic transformation by outlining the efficiency cascade of clone production beginning with co-transformation rates through to the average rate of fertility achieved for transgenic events. Summarized in this way, these results offer an example of a production framework upon which initial output goals can be based should this protocol be implemented for research purposes.

Finally, we have demonstrated that bombarding immature zygotic embryos derived from field-grown plants resulted in a higher efficiency of transformation than that achieved for our greenhouse-derived immature zygotic embryos. In the absence of field-derived embryo explants, and in the greenhouse conditions under which this study was conducted, Type II callus provided a more reliable target tissue than greenhouse-derived embryo explants. In our experience, greenhouse-grown immature zygotic embryos have shown a highly variable response in bombardment experiments, with transformation rates ranging from 11% to close to zero (data not shown). In contrast, we have achieved consistent, high transformation rates of field embryos over two field seasons. The relatively poor transformation rate of our greenhouse embryos is likely attributable to the sub-optimal conditions in which the source plants are grown. Increasing the number and quality of light fixtures per square foot in the greenhouse to improve light intensity and distribution may favor greenhouse embryo transformation rates. For example, our average transformation rate for greenhouse

embryos harvested in late March through June is four-fold higher than that achieved from October through December (data not shown). This suggests that variability in transformation rate may be associated with reduced source-plant vigor as supplementation of the greenhouse lighting by ambient sunlight decreases with shortening day lengths in the fall and early winter. In addition, we have observed delayed flowering of transgenic and non-transgenic plants grown in a subsection of our greenhouse that is consistently cooler than the main greenhouse. Maintaining constant optimum temperatures to help promote timely flowering of source plants may also increase the vigor of our greenhouse-derived embryos. Finally, we have observed distinct differences in the volume of root mass developed by donor plants grown under different soil/fertility regimes. Liquid fertilizer (and the potential over-watering regime dictated by it), in conjunction with the use of a relatively dense 'house-mix' soil for potting resulted in mature plants with visibly reduced root mass when compared with plants grown under the soil/fertility regime outlined in Materials and Methods. Plants with reduced root mass underwent a daily wilting cycle even when their soil was drenched. These observations emphasize the importance of providing well-aerated soil and not over-watering plants during greenhouse plant development. Improving or further optimizing greenhouse environmental conditions such as these could help reduce the difference between transformation rates of field and greenhouse zygotic embryos reported here.

High-efficiency *Agrobacterium*-mediated transformation of maize has recently been demonstrated (Ishida et al., 1996; Zhao et al., 1998) although its routine application has not yet been widely reported. This biological transformation technology appears to favor the recovery of a higher proportion of single or low-copy number events in comparison to events recovered from maize biolistic transformation (Zhao et al., 1998). Such an attribute is particularly beneficial should low-copy number events lead to greater stability of transgene expression in progeny (Gordon-Kamm et al., 1999). Multiple copies of the transgene are common in transformation events produced using the biolistic gun (Register et al., 1994; Kohli et al., 1998; Zhao et al., 1998). In our experience, screening clones at the callus stage for copy number or transgene expression (where feasible), and regenerating a desired subset of transgenic events has helped overcome this limitation.

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