

Chapter 3

Biolistic Gun-Mediated Maize Genetic Transformation

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Summary

Biolistic gun-mediated transformation is one of the two most effective and popular methods for introducing genes into maize. In this chapter, we describe a detailed protocol for genetic transformation of the maize genotype, Hi II. The protocol uses 0.6- μm gold particles as microcarriers and the herbicide resistance *bar* gene as a selective marker. Both immature zygotic embryos and immature embryo-derived callus cultures can be transformed using this protocol. To ensure successful reproduction of this protocol, we provide step-by-step laboratory transformation procedures as well as details on growing and caring for transgenic plants in the greenhouse.

Keywords: *Zea mays*, Maize, Genetic transformation, Biolistic gun

1. Introduction

Biolistic gun (or particle gun)-mediated DNA delivery was the first successful method used in producing fertile transgenic maize plants (1). “Biolistic” is a generic term for microparticle bombardment and derives from biological ballistic (2). Currently, microprojectile bombardment is among the most reliable and efficient direct DNA delivery systems for monocots. The original design of the biolistic gun used a gunpowder charge to propel the DNA-coated tungsten particles (microparticles) through a vacuum chamber into target cells (3). The current biolistic device (Biolistics PDS-1000/He particle delivery system) uses DNA-coated gold particles driven by helium gas (4).

According to BioRad PDS-1000/He manual (http://140.226.65.22/Davis_lab/Helminth_Protocols/PDS_1000_Manual.pdf), “The Biolistic PDS-1000/He system uses high pressure helium,

released by a rupture disk, and partial vacuum to propel a macrocarrier sheet loaded with millions of microscopic tungsten or gold microcarriers toward target cells at high velocity. The microcarriers are coated with DNA or other biological material for transformation. The macrocarrier is halted after a short distance by a stopping screen. The DNA-coated microcarriers continue traveling toward the target to penetrate and transform the cells.”

The velocity at which the “DNA bullets” (microcarriers) strike the target cells can be adjusted by (1) changing the helium pressure (psi of chosen rupture disk), (2) the amount of vacuum in the bombardment chamber, (3) the distance from the rupture disk to the macrocarrier, (4) the macrocarrier travel distance to the stopping screen, and (5) the distance between the stopping screen and target cells.

These parameter choices are critical to the success of gene delivery for recovering stable transgenic events. Typically, researchers use transient expression of a visual marker gene (such as the *gus* or *gfp* gene) for optimizing these conditions. Although transient assays can serve as an indicator of whether DNA delivery has occurred, they cannot be the only criteria used for optimization of stable transformation protocols.

Other particle acceleration methods based on similar concepts have also been developed. The ACCELL™ system uses an electrical impulse to accelerate the particles. The greater control of particle penetration by this device makes it a versatile tool for delivering DNA to a variety of crops, genotypes, and tissues (5). The particle inflow gun (PIG) (6, 7) accelerates DNA-coated particles with a gentle burst of gas without the use of a macrocarrier, thus having the advantage of being less damaging to the target.

In establishing a protocol for maize transformation, the following factors should be considered: effectiveness of gene delivery methods, competence of targeted plant materials, and robustness of the selection systems used for recovery of transformed events. While the biolistic delivery method possesses obvious advantages for its ability to penetrate walled plant cells readily, thereby significantly expanding the range of transformation-competent plant tissues, its major disadvantage is that it generates a high frequency of multiple transgene insertion copies in the plant genome. Compared with low copy number insertion events, high copy number insertion events are prone to multisequence-induced gene silencing (8) and have been shown to cause lower stable transgene expression over generations (9). On the other hand, because the biolistic delivery method offers greater flexibility in choice of plant target materials, it remains an efficient and powerful transformation method for many recalcitrant plant species.

The maize biolistic transformation method described here is based on Songstad et al.'s protocol of 1996 (10) and our previously published protocol in 2000 (11). Our current average transformation frequency is 18% (transformation frequency is defined as the number of independent bialaphos-resistant calli per 100 bombarded embryos). Typically, bialaphos-resistant callus events can be identified 2–3 months after the day of bombardment experiment. Transgenic plantlets are regenerated 1–2 months later and seeds can be harvested in another 2–3 months. The total duration of this protocol (the day of bombardment to seed harvest) is about 8.5 months.

2. Materials

2.1. DNA Constructs

A typical DNA construct used in our biolistic-mediated maize transformation system contains the *bar* gene selectable marker cassette, which confers resistance to phosphinothrycin, the active ingredient in bialaphos (12). We have used both the double 35S CaMV promoter (13) in construct pTF101.1 (14) and the maize ubiquitin promoter (15) in construct pBar184 (11) to drive the *bar* gene.

2.2. Plant Material

1. Maize Hi II F1 seeds (*see Note 1*): Ears of the maize Hi II genotype [A188 × B73 origin (16) harvested from green house grown embryo donor plants 9–12 days after pollination].
2. Maize B73 seeds (*see Note 1*): as pollen donor plant.

2.3. Stock Solutions

1. N6 Vitamin stock (1,000×): 1.0 g/L thiamine HCl, 0.5 g/L pyridoxine HCl, 0.5 g/L nicotinic acid, 2.0 g/L glycine. Store in 50 mL/L aliquots in Falcon tubes at –20°C. Thaw one tube at a time and store at 4°C.
2. MS Vitamin (modified) Stock (1,000×): 0.5 g/L thiamine HCl, 0.5 g/L pyridoxine HCl, 0.05 g/L nicotinic acid, 2.0 g/L glycine. Store 50 mL/L aliquots in Falcon tubes at –20°C. Thaw one tube at a time and store at 4°C.
3. 2,4-D, that is, (2,4-dichlorophenoxy acetic acid) stock: Weigh 0.25 g/L 2,4-D in a fume hood, and dissolve in 1 N KOH (10 mL/L) on low heat. When dissolved, bring up to 250 mL/L final volume with ddH₂O water. Store at 4°C in Duran bottle.
4. Silver nitrate stock: Dissolve 0.85 g/L silver nitrate (Fisher) in 100 mL/L of ddH₂O. Filter-sterilize the stock solution (50 mM) and store at 4°C for up to 1 year in a foil-wrapped container to avoid exposure to the light.

5. Bialaphos stock: Dissolve 100 mg of bialaphos (Duchefa Plant Biotechnology Products, *see Note 2*) in 100-mL/L ddH₂O. Filter-sterilized stock solution (1 mg/mL) is stored at 4°C for up to 4 months in 50-mL/L Falcon tubes.
6. Glufosinate stock: Dissolve 100 mg of glufosinate ammonia (Sigma, Cat. # D5417) in 100 mL of ddH₂O. Stock solution (1 mg/mL) is filter sterilized and stored in 50-mL Falcon tubes at 4°C.

2.4. Culture Media (*see Notes 3 and 4*)

1. Callus initiation (N6E) medium: 4 g/L N6 salts [Phytotech Labs; (17)], 1 mL/L N6 vitamin stock, 2 mL/L 2,4-D, 2.8 g/L L-proline, 30 g/L sucrose, 100 mg/L casein hydrolysate, 100 mg/L myo-inositol, 2.5 g/L gelrite; adjust pH to 5.8 using 1 N KOH. Silver nitrate stock (0.5 ml/L for a final concentration of 25 µM) is added after autoclaving.
2. Osmotic (N6OSM) medium: 4 g/L N6 salts, 1 mL/L N6 vitamin stock, 2 mL/L 2,4-D, 0.7 g/L L-proline, 30 g/L sucrose, 100 mg/L casein hydrolysate, 100 mg/L myo-inositol, 36.4 g/L sorbitol, 36.4 g/L mannitol, 2.5 g/L gelrite; adjust pH to 5.8 using 1 N KOH. Silver nitrate stock (0.5 ml/L for a final concentration of 25 µM) is added after autoclaving.
3. Selection (N6S) medium: 4 g/L N6 salts, 1 mL/L N6 vitamin stock, 2 mL/L 2,4-D, 100 mg/L myo-inositol, 30 g/L sucrose, 2.5 g/L gelrite; adjust pH to 5.8 using 1 N KOH. Bialaphos (2 mL/L) and silver nitrate stock (0.1 ml/L for a final concentration of 5 µM) are added after autoclaving.
4. Regeneration medium I: 4.3 g/L MS salts [Phytotech Labs; (18)], 1 mL/L modified MS vitamin stock, 100 mg/L myo-inositol, 0.25 mL/L 2,4-D, 30 g/L sucrose, 3 g/L gelrite; adjust pH to 5.8 using 1 N KOH. Bialaphos (2 ml/L) is added after autoclaving when medium is cooled
5. Regeneration medium II: 4.3 g/L MS salts (Phytotech Labs), 1 ml/L modified MS vitamin stock, 100 mg/L myo-inositol, 60 g/L sucrose, 3 g/L gelrite; adjust pH to 5.8 using 1 N KOH. Filter-sterilized glufosinate ammonia (6 ml/L) is added after autoclaving.
6. Regeneration medium III: 4.3 g/L MS salts, 1 mL/L modified MS vitamin stock, 100 mg/L myo-inositol, 30 g/L sucrose, 3 g/L gelrite; adjust pH to 5.8 using 1 N KOH.

2.5. Supplies for Biolistic Bombardment

1. 0.6-µm Gold particles (Bio-Rad, Hercules, CA).
2. Macrocarrier holders (Bio-Rad, Hercules, CA).
3. Macrocarriers (Bio-Rad, Hercules, CA).
4. 150-µm mesh screen (McMaster Carr, Elmhurst IL).

5. 100% Ethanol.
6. 2.5 M CaCl₂, filter-sterilized, 4°C.
7. 0.1 M Spermidine, free-base, filter-sterilized, aliquoted, and frozen at -20°C.
8. Ultrasonicator (Fisher, 18 oz. FS6 single channel sonic cleaner).
9. Vortex Genie 2 (Fisher).

2.6. Other Supplies and Reagents

1. Sterilizing solution: 50% commercial bleach (5.25% hypochlorite) and one drop of surfactant Tween 20 per liter.
2. Redi-Earth (Hummert, 4500 Earth City Expressway, Earth City, MO 63045, USA; Cat. # 10-2030-1).
3. Sunshine Universal Mix SB300 (Fosters Inc., P.O. Box 2674, Waterloo, IA 50704, USA).
4. Greenhouse flat with drainage holes (holds 32 small pots) (Hummert, Cat. # 11-3000-1).
5. Small pot (6.4 cm² each in four packs) (Hummert, Cat. # 11-0300-1).
6. Humi-dome (plastic, transparent) (Hummert, Cat. # 14-3850-1).
7. Large pot for Hi II (2-gal nursery pot with four drainage holes) (Hummert, Cat. # 14-9606-1).
8. Large pot for B73 pollen donor plants (3-gal nursery pot with four drainage holes) (Hummert, Cat. # 14-9612-1).
9. Osmocote Plus 16-8-12 (controlled release fertilizer tablets with trace elements) (Hummert, Cat. # 07-6450-1).
10. Calcium/magnesium solution (Dr. C. Block, USDA-ARS, North Central Regional Plant Introduction Station, Ames, IA, USA): First make two separate stock solutions as follows:
 - (a) Stock #1: 720 g/L of Ca(NO₃)₂·4H₂O.
 - (b) Stock #2: 370 g/L of MgSO₄·7H₂O (Epsom salts).
 - (c) To prepare working solution, add 5 mL of each Stock #1 and Stock #2 into 1 gal (~4 L) of H₂O (*see Note 5*).
11. Miracle Gro Excel 15-5-15 (water-soluble fertilizer supplemented with calcium and magnesium Hummert, Cat. # 07-5660-1).
12. Marathon® (restricted use pesticide for aphid control) (Hummert Cat. # 01-1118-1).
13. Whitemire yellow monitoring cards (for fungus gnat control) (Hummert Cat. # 01-2700-1).

14. Shoot bags (Lawson Bags, 318 Happ road, P.O. Box 8577, Northfield, IL 60093, Cat. # 217).
15. Striped (red or green) tassel bags (Lawson, Cat. # 404).
16. Plain (brown) tassel bags (Lawson, Cat. # 404).
17. Vent tape (1 in.) (Fisher, Cat. # 19-027-761).
18. Filter paper (Whatman No. 4, 5.5 cm) (Fisher, Cat. # 09-825-K).
19. Drierite (8 mesh indicating) (Fisher, Cat. # 07-578-3B).

3. Methods

3.1. Preparing Plant Materials for Biolistic Gun Transformation

3.1.1. Embryo Dissection

Both immature zygotic embryos (IZE) and IZE-derived embryogenic callus cultures from Hi II germplasm can be used for biolistic transformation.

1. Maize ears are harvested 9 (summer) to 12 (winter) days after pollination in the greenhouse. The ideal size of immature embryo for transformation should be between 1.2 and 2 mm (*see Note 6*).
2. Dehusk ear. Cut off and discard top 1 cm of ear and insert a straight-nosed forceps into the top end of the ear. This “handle” facilitates aseptic handling of the cob during embryo dissection. Place impaled ear and forceps into a sterilized mason jar in a laminar flow bench. If necessary, sterilize up to four ears in one mason jar.
3. Add ~700 mL of sterilizing solution to cover ear. During the 15–20 min disinfection, occasionally swirl the ears and tap the mason jar on the surface of the flow bench to dislodge air bubbles for thorough surface sterilization of ear. Holding on to the forceps, pour off bleach solution and rinse the ears three times in generous amounts of sterilized water. The final rinse is drained off and the ears are ready for embryo dissection (*see Notes 7 and 8*).
4. In a large (150 mm × 15 mm) sterile Petri plate, cut off the kernel crowns (the top 1–2 mm) with a sharp scalpel blade. Use sterilizing ovens for intermittent resterilizing of utensils throughout this protocol (*see Note 9*).
5. Excise the embryos by inserting the narrow end of a sharpened spatula between the endosperm and pericarp at the basipetal side of the kernel (toward the bottom of the cob) popping the endosperm out of the seed coat. This exposes the untouched embryo, which sits at the top side of the kernel, close to the kernel base. The embryo is

gently coaxed onto the spatula tip and plated with the embryo-axis side down (scutellum side up) onto a filter paper overlaying the N6E media in a 2 cm × 2 cm grid (30 embryos/plate).

6. Wrap the plate with a vent tape and incubate at 28°C in the dark for 2 or 3 days before bombardment, whichever is most convenient.
7. Alternatively, these embryos can also be kept in 28°C in the dark for 2 weeks to initiate Type II callus.

3.1.2. Type II Callus Initiation

1. After 2 weeks, friable, rapidly growing embryogenic callus can be seen proliferating from the embryo scutellar tissue of ~100% of the IZE explants. This tissue is subcultured to fresh N6E medium, and plates are wrapped with parafilm (28°C, dark).
2. Callus lines, each originating from an independent IZE source are developed for bombardment over a 6–8-week period by weekly subculturing of this material to fresh N6E medium (*see Note 10*).

3.2. Gold Particle Preparation

3.2.1. Washing Gold

1. Weigh 15-mg gold (0.6 μm) particles and transfer to sterile, 1.5 mL microcentrifuge tubes. These tubes are considered 10× gold quantities (*see Note 11*).
2. In the laminar flow hood, add 500 μL 100% ethanol, straight from the freezer, to each tube of 15-mg gold and sonicate in an ultrasonic water bath for 15 s. Tap the closed tube on the bench to gather all droplets to the tube bottom and let the tube sit until all the particles have settled out (up to 30 min).
3. Spin in a tabletop centrifuge for 60 s at 3,000 rpm, and remove the ethanol supernatant (keep teardrop-shaped pellet facing down or it will fall into the pipet).
4. To rinse, add 1 mL ice cold, sterile ddH₂O by dribbling the water down the side of the microcentrifuge tube. Slightly disturb the pellet by finger vortexing, and then let the gold settle out again.
5. Spin at 3,000 rpm for 60 s. Repeat the rinse step two more times, the third time centrifuging at 5,000 rpm for 15 s.
6. After removing the final wash, suspend the pellet in 500 μL sterile water.
7. Ultrasonicate this suspension for 15 s, then immediately place the tube on a vortex to keep it shaking as rapidly as possible (vortex setting of 3).
8. Leave the tube shaking at this setting while you open it and aliquot the washed gold for storage.

3.2.2. Aliquoting Gold to 1× Tubes

1. For each 10× tube of washed gold, set out ten, 1.5 mL microcentrifuge tubes in a microcentrifuge tube rack.
2. While the 10× tube is shaking, aliquot 250 μL of the gold suspension to each of the 10 tubes. Then, beginning with the last tube, start backward, aliquoting another 250 μL of the gold suspension to each tube.
3. When finished, each “1×” tube of gold should contain 1.5-mg gold in 50 μL water. Label the top of each tube as 1×, close, and freeze (−20°C) until use.

3.2.3. Coating the Gold with DNA

1. On bombardment day, leave the gold in the freezer until just before you begin the gold coating procedure. *Briefly*, thaw one, 1× tube of gold for 8–12 plates to be bombarded.
2. Ultrasonicate the tube for 15 s.
3. In the flow bench, add, in sequence, the appropriate quantities of selection construct and GOI construct (*see Note 12*), finger-vortexing the tube after each.
4. Finger-vortex the tube well and then tap it on the bench top to gather all the droplets to the bottom of the tube.
5. Add 50 μL CaCl₂. Using the same pipet tip, gently suck the suspension up and down once, then place the tube on the vortex at low speed (setting 2–3 shaking on Vortex Genie 2).
6. Add 20 μL spermidine while the tube is still shaking on the vortex. Wait 30 s, close the tube, and finger-vortex well. Return the tube to the vortex and let it shake for 10 min.
7. Remove the tube from the vortex and let the gold settle out for several minutes.
8. Centrifuge for 15 s at 5,000 rpm (just enough to pellet the gold), and then pipet off the supernatant.
9. Take the 100% ethanol from the freezer (*see Note 13*) and add 250 μL cold ethanol to the 1× pellet.
10. Finger-vortex to dislodge the pellet, and then rock the tube back and forth until the gold achieves a very “silty” smooth consistency dispersed on the base of the tube.
11. Let the tubes sit until the gold settles out (3–5 min). Centrifuge for 15 s at 5,000 rpm. Remove the supernatant and add 140 μL 100% ethanol.
12. Finger-vortex well (do not sonicate) to ensure complete suspension of the gold pellet and place on the vortex (setting 2–3) for loading the macrocarriers.

3.2.4. Loading the Macrocarriers

1. Fit presterilized macrocarriers (*see Note 14*) into their stainless steel holders in a sterile dish surrounded by indicating Drierite (*see Note 15*).
2. While the 1× tube of DNA-coated gold particles is still shaking, open the tube and aliquot 10 μL of the suspension onto the center of each macrocarrier (*see Note 16*).
3. After loading the macros, let them sit for 5–10 min to be sure that they are dry before bombardment.

3.3. Bombardment

3.3.1. Embryo Bombardment

1. After 2–3 days of preculture on N6E, the embryos will be ridged and swollen as Type II callus initiation has begun. This is an appropriate stage for bombardment.
2. Draw a 3.5-cm diameter circle on the bottom of a plate of N6OSM medium.
3. Four hours prior to bombardment, use sterile forceps to transfer the embryos and filter paper onto the N6OSM medium (*see Note 17*). Embryos should be facing scutellum side up at bombardment since it is from this surface that subsequent callus initiation begins from which transformed cells are then selected.
4. Load 650-psi rupture disk.
5. Assemble the macrocarrier launch assembly by first laying in place a stopping screen followed by an inverted, preloaded macrocarrier holder (*see steps 1–3 in Subheading 3.2.4*), which is held in place by screwing on the launch assembly lid.
6. Slide the launch assembly into place immediately below the helium nozzle. Pre-set gap distance to 6 mm.
7. Slide a sterilized 150-μm mesh screen onto the shelf directly below the launch assembly. This screen is supported on a second plexiglass stage (like the one that holds the Petri plate at bombardment) with a 3.8-cm-diameter hole cut in the middle of it (*see Note 18*).
8. Slide the opened Petri dish containing the target tissue onto the shelf at a selected distance from the stopping screen (6 cm).
9. The vacuum chamber is closed, a vacuum pulled, and the gun fired in time for the rupture disk to break as soon as the vacuum reaches 28 in. of Hg.
10. The chamber is vented, the plate containing the bombarded tissue is removed, and the gun is prepared for the next bombardment by replacing the spent rupture disk, macrocarrier, and stopping screen (disposables). All plasmid waste is disposed of in biohazard bags for autoclaving.

11. Repeat **steps 4–10** for each shot.
12. The bombarded embryos (still on filter paper on N6OSM) are gently wrapped with vent tape and incubated in 28°C in the dark.
13. The next day (16–20 h after bombardment), embryos are transferred off the filter paper on N6OSM to the surface of N6E media with no filter paper to continue callus initiation. Embryos are again oriented scutellum side up and plates are wrapped with vent tape.

3.3.2. Callus Bombardment

1. Typically, 6–8 weeks after callus initiation, friable and rapidly growing embryogenic Type II callus derived from the embryo scutellar tissue can be ready for bombardment.
2. Draw a 3.5-cm-diameter circle on the bottom of a plate of N6OSM medium. This defines the target area to which callus pieces are loaded for bombardment.
3. Four hours prior to the bombardment, use a microscope to transfer 30 callus pieces (4 mm) from a friable, rapidly growing callus line to the target area.
4. Bombardment parameters for Hi II callus are identical to those used for Hi II IZE (refer to **steps 4–11** in **Subheading 3.3.1** above).
5. The bombarded callus is left on N6OSM medium for 1 h after bombardment and then transferred to N6E medium maintaining the integrity of each bombarded piece.
6. Plates are wrapped with vent tape (28°C, dark).

3.4. Selection for Stable Transgenic Events from Bombarded Embryos or Callus

1. After 7 days on initiation medium (N6E) the bombarded embryos or callus pieces are transferred to N6S selection medium (2.0 mg/L bialaphos) to begin the recovery of transformed cells.
2. Plates are wrapped with parafilm throughout selection, and all embryo explants or callus pieces are subcultured intact throughout selection (*see Note 19*).
3. Two weeks later, embryos or callus pieces are transferred to fresh N6S. This step is repeated once more before bialaphos-resistant events are visible emerging from selected immature embryos or callus pieces (within 6–8 weeks from date of bombardment). Each of these proliferating callus clumps is considered an independent putative transgenic event (*see Note 20*).

3.5. Regeneration of Hi II Transgenic Plants

1. Regeneration of transgenic Type II callus (friable, stocked somatic embryos present) is accomplished by subculturing

about 12 pieces (10-mm diameter) of embryogenic callus (use a 40× dissecting scope) to regeneration medium I.

2. Plates are wrapped with vent tape and incubated (dark, 25°C) for 10 to 14 days after which 15 small pieces of callus (approximately 4 mm) enriched with stocked somatic embryos are subcultured (again using the scope) from callus on regeneration medium I to regeneration medium II.
3. These plates are incubated for 2–3 weeks at 25°C in the dark (*see Note 21*). Petri plates are wrapped with vent tape.
4. After this maturation period (2–3 weeks), many of the somatic embryos on regeneration medium II are opaque and white with a clearly defined scutellar region. From some, the coleoptile is already emerging. Again using a dissecting scope, transfer these mature somatic embryos (~12 pieces per plate) to the surface of regeneration medium III for germination in the light (25°C, 80–100 $\mu\text{E}/\text{m}^2/\text{s}$ light intensity, 16:8 photoperiod). Again wrap the plates with vent tape.
5. Each somatic embryo germinates to form leaves and roots on regeneration medium III within 1 week and plantlets are ready for transfer to soil within 10 days (*see Note 22*).

3.6. Transplanting and Acclimation

1. In a laminar flow hood, use sterile forceps to transfer plantlets (a good-sized plantlet measures about ~5 cm) from the Petri plate to the soil surface of a small pot filled with Redi-Earth (*see Note 23*).
2. Remove any medium still clinging to the roots. Plants should be handled with extreme care to avoid breaking off the leaf.
3. Plantlet roots are gently pressed into the soil and covered. Place small pots into greenhouse flat with drainage holes. Thoroughly soak the flat with a gentle stream of water so as not to dislodge the transplants.
4. Place the flat in the growth chamber and cover it with a humi-dome in which one ventilation hole has been cut (*see Note 24*).
5. Flats should not need water for 48 h if thorough soaking was done at transplant. After that, water individual plants only as needed.
6. Remove the humi-dome when the plants are tall enough to touch it, and 1 week later move the flat from the growth chamber to the greenhouse (*see Note 25*).

3.7. Greenhouse Care of Transgenic Plants

1. Once transgenic plants have been moved to the greenhouse continue to monitor soil moisture on a per plant basis. Water only if dry, using a watering can with a well-defined spout.

2. Transgenic plantlets are fertilized once using liquid Miracle Gro Excel 15-5-15 (20 ppm), a low concentration water-soluble fertilizer, before transplanting to big pots (*see Note 26*).
3. At transplant to big pots, add one (15 g per 2 gallon pot) Osmocote Plus 16-8-12 tablet to bottom half of soil profile. At 6-7 leaf stage, add an additional tablet to soil surface of each pot.
4. After transplant to big pots, plants are watered three times with Miracle Gro Excel 15-5-15 (50-100 ppm). Following this, calcium/magnesium solution is given to all plants once every 2 weeks.
5. We cross all our R_0 female transgenic plants by pollinating them with nontransgenic donor pollen.
6. To provide nontransgenic donor pollen to pollinate transgenic ears, begin by planting 2 donor seeds twice per week (4 seeds per week) as soon as the first transgenic material is transferred to the light on regenerate medium I.
7. Tassels of all R_0 transgenic plants are bagged as soon as tassels emerge to minimize transgenic pollen flow in the greenhouse. In addition, transgenic plants are grown in a separate room from the nontransgenic, pollen donor plants.
8. After pollination, watering is continued as needed until 21–25 days later at which time watering is stopped altogether, and plants are moved to a dry-down area.
9. To further aid in cob dry-down, lift the pollination bag off the ear 15 days postpollination. Ten to 15 days later pull down the husks to facilitate further drying of the kernels (*see Notes 27 and 28*).
10. Forty to 45 days after pollination, harvest the seed (*see Note 29*). Seed is inventoried and securely stored in the cold (0–3°C, 60% relative humidity).

4. Notes

1. We typically plant Hi II F_1 seeds year round in the greenhouse and obtain F_2 immature embryos (by self- or sib-pollination of F_1 plants) for transformation experiments. F_1 seeds are produced in the field by pollinating Hi II parent A silks with Hi II parent B pollen. These parent lines and B73 can be obtained from the Maize Genetics Cooperation Stock Center (<https://maizecoop.cropsci.uiuc.edu/request/>) and are increased and

maintained in our field by sib-pollination when feasible. Hi II and B73 plants generally take 60 and 70 days (depending on the season), respectively, to flower in the greenhouse.

Corn plants in our greenhouse are placed in pots on the ground beginning 2 weeks after transplant to large pots. Our greenhouse operates on a 16:8 photoperiod. The average temperature is 28°C (day) and 21°C (night). The light intensity (230 $\mu\text{E}/\text{m}^2/\text{s}$ at 3.5 ft above ground) was measured in February on a slightly overcast day, therefore, does not factor in any additional sunlight.

To provide a steady flow of immature embryos for transformation experiments, 10 Hi II F_1 seeds are planted twice per week to guarantee 15 ears per week to the lab.

2. Gold Bio Technology Inc. (Duchefa Plant Biotechnology Products), St. Louis, MO, USA at <http://www.goldbio.com>, Cat. # B0178.
3. Media described in **steps 1–3** in **Subheading 2.4** use 100 × 15 Petri plates (Fisher) and are poured at a volume of 40 plates per liter. These media are derived from Songstad et al. (19) and Vain et al. (20) (for step 2 in Subheading 2.4).
4. Regeneration media described in steps 4–6 in **Subheading 2.4** use 100 × 25 Petri plates (Fisher) and are poured at a volume of 32 plates per liter. Regeneration medium II is after Armstrong and Green (21) and McCain et al. (22). All media are dried thoroughly before storage at room temperature in the dark.
5. The stock solutions should be made separately rather than adding both salts to one bottle of water. If they are not made separately, gypsum will immediately be formed.
6. Immature ears harvested from the greenhouse (or field) are stored in their husks and pollination bags in a larger plastic bag at 4°C. If there are not enough freshly harvested ears for an experiment, ears stored from Friday through Sunday, or Tuesday through Thursday, are used for experiments on Monday and Friday, respectively. We have not experimented with ears stored for longer than 5 days.
7. For surface sterilizing a large number of ears, we use a preautoclaved 4-L beaker that will hold up to 20 ears at a time.
8. Greenhouse ears can be surface sterilized for as little as 15 min, but we always use the full 20 min when sterilizing field ears from which we routinely encounter more problems with fungal or bacterial contamination in experiments.
9. Intermittent resterilization of all utensils used for dissection is accomplished using a Steriguard 350 bead sterilizer (Inotech Biosystems International, Rockville, MD, USA).

10. Callus lines are discarded 4 months after initiation to minimize detrimental effects (such as reduced regenerability and poor fertility) that may result from extended time in tissue culture.
11. At least two tubes of gold are always washed at one time so that they balance each other in the centrifuge steps.
12. We currently use 0.03 ug of selectable marker per 1× gold tube + threefold more GOI, adjusted for molar equivalents depending on relative sizes of the plasmids.
13. Ethanol (100%) is properly sealed and stored in the freezer when not in use.
14. We sterilize macrocarriers by soaking 10 min in 70% ethanol and then air-dry over night.
15. After loading, macrocarriers are laid over a bed of Drierite to maintain dryness during bombardment.
16. While aliquoting the suspension, draw a half-spiral with the pipet tip from the center and outward of each macrocarrier to ensure the even distribution of the suspension over the inner, target circle. *Important!* Work quickly to avoid evaporation of the remaining suspension.
17. Center the embryo grid, not the filter paper, on the 3.5-cm-diameter circle drawn on the bottom center of the plate.
18. The mesh screen is sterilized by autoclaving. We typically reuse this screen for 8–12 shots (with same DNA construct) before discarding.
19. Both bombarded IZE and callus pieces are transferred intact throughout selection – there is no need to break proliferating callus into pieces during selection. This saves time and confusion about which piece derives from which explant.
20. Using the described protocol, at an average frequency of 18%, we expect to recover eighteen independent, bialaphos-resistant calli per 100 bombarded embryos. When using Hi II germplasm in which we recover few to no escapes (events that do not carry the *bar* selectable marker gene), we calculate transformation frequency as (number of bialaphos-resistant calli recovered ÷ total number of embryos bombarded) × 100.
21. Glufosinate ammonium contains the same active ingredient (phosphinothrycin) as bialaphos. It is used instead of bialaphos during this regeneration step to save cost. In our experience, glufosinate must be used at higher concentrations than bialaphos to achieve the same effect. Imposing continued selection pressure during this regeneration step (whether glufosinate or bialaphos) is effective because non-transgenic callus does not form mature somatic embryos on

this medium. As such, only callus containing the *bar* gene is advanced to the light after this final in vitro selection step.

22. Using this protocol we expect an average of 8 out of 10 callus events to produce healthy plantlets.
23. Typically, plantlets on each plate germinate at different rates. After transplanting the large plantlets, smaller plantlets in the same Petri plate are returned to the light until they too are large enough for transplant to soil.
24. A Conviron (Controlled Environments Limited, 590 Berry St. Winnipeg, MB, Canada) growth chamber is used for this intermediate step. The conditions are 16:8 photoperiod; 350 $\mu\text{E}/\text{m}^2/\text{s}$ light intensity (plant height of 30 cm) with a combination of fluorescent and incandescent bulbs; 26°C (day) and 22°C (night).
25. If the greenhouse is in a different building than the laboratory and the outdoor temperature is below freezing or very cold, special care is needed during this step. Cover the flat with a humi-dome, wrap it in a plastic garbage bag, and transport it to the greenhouse in a preheated vehicle.
26. A plantlet is ready to be transplanted to a big pot if soil adheres to its root ball when lifted out of the small pot. Plantlets are generally over 16-cm tall at this stage.
27. If seeds are contaminated by fungus, use 70% ethanol to clean the surface before storage.
28. Common pests found in our greenhouse are aphids (on the tassels or on the underside of leaves), spider mites (on the underside of leaves), and thrips (on the leaf whorl). We spray for mites or thrips once a month, or as needed, using Floramite[®], Pylon[®], Akari[®], Avid[®], or Samite[®] for spider mite control and Conserve[®] for thrip control (all from Hummert). Fungus gnats are another common pest and often become a problem when plants are overwatered. Place yellow monitoring cards (to which the air-borne gnats stick) around the greenhouse to control them. Another disease that we see although infrequently is smut. Smutted plants are immediately discarded. To limit disease onset, the greenhouse must be kept clean. The floor should be frequently swept or sprayed clean; the drain hole should always be left unclogged, and fallen and dead leaves still clinging to the plants should be removed. Garbage should be emptied once a week. Mice moving in from the field in the fall will feed on maize seed (transgenic or otherwise). To solve this problem, we set out mice traps baited with peanut butter each autumn.
29. On an average, 70% of regenerated events will produce >50 kernels (we take 3–4 plants per event to the greenhouse). Because we have observed consistently poor transgenic seed

sets for plants flowering in the greenhouse in July (in Ames, IA) we now avoid February transformations for constructs going to seed.

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