Chapter 8

Maize (*Zea mays* L.)

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Abstract

Agrobacterium tumefaciens-mediated transformation is an effective method for introducing genes into maize. In this chapter, we describe a detailed protocol for genetic transformation of the maize genotype Hi II. Our starting plant material is immature embryos cocultivated with an *Agrobacterium* strain carrying a standard binary vector. In addition to step-by-step laboratory transformation procedures, we include extensive details in growing donor plants and caring for transgenic plants in the greenhouse.

Key words *Agrobacterium*-mediated, *Agrobacterium tumefaciens*, Genetic transformation, Maize, Standard binary vector, *Zea mays*

1 Introduction

Transgenic maize is among one of the first biotechnology crops globally commercialized. A number of gene delivery systems such as the biolistic gun, electroporation, silicon carbide whiskers, and *Agrobacterium tumefaciens* infection can be used for maize transformation [1]. One of the greatest advantages of using the *Agrobacterium*-mediated transformation method is its ability to generate large numbers of maize events with single or relatively low transgene copy numbers [2–4]. In general, simple transgene insertion is preferred because these transgenic plants are less prone to multi-sequence-induced gene silencing [5] and have been shown to maintain higher and more stable transgene expression over generations [4]. However, one of the major challenges in implementing this method is that it involves balancing interactions between two living organisms, the plant and the bacteria, to achieve success. Cells of one maize genotype or tissue type may be transformable using the biolistic gun, but not readily amenable to transformation using the *Agrobacterium* method if they are not susceptible to infection by this biological delivery agent. For example, we have achieved stably transformed plants from immature embryo scutellum cells of the inbred line Oh43 using the biolistic

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gun but have been unsuccessful in achieving transient or stable transformation of the same genotype using the *Agrobacterium* method (B. Frame, unpublished).

To date, the most widely targeted explant for *Agrobacterium-mediated* stable transformation of maize is the immature zygotic embryo [2, 3, 6, 7]. Efficient, stable transformation has also been achieved using seedling-derived Type I embryogenic callus which was successfully transformed and progeny produced, using *Agrobacterium* [8]. In this chapter, we describe the *Agrobacterium*-mediated maize transformation protocol used routinely in our laboratory [6] to transform immature embryos of the maize Hi II genotype. *A. tumefaciens* strain EHA101 harboring a standard binary vector is used to infect the embryos and deliver transgenes into plant cells. Type II callus cultures induced from the infected embryos are selected on bialaphos-containing media, and putative transgenic plants are regenerated from bialaphos-resistant, somatic embryogenic callus. The process (from non-transgenic donor embryos to transgenic maize seed) requires approximately 210 days (Fig. 1). The average transformation frequency for this system is 7 % (defined as seven bialaphos-resistant callus lines recovered from 100 infected immature embryos) and ranges from 1 to 25 % depending on the gene of interest.

2 Materials

2.1 Agrobacterium tumefaciens *Strain and Vector*

Agrobacterium tumefaciens strain EHA101 [9] carrying the 9,186 bp standard binary vector pTF101.1 [10] was derived from pPZP [11]. This vector contains the *bar* gene selectable marker cassette which confers resistance to phosphinothricin, the active ingredient in bialaphos [12]. The *bar* gene is driven by the double 35S CaMV promoter [13] and is flanked on the 5′ end by a tobacco etch virus (TEV) translational enhancer [14] and on the 3′ end by the soybean vegetative storage protein terminator [15]. pTF101.1 also contains a multiple cloning site (MCS) for insertion of a gene of interest (GOI) (*see* **Note 1**).

2.2 Plant Material

1. Maize Hi II F1 seeds (*see* **Note 2**): ears of the maize Hi1 II genotype (A188 × B73 origin) [16] harvested from greenhouse-grown embryo donor plants 8–13 days after pollination. Immature zygotic embryos (1.2–1.8 mm) aseptically dissected from these ears are targeted for *Agrobacterium*-mediated transformation [6, 17] without pre-culture.
2. Maize B73 seeds: used as pollen donor plant.

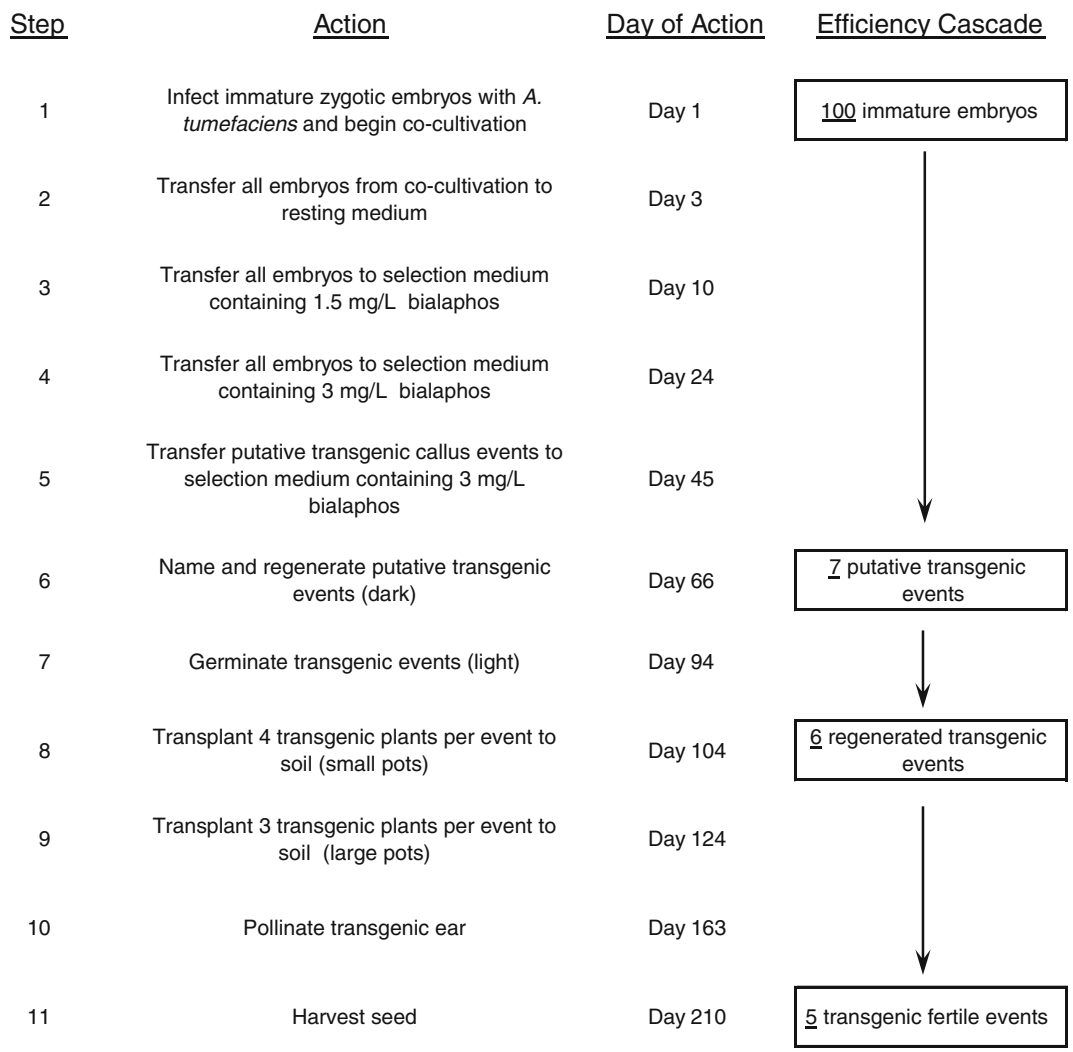

Fig. 1 Time line and frequency cascade for production of fertile transgenic plants from Hi II immature embryos transformed with *A. tumefaciens*. After cocultivation, all immature embryo explants are subcultured through resting and selection (steps 2–4). Identification of putative transgenic events begins at about Day 45 (step 5) but can continue for as long as 10 weeks after infection. We recover an average of seven putative, independent transgenic events (bialaphos-resistant calli) from 100 *Agrobacterium*-infected immature embryos. Ninety percent of these Hi II putative transgenic callus events are successfully regenerated to plants and grown to maturity in the greenhouse. We typically cross three transgenic plants per event to produce >50 seed from three of every four transgenic events taken to seed

2.3 Stock Solutions

2.3.1 Vitamins and Phytohormones

1. N6 vitamin stock (1,000×): 1.0 g/L thiamine HCl, 0.5 g/L pyridoxine HCl, 0.5 g/L nicotinic acid, and 2.0 g/L glycine. Store in 50 mL aliquots in Falcon tubes at –20 °C. Thaw one tube at a time and store at 4 °C.
2. MS vitamin stock (modified, 1,000×): 0.5 g/L thiamine HCl, 0.5 g/L pyridoxine HCl, 0.05 g/L nicotinic acid, and 2.0 g/L glycine. Store 50 mL aliquots in Falcon tubes at –20 °C. Thaw one tube at a time and store at 4 °C.

3. 2,4-Dichlorophenoxy acetic acid (2,4-D): weigh 0.25 g 2,4-D in a fume hood, and dissolve in 1 N KOH (10 mL) on low heat. When dissolved, bring up to 250 mL final volume with ddH_2O water. Store at 4 °C in Duran bottle.

2.3.2 Antibiotics and Selective Agents

1. Kanamycin sulfate: 10 mg/mL stock in ddH_2O. Filter-sterilize through a 0.2 μm membrane. Store 0.25 mL aliquots in Eppendorf tubes at −20 °C.
2. Spectinomycin sulfate: 50 mg/mL stock in ddH_2O. Filter-sterilize and store 0.10 mL aliquots in Eppendorf tubes at −20 °C.
3. Bialaphos: dissolve 100 mg of bialaphos (Gold Biotech) in 100 mL ddH_2O. Filter-sterilized stock solution (1 mg/mL) is stored at 4 °C for up to 4 months in 50 mL Falcon tubes.
4. Glufosinate: dissolve 100 mg of glufosinate ammonia (Sigma) in 100 mL of ddH_2O. Stock solution (1 mg/mL) is filter-sterilized and stored in 50 mL Falcon tubes at 4 °C.
5. Cefotaxime: dissolve 1.0 g of cefotaxime (PhytoTechnology Laboratories) in 5 mL ddH_2O. Store filter-sterilized stock solution (200 mg/mL) in 0.25 mL, aliquots, and store at −20 °C.
6. Vancomycin: dissolve 1.0 g of vancomycin (PhytoTechnology Laboratories) in 5 mL ddH_2O. Store filter-sterilized stock solution (200 mg/mL) in 0.25 mL aliquots at −20 °C (*see* **Note 3**).

2.3.3 Other

1. Acetosyringone (AS): stock solution (100 mM) is prepared by dissolving 0.392 g of AS in 10 mL of dimethyl sulfoxide (DMSO) and then diluting this 200 mM solution 1:1 with ddH_2O before filter-sterilizing. Store 0.5 mL aliquots in Eppendorf tubes at −20 °C.
2. L-Cysteine: dissolve 100 mg/mL L-cysteine in ddH_2O and filter-sterilize. Use the same day (*see* **Note 4**).
3. Silver nitrate: Dissolve 0.85 g silver nitrate in 100 mL of ddH_2O. 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.

2.4 Culture Media

2.4.1 For Agrobacterium

1. YEP medium [18]: 5 g/L yeast extract, 10 g/L peptone, 5 g/L $NaCl_2$, and pH 6.8 with NaOH. Add Bacto agar (15 g/L) to prepare solid YEP medium. For growing pTF101.1 in EHA101, the final antibiotic concentrations in YEP are 50 mg/L kanamycin (disarmed Ti plasmid pEHA101) and 100 mg/L spectinomycin (binary vector plasmid pTF101.1). Pour to 100 × 15 mL Petri plates.

*2.4.2 For Maize (See **Note 5**)*

1. Infection (liquid) medium: 4 g/L N6 salts [19], 1 mL/L N6 vitamin stock, 1.5 mg/L 2,4-D, 0.7 g/L L-proline, 68.4 g/L sucrose, and 36 g/L glucose, adjust pH to 5.2 using 1 N KOH. Filter-sterilize and store at 4 °C. Acetosyringone (AS) is added immediately prior to use for a final concentration of 100 μM.
2. Cocultivation medium: 4 g/L N6 salts, 1.5 mg/L 2,4-D, 0.7 g/L L-proline, 30 g/L sucrose, and 3 g/L Gelrite, adjust pH to 5.2 using 1 N KOH. Filter-sterilized N6 vitamin stock (1 mL/L), silver nitrate (5 μM), AS (100 μM), cefotaxime (100 mg/L) (*see* **Note 6**), and L-cysteine (300 mg/L) are added after autoclaving when medium has cooled. This medium is used within 4 days of being made.
3. Resting medium: 4 g/L N6 salts, 1.5 mg/L 2,4-D, 0.7 g/L L-proline, 30 g/L sucrose, 0.5 g/L 2-(4-morpholino)-ethane sulfonic acid (MES), and 8 g/L purified agar, adjust pH to 5.2 using 10 N KOH. Filter-sterilized N6 vitamins (1 mL/L), cefotaxime (100 mg/L), vancomycin (100 mg/L), and silver nitrate (20 μM) are added after autoclaving when medium is cooled.
4. Selection medium I: 4 g/L N6 salts, 1.5 mg/L 2,4-D, 0.7 g/L L-proline, 30 g/L sucrose, 0.5 g/L MES, and 8 g/L purified agar, adjust pH to 5.8 using 10 N KOH. Filter-sterilized N6 vitamins (1 mL/L), cefotaxime (100 mg/L), vancomycin (100 mg/L), silver nitrate (20 μM), and bialaphos (1.5 mg/L) are added after autoclaving when medium is cooled.
5. Selection medium II: the same as Selection medium I except that bialaphos concentration is increased to 3 mg/L.
6. Regeneration medium I: 4.3 g/L MS salts [20], 1 mL/L MS vitamin stock, 100 mg/L myo-inositol, 0.25 mg/L 2,4-D, 30 g/L sucrose, and 3 g/L Gelrite, adjust pH to 5.8 using 1 N KOH. Filter-sterilized bialaphos (2 mg/L) and cefotaxime (100 mg/L) are added after autoclaving when medium is cooled.
7. Regeneration medium II: 4.3 g/L MS salts, 1 mL/L MS vitamin stock, 100 mg/L myo-inositol, 60 g/L sucrose, and 3 g/L Gelrite, adjust pH to 5.8 using 1 N KOH. Filter-sterilized glufosinate ammonia (6 mg/L) and cefotaxime (100 mg/L) are added after autoclaving when medium is cooled.
8. Regeneration medium III: 4.3 g/L MS salts, 1 mL/L MS vitamin stock, 100 mg/L myo-inositol, 30 g/L sucrose, and 3 g/L Gelrite, adjust pH to 5.8 using 1 N KOH.

2.5 Other Supplies and Reagents

1. Sterilizing solution: 60 % commercial bleach (5.25 % hypochlorite), 1 drop of surfactant Tween 20 per liter.
2. Redi-Earth: Hummert Cat. # 10-2030-1, Hummert (4500 Earth City Expressway, Earth City, MO 63045).
3. Metro-Mix 900: Sun Gro Horticulture (770 Silver Street, Agawam, MA 01001).
4. Greenhouse flat with drainage holes (holds 32 small pots): Hummert Cat. # 11-3000-1.
5. Small pot (6.4 cm^2 each in 4-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. # 14-9644-1.
8. Large pot for B73 pollen donor plants (3-gal nursery pot with four drainage holes). Hummert Cat. # 14-9637-1.
9. Osmocote Plus 15-8-11 (controlled release fertilizer tablets with trace elements): Hummert Cat. # 07-6455-1.
10. Calcium/magnesium solution (Dr. C. Block, USDA-ARS, North Central Regional Plant Introduction Station, Ames, Iowa, USA): first, make two separate stock solutions. Stock #1, 720 g/L of $Ca(NO_3)_2{\cdot}4H_2O$; Stock #2, 370 g/L of $MgSO_4{\cdot}7H_2O$ (Epsom salts). To prepare a working solution, add 9 mL of each Stock #1 and Stock #2 into 1 gal (~4 L) of H_2O (*see* **Note 7**).
11. Peters Excel Cal-Mag 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. Pestrap sticky cards (for insect monitoring): Hummert Cat. # 01-3730-1.
14. Shoot bags: Lawson Cat. # 217, Lawson Bags (318 Happ road, P.O. Box 8577, Northfield, IL 60093).
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. pH/EC meter: Hummert Cat. # 45-5008-1.

3 Methods

3.1 Growing Donor Plants for Immature Embryo Production

1. Seed germination is conducted in the greenhouse (*see* **Notes 8** and **9**).
2. Fill a plastic 4-pack with Metro-Mix 900. Water until the soil is completely wet.
3. Bury one corn seed 2.5 cm deep in the middle of each pot. Place the 4 packs in a greenhouse flat with drainage holes under a plastic Humi-Dome to avoid excessive evaporation of moisture from the soil.
4. Seeds should germinate in approximately 4 days. Check moisture daily; water the plants only when the soil is dry.
5. After 8–9 days, or just before the plants reach the top of the dome, remove the plastic dome and continue to water as needed.
6. After approximately 2 weeks, transplant each seedling (~4–5 leaf stage) into a large pot.
7. To begin, half fill a 2 gal (7.6 L) pot with Metro-Mix 900, and add 1 tablet (7.5 g) of Osmocote Plus. Add Metro-Mix to 80 % of the pot (~5 cm from top edge of the pot, *see* **Note 10**).
8. Drench the soil by filling until the water level reaches to the top edge of the pot. Let it drain completely and drench again.
9. To transplant, move corn plantlet with soil adhering to the root ball from the small pot and lay it on the wetted soil surface in middle of the big pot. The roots are pressed into the soil and buried to a depth that keeps the plant from falling over as it grows (*see* **Notes 11** and **12**).
10. Avoid overwatering young plants (*see* **Notes 13–15**).
11. Calcium deficiency symptoms may become visible after transplant to big pots (*see* **Note 16**).
12. Prior to internode elongation (approximately 2 weeks after transplant), place one more tablet of Osmocote Plus fertilizer onto the soil surface of each pot. For B73 plants, a third controlled release fertilizer application may be needed.
13. In addition, all non-transgenic pollen or embryo donor maize plants are fertilized on a continual feed basis at a rate of 100 ppm Peters Excel Cal-Mag 15-5-15 from seed sowing until 4.5 weeks. After 4.5 weeks, all donor maize plants are fertilized on a continual feed basis at a rate of 200 ppm Peters Excel Cal-Mag 15-5-15. Plants are checked daily and watered on an as needed basis.
14. Fifty-five to sixty days after germination, embryo donor plants are sib-pollinated for producing embryo donor ears for transformation.

15. Cover any emerging ears with shoot bags to prevent contamination of the silks before controlled pollination (*see* **Note 17**).
16. After the silks have been visible (under the shoot bag) for 1–2 days, cut them and the top inch of the cob off to prepare a uniform surface of silks for pollination the next day (*see* **Notes 18** and **19**). Use white or light yellow pollen from sibling Hi II plants for pollination (*see* **Note 20**).
17. Cover the pollinated silks with a plain pollination bag and label with the plant ID and cross date. Ears can be harvested 8 (summer) to 13 (winter) days after pollination. The size of immature embryo used for transformation ranges between 1.2 and 1.8 mm (*see* **Note 21**).

3.2 Preparation of Agrobacterium *Culture for Infection*

1. The vector, pTF101.1, in strain EHA101 is maintained on solid YEP with antibiotics at 4 °C for 1 month (mother plate) before it is refreshed from long-term −80 °C glycerol stocks.
2. To initiate a maize transformation experiment, streak *A. tumefaciens* from 4 °C mother plate to solid YEP with antibiotics and grow for 3 days at 19 °C (*see* **Note 22**).
3. On the day of infection, inoculate one loop full (3 mm) of this bacteria culture into 6 mL infection medium supplemented with 100 μM AS in a 50 mL Falcon tube.
4. The culture is shaken gently for 2 h by taping it horizontally to a Vortex Genie (Fisher) platform set on low speed (~75 rpm).

3.3 Ear Sterilization and Embryo Dissection

1. While the *A. tumefaciens* is being pre-cultured, surface sterilize maize ears. To begin, cut off and discard the top 1 cm of a de-husked maize cob (silk end). Insert the tip of a straight nosed forceps into this end to secure the cob for aseptic manipulation during embryo dissection. In a laminar flow bench, place up to 4 ears in sterile Mason jar, with forceps handles protruding.
2. Add ~700 mL (enough to cover ears) of sterilizing solution. During the 25-min disinfection, occasionally tap the Mason jar on the bench surface to dislodge air bubbles. Pour off bleach solution and rinse the ears three times in generous amounts of sterilized water (*see* **Note 23**).
3. In a large (150 × 15 mm) sterile Petri plate, cut off top 1–2 mm of kernel crowns using a new (sharp) scalpel blade (*see* **Note 24**).
4. Insert the end of a sharpened spatula straight down (do not insert at angle or you may split the embryo in half) between the endosperm and pericarp at the basipetal side of the kernel (toward the bottom of the cob).
5. Pop the endosperm out of the seed coat by gently wiggling the spatula (if you dislodge the kernel from the cob instead,

you have inserted the spatula too deeply). This exposes the untouched embryo which is then gently coaxed onto the spatula tip and transferred directly to liquid infection medium.

3.4 Infection, Cocultivation, and Resting

1. Up to 50 immature zygotic embryos are dissected directly into a 2 mL Eppendorf tube filled with bacteria-free infection medium (with 100 μM AS).
2. Remove this first wash using a 1 mL Pipetman and wash embryos a second time with the same medium (1 mL) which is stored at 4 °C in the fridge throughout the experiment.
3. After removing the final wash, add 1 mL of pre-cultured *A. tumefaciens* suspension (adjusted to $OD_{550} = 0.30–0.40$ using a spectrophotometer and stored at 4 °C in the fridge throughout the experiment) to the embryos. The tube is gently inverted (not vortexed) 20 times before resting it for 5 min on its side on the bench with all embryos submerged.
4. After infection, transfer embryos, along with the *A. tumefaciens* suspension, to the surface of cocultivation medium (300 mg/L L-cysteine) using a 1 mL pipet tip (cut off to enlarge the bore size). Carefully pipet-off any excess *A. tumefaciens* suspension surrounding the embryos using an uncut tip.
5. Orient infected embryos scutellum side up with the aid of a dissecting scope. Wrap plates with vent tape and incubate at 20 °C (dark) for 3 days.
6. Transfer all embryos from cocultivation medium to resting medium (35 embryos per plate). Wrap plates with vent tape and incubate at 28 °C (dark) for 7 days (*see* **Note 25**).

3.5 Selection for Putative Transgenic Callus Events

1. After the 1 week resting period, transfer all embryos to Selection I medium (1.5 mg/L bialaphos) to begin selection. Plates are wrapped with Parafilm and incubated at 28 °C (dark).
2. Two weeks later, selection pressure is enhanced by transferring embryos to Selection II medium (3 mg/L bialaphos) for 3 weeks.
3. About 6 weeks after infection, a rapidly growing, embryogenic sector of callus emerges on a subset of infected embryos while no further callus proliferation, and in some cases browning, occurs on the majority of other embryos. Each of these proliferating calli is considered an independent putative transgenic event (*see* **Note 26**).
4. Subculture each putative event to fresh Selection medium II for bulking and naming.
5. Molecular biological analyses such as Southern, Northern blot hybridization, or histochemical GUS assays can be performed at this stage.

3.6 Regeneration of Transgenic Plants

1. Uniquely identified putative callus events are transferred to Regeneration medium I (one event per plate). Plates are wrapped with Parafilm and incubated at 25 °C for 10–14 days.
2. With the aid of a 40× dissecting microscope, up to 15 pieces (4 mm) of bialaphos-resistant, embryogenic callus (stocked embryos suspended in a friable callus matrix) from one independent transformation event are transferred to Regeneration medium II (*see* **Note 27**). Plates are wrapped with vent tape and incubated at 25 °C (dark) for 2–3 weeks.
3. After this maturation period, many somatic embryos are swollen, opaque, and white, and, from some pieces, the coleoptile is already emerging. Again using a dissecting microscope, transfer these mature somatic embryos (~12 pieces) to the surface of Regeneration medium III for germination in the light (25 °C, 80–100 μE/m^2/s light intensity, 16:8 photoperiod) and wrap the plates with vent tape.
4. Somatic embryos germinate (sprouting leaves and roots) on Regeneration medium III within 1 week, and plantlets are ready for transfer to soil within 10 days.

3.7 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 28**).
2. Any medium still clinging to the roots is removed, and plants are handled with extreme care to avoid breaking off the leaf. A premade tag marked with the construct, event, and plant number of each plantlet is inserted into each small pot at this time and accompanies the plant through to maturity in the greenhouse.
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 29**).
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 Humi-Dome when plants are tall enough to touch it, and 1 week later, move the flat from the growth chamber to the greenhouse (*see* **Note 30**).

3.8 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 as needed on a continual feed basis with 100 ppm Excel Cal-Mag 15-5-15 water-soluble fertilizer before transplanting to big pots.
3. 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 15 cm tall at this stage.
4. Follow **steps 7–9** in Subheading 3.1 for transplanting transgenic plants from small pot to big pot.
5. After transplant to big pots, plants are watered as needed on a continual feed basis with 100 ppm Excel Cal-Mag 15-5-15.
6. Follow **steps 7** and **12** in Subheading 3.1 for osmocote fertilization instructions.
7. Molecular analysis can be performed at this stage.

3.9 Transgenic Seed Production

1. We cross all our R_0 female transgenic plants by pollinating them with non-transgenic donor pollen.
2. To provide non-transgenic donor pollen to pollinate transgenic ears, begin by planting 4–8 donor seeds twice per week (8–16 per week) as soon as the first transgenic material is transferred to Regeneration II medium (**step 2** of Subheading 3.6).
3. Follow **steps 15–17** in Subheading 3.1 for controlled pollination.
4. Striped pollination bags are used for differentiating transgenic and non-transgenic crosses in the greenhouse, and all bags are labeled with the cross ID (female plant ID × male plant ID) and date using a thick, black permanent marker.
5. Tassels of all R_0 transgenic plants are removed as soon as tassels emerge to eliminate transgenic pollen flow in the greenhouse. In addition, transgenic plants are grown in a separate room from the non-transgenic, pollen donor plants.
6. After pollination, watering at 100 ppm is continued as needed basis until 25 days later at which time watering is stopped altogether, and plants are moved to a dry-down area.
7. To further aid in cob dry-down, the pollination bag is lifted off the ear 10 days post-pollination. Fifteen days later, the husks are pulled down to facilitate further drying of the kernels.
8. Forty days after pollination, the seed is harvested (*see* **Note 31**). Seed is inventoried and securely stored in the cold (0–4 °C, 60 % relative humidity).

3.10 Greenhouse Operations

1. To prevent and manage greenhouse pests and disease, chemical spraying and rigorous cultural practices are followed (*see* **Note 32**).

2. To maintain yearlong immature embryo and transgenic seed production flow, the greenhouse is cooled by an internal air handler during the summer (*see* **Note 33**).
3. Soil EC (electrical conductivity) and pH are monitored weekly to ensure that nutrient delivery and uptake are optimized (*see* **Note 34**).

4 Notes

1. In addition, we have recovered transgenic events using construct pTF101.1 and constructs derived from the pTF101.1 vector [i.e., pTF102 [6]] in strains LBA4404 [21], GV3101::pMP90 [22, 23], AGL-0 and AGL-1 [24], and C58 Z707 [25].
2. F_1 seeds planted year-round in the greenhouse are produced in the field or greenhouse by pollinating Hi II parent A ears with Hi II parent B pollen. These parent lines can be obtained from the Maize Genetics Cooperation Stock Center (http://w3.aces.uiuc.edu/maize-coop/) and are increased and maintained in the greenhouse or field by sib-pollination. Hi II plants take approximately 60 days (depending on the season) to flower in the greenhouse.
3. If the two formulations of cefotaxime and vancomycin are mixed together before adding to the medium, they will form a precipitate, so add separately, with intermediate stirring, to the cooled medium.
4. The L-cysteine stock comes out of solution if left overnight at 4 °C. As such, we make this stock fresh each time we make cocultivation medium.
5. All media described in Subheading 2.4.2 (except liquid infection medium and Regeneration medium I) use 100 × 25 vented lid Petri plates (Fisher) and are poured to a volume of 32 plates/L. Regeneration medium I uses 100 × 15 Petri plates (Fisher) and is poured to a volume of 20 plates/L. Media 1–5 are after Zhao et al. [3] with the addition of both cysteine (300 mg/L) and cefotaxime (100 mg/L) to cocultivation medium and the use of cefotaxime and vancomycin instead of carbenicillin for counterselection of *A. tumefaciens* after cocultivation. Regeneration medium II is after Armstrong and Green [26] and McCain et al. [27]. All media are dried thoroughly before storage at room temperature in the dark.
6. Cefotaxime (100 mg/L) is added to cocultivation medium to limit non-*Agrobacterium*, bacterial contamination (e.g., *Serratia*) not eliminated by surface sterilization of the ear.

7. 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.
8. To provide a steady flow of immature embryos for *Agrobacterium* transformation experiments, 12 Hi II F_1 seeds are planted twice per week to ensure 15 ears per week to the lab.
9. Maize 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 μE/m^2/s at 3.5 ft above ground) was measured in February on a slightly overcast day, therefore, does not factor in any additional sunlight.
10. If pots are filled with too little soil, they will dry out quickly between waterings. Conversely, if filled too full, thorough drenching is prevented at watering.
11. Marathon® or Mantra®, for aphid control, is added at this stage as part of the transplant step.
12. The soil should cover all the roots. Be sure to plant the young plants deep enough or they will tip over when they grow taller and before their brace roots have formed.
13. Normally, no watering is needed for 1 week if plants are drenched thoroughly at transplant (7–14 days later, they are watered as needed). Check the soil moisture daily by lifting the pot and feeling its weight. Water when the pot can be lifted easily with one hand. Watering may not be needed if only the surface layer of soil is dry. If the water pressure from the hose sends soil flying out of the pot, turn it down to avoid leaving excess dirt on the greenhouse floor after each watering.
14. Pay special attention to small plants. Young plants will begin to develop a strong root system if not over watered.
15. Plants need more water as they mature. During the summer, fully grown plants may need to be watered twice a day.
16. Calcium deficiency is a chronic problem in our greenhouse and may begin as soon as plantlets are transplanted to big pots. Plants develop rippled edges and unpigmented patches or lesions on the leaves and may also be severely stunted. In the worst cases, the leaves in the whorl wrap tightly around each other and eventually rot. To prevent or treat calcium deficiency symptoms, drench plants with calcium/magnesium solution (Subheading 2.5) after transplant and once a week thereafter.
17. Be sure shoot bags are firmly pulled over ear shoots otherwise they will fall off and the emerging silks will be "contaminated" with unwanted pollen.

18. Prior to silk cut back, clean scissors with 70 % ethanol to prevent cross-contaminating ears with mold.
19. If silks have been emerged for as long as 5–7 days, they can still be cut back and recovered with the shoot bag, and the fresh "stubs" pollinated the next day.
20. Do not use old and crystallized yellow pollen because it is not viable, although pollen color will vary depending on genotype. Pollen donor plants are labeled with the date of first pollen emergence and discarded 5 days after this date. Be careful with B73 tassels, they are easy to accidentally break off, and maneuvering the tall plant to obtain pollen can be difficult.
21. Ears harvested from the greenhouse (or field) are stored in their husks and pollination bags in a refrigerator crisper at 4 °C. 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.
22. We occasionally grow *Agrobacterium* for 2 days at 28 °C in preparation for an experiment. In initial side by side comparisons, the 19 °C/3 day combination resulted in higher, but not significantly different transformation efficiencies than 28 °C/2 days so we have continued to use the former (B. Frame, unpublished). T-DNA transfer machinery was reported to function optimally at 19 °C when compared to 28 °C [28].
23. Surface sterilizing a large number of ears saves time and resources by using a pre-autoclaved 4 L beaker which will hold up to 20 ears at a time.
24. Intermittent re-sterilization of all utensils used for dissection is accomplished using a Steriguard 350 bead sterilizer (Inotech Biosystems International, Rockville, MD, USA).
25. After 3 days on freshly prepared cocultivation containing 300 mg/L-cysteine, embryos smaller than 1.5 mm are often flaccid and slow to produce callus. Nevertheless, transfer all embryos from cocultivation medium to resting and from resting to selection media, regardless of their appearance.
26. Using the described protocol, at an average frequency of 7 %, we expect to recover seven independent, bialaphos-resistant calli per 100 infected 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 infected) × 100.
27. Glufosinate ammonium contains the same active ingredient (phosphinothricin) as bialaphos and was substituted for bialaphos initially because it was easier to obtain. Imposing continued selection pressure during this first 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 expressing the *bar* gene is advanced to the light after this final in vitro selection step.

28. We typically drench a tub of Redi-Earth first with warm water (as it wets the soil better) followed by cold water and fill the small pots with thoroughly pre-wetted Redi-Earth.
29. We use a Conviron (Controlled Environments Limited, 590 Berry St. Winnipeg, Manitoba, Canada) growth chamber for this stage. The conditions are 16:8 photoperiod, 350 μE/m^2/s light intensity (plant height of 30 cm) with a combination of fluorescent and incandescent bulbs, and 26 °C (day) and 22 °C (night).
30. 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.
31. If fungus is visible on the cob surface, wipe with 70 % ethanol to clean the surface before storage. Discard any discolored or damaged seed.
32. Common pests found in our greenhouse are aphids (on the tassels or on the underside of leaves), spider mites (on the leaf back), and thrips (on the leaf whorl). We spray for mites or thrips as needed, using Shuttle O®, Judo®, TetraSan®, Floramite®, Pylon®, Avid®, or Samite® for spider mite control and Conserve®, Overture®, Pedestal®, Pylon®, Azatin®, and Talstar® for thrip control (all from Hummert). Fungus gnats often become a problem when plants are over watered. Place Pestrap yellow monitoring cards (to which the airborne gnats stick) at various soil levels in the greenhouse to monitor them. Smutted plants are seen infrequently and are immediately discarded. In order to limit disease onset, the greenhouse must be kept clean. Floors are swept frequently or sprayed clean, drain holes are kept free of clogging debris, and fallen and dead leaves still clinging to the plants are routinely removed. Floors are sanitized using Green-Shield, or a similar disinfectant, 1–4 times per month. Garbage is emptied at least once a week. Mice will feed on maize seed (transgenic or otherwise). Mouse traps baited with peanut butter are set out each autumn and spring.
33. From late May to September, a large convection tube that runs down the center of each greenhouse bay is used for additional cooling. These tubes are connected to internal air handlers with an output of 800–10,000 cfm (cubic feet per minute). During this period, the supplemental greenhouse lights are adjusted so that they automatically turn off when the large coolers are running, thereby reducing greenhouse

temperature further. When the large coolers are off, the lights automatically turn back on. These large coolers only run during peak times of heat/sun during summer days.

34. Once a week, we test five pots (2 gal size) per greenhouse bay for pH and EC using the PourThru method [29, 30] as follows: 1 h before testing, water or fertilize each pot as normal, being sure to saturate the substrate. After 1 h, place a heavy-duty foam paper plate under the container. Pour distilled water on the surface of the substrate to collect 50 mL of leachate. For a 2 gal pot, apply approximately 80–90 mL of distilled water to achieve 50–60 mL of leachate. Leachate of more than 60 mL will dilute the sample and provide low EC readings. Pour the collected leachate from the foam plate into a plastic beaker. Calibrate the pH/EC meter according to the manufacturer's instructions. Measure and record the pH/EC of each sample, taking the average of the samples. The EC of each sample will not vary over time, but pH will begin to drift after 2 h. pH range for greenhouse maize should be between 5.5 and 6.0, while EC range should be between 2.6 and 4.6 mS (siemens)/cm.

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