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Trevor A. Thorpe
Edward C. Yeung *Editors*

Plant Embryo Culture

Methods and Protocols

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Methods and Protocols

Edited by

Trevor A. Thorpe and Edward C. Yeung

Department of Biological Sciences, University of Calgary,
Calgary, Alberta, Canada

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Editors
Trevor A. Thorpe
Department of Biological Sciences
University of Calgary
Calgary, Alberta
Canada
tthorpe@ucalgary.ca

Edward C. Yeung
Department of Biological Sciences
University of Calgary
Calgary, Alberta
Canada
yeung@ucalgary.ca

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Chapter 22

Genetic Transformation Using Maize Immature Zygotic Embryos

Bronwyn Frame, Marcy Main, Rosemarie Schick, and Kan Wang

Abstract

Epidermal and subepidermal cells in the abaxial, basal region of the maize (*Zea mays* L.) immature zygotic embryo (IZE) scutellum can be induced by exogenous auxin to proliferate and undergo somatic embryogenesis. Successful genetic transformation of IZEs depends not only on optimizing transformation parameters for these totipotent cells, but also on achieving high embryogenic callus induction frequency (ECIF) in a population of targeted explants. In maize, ECIF is strongly influenced by genotype, the tissue culture media used, and the interaction of these two factors. Altering tissue culture media components to increase ECIF and/or transformation frequency (TF) has been one approach used to expand the range of maize genotypes amenable to genetic transformation using the IZE. This chapter outlines such an approach – an *Agrobacterium*-mediated transformation protocol is used for direct-targeting IZEs of the hybrid Hi Type II and inbred B104 lines. Two different media regimes are used for successful culture and transformation of two distinct genotypes.

Key words: *Agrobacterium tumefaciens*, B104, Callus induction frequency, Embryogenic callus, Genetic transformation, Hi II, Immature zygotic embryo, Maize

1. Introduction

Evidence that scutellar cells of the maize (*Zea mays* L.) immature zygotic embryo (IZE) can be induced to produce embryogenic callus in the presence of exogenous auxin was first reported over 30 years ago (1). Regeneration of fertile plants from this callus (2) demonstrated the totipotent nature of these epidermal and subepidermal meristematic cells found in the abaxial, basal region of the IZE scutellum (3). Both compact Type I and friable Type II embryogenic callus phenotypes (4) were observed to originate from these scutellar cells (3).

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In the last two decades, numerous studies have shown that callus and cell suspensions derived from these totipotent cells are also transformation competent (5, 6). Of particular impact were reports demonstrating that the IZE scutellum can itself be directly targeted for genetic transformation using electroporation (7), the biolistic gun (8, 9) and *Agrobacterium*-mediated methods (10–12). Expected progeny segregation ratios for the inherited transgene provided evidence that, whether targeted embryos formed Type I (7, 10, 13) or Type II (9, 12, 14) callus, transformation occurred in a single cell in this meristematic region of the embryo scutellum.

A major benefit of direct-targeting the IZE for genetic transformation is a reduction in the in vitro culture period required to recover transgenic plants (8). This not only reduces the amount of labor required for routine maintenance of cell cultures, it also minimizes aberrant plant phenotypes caused by culture-induced somaclonal variation thereby favoring transgenic plant fertility (9). A practical drawback to using immature embryos for transformation is the dependency on year-round, high quality greenhouse space for growing embryo donor plants. Perhaps the greatest hurdle to using maize IZEs for transformation is achieving an adequate Type I or Type II embryogenic callus induction frequency (ECIF) in a targeted explant population (9, 13, 15, 16). While high ECIF does not guarantee success (13, 17) it is a necessary prerequisite for achieving a robust transformation protocol using the IZE. In maize, the frequency of embryogenic callus induction is genotype specific (18–20) and influenced by factors such as tissue culture media components (4, 19, 21, 22), embryo size (23), and environmental conditions of the embryo donor plants (23). Transformation and cocultivation parameters can themselves affect ECIF and need to be optimized while maintaining adequate ECIF after transgene delivery (9, 12, 15, 16, 24). Maize genotypes which exhibit high ECIF (~100% Type I or Type II callus phenotype) in culture such as the hybrid genotype Hi Type II or Hi II (25), and inbred lines A188 or H99 (20) have been successfully transformed using super-binary (10, 14, 16) or standard-binary (12, 26) *Agrobacterium* vectors to direct-target IZEs. Efforts to extend these routine transformation protocols to elite or diverse inbred lines have focused on breeding responsiveness into the genotype of choice (27), optimizing an array of transformation parameters (14, 28, 29), or altering culture media components to improve ECIF (13,17) or transformation frequency (TF) (12–14, 16, 26).

This chapter describes side by side protocols for using a standard-binary *Agrobacterium* vector and two media regimes to transform IZEs from two distinct maize genotypes: the Hi II hybrid line (25) and inbred line B104 (30).

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2. Materials

2.1. Plant Materials

1. Hi II. F_1 seed of the hybrid Hi II line (25) is produced in the field (Ames, IA) each summer by pollinating Hi II parent A silks with Hi II parent B pollen (Hi II pA x Hi II pB, see Note 1). These two parent seed germplasms were obtained from the Maize Genetics Coop (https://maizecoop.cropsci.uiuc.edu/request/). F_2 IZEs used for all Hi II transformation experiments are produced from sib-pollinated F_1 plants grown year round in the ISU Plant Transformation Facility greenhouse in Ames, Iowa as described in our greenhouse protocol at: http://www.agron.iastate.edu/ptf/protocol/Greenhouse%20Protocol.pdf. Nine (in summer) to eleven (in winter) day-old ears are harvested when embryo size is between 1.2 and 1.8 mm. After harvest, maize ears (in their husks and inside their pollination bag) are stored in the refrigerator (4°C) in a loosely sealed dark plastic bag. Ears are stored for at least 1 and at most 4 days before being used for *Agrobacterium*-mediated genetic transformation experiments (see Notes 2 and 3).
2. B104. Greenhouse or field grown (see Note 4) embryo donor ears of maize inbred line B104 (30) are harvested 10 (from summer greenhouse) to 13 (from summer field) days after cross pollination when IZEs are 1.5–2 mm long (see Note 5). B104 seed can be obtained from the Iowa State University Committee for Agriculture Development (http://www.ag.iastate.edu/centers/cad/corn.html). Greenhouse care of B104 plants and storage of ears after harvest are identical to that described for Hi II (see Note 6).

2.2. Plasmids and A. tumefaciens Strains Used for Hi II and B104 Genetic Transformation

The cloning vector used routinely for Hi II and B104 *Agrobacterium*-mediated transformation of IZEs is pTF101.1 (31) – a derivative of the pPZP binary vector with a broad host range pVS1 origin of replication (32). pTF101.1 is an 11.6 kb standard binary vector in *A. tumefaciens* strain EHA101 (33) and contains a spectinomycin-resistant marker gene (*aadA*) for bacterial selection. In this vector, the herbicide resistant *bar* selectable marker gene (34) is driven by the cauliflower mosaic virus (CaMV) double 35S promoter (2×P35S). The tobacco etch virus (TEV) translational enhancer (35) was inserted at the 5′ end of the *bar* gene and the soybean vegetative storage protein terminator (36) was cloned to its 3′ end. A multiple cloning site for introducing any gene of interest (GOI) into pTF101.1 between the right border region and the plant selectable marker gene carries unique restriction sites for *Bam*H I, *Eco*R I, *Hin*d III, *Sac* I, *Sma* I, and *Xba* I (see Note 7). The stock solutions and culture media for *A. tumefaciens* are as follows.

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1. Spectinomycin sulfate (Sigma, St. Louis, MO, USA): 100 mg/mL stock in ddH_2O. Sterilize by filtration through a 0.2 µm membrane (Fisher Scientific Inc, Pittsburgh, PA, USA), aliquot (0.05 mL) and store at –20°C for up to 6 months (see Note 8).
2. Kanamycin sulfate (Sigma): 10 mg/mL stock in ddH_2O. Sterilize by filtration. Dispense in 0.25 mL aliquots in eppendorf tubes and store at –20°C for up to 6 months.
3. YEP medium (37): 5 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl, pH 6.8. For solid medium, add 15 g/L Bacto agar. Appropriate antibiotics are added to autoclaved medium after it cools to 50°C. For the strain EHA101 containing pTF101.1, the final antibiotic concentrations are: 50 mg/L kanamycin (for maintaining of the disarmed Ti plasmid pEHA101), 100 mg/L spectinomycin (for maintaining the binary vector plasmid pTF101.1).

2.3. Culture Media for Maize Transformation

2.3.1. Stock Solutions for Transformation of Hi II and B104

1. N6 vitamin stock (38): 1.0 g glycine, 0.5 g thiamine HCl, 0.25 g pyridoxine HCl, and 0.25 g nicotinic acid are dissolved in 500 mL ddH_2O. This stock solution (1,000×) is filter sterilized, and stored at –20°C in 40 mL aliquots, which are thawed and used over a period of weeks as needed.
2. MS vitamin stock (39) (modified, see Note 9): 1.0 g glycine, 0.25 g thiamine HCl, 0.25 g pyridoxine HCl, and 0.025 g nicotinic acid are dissolved in 500 mL ddH_2O. This stock solution (1,000×) is filter sterilized, and stored at –20°C in 40 mL aliquots which are thawed and used over a period of weeks.
3. 2,4-D: 200 mg of powdered 2,4-dichlorophenoxyacetic acid (2,4-D) is dissolved in 5 mL of 1 N KOH on low heat and brought up to a final volume of 200 mL with ddH_2O. The stock solution (1 mg/mL) is stored at 4°C (see Note 10).
4. Dicamba: 0.0663 g of Dicamba (3,6,dichloro-*o*-anisic acid) is dissolved in 1 mL 1 N KOH on low heat and brought up to a final volume of 10 mL with ddH_2O. The stock solution (30 mM) is stored at 4°C.
5. Bialaphos: 100 mg of Bialaphos (Gold Biotechnology, Duchefa, St. Louis, USA) is dissolved in 100 mL of ddH_2O. The stock solution (1 mg/mL) is filter sterilized and stored at 4°C for up to 6 months.
6. Glufosinate: 100 mg of glufosinate ammonia is dissolved in 100 mL of ddH_2O. The stock solution (1 mg/mL) is filter sterilized and stored at 4°C for up to 6 months.
7. Acetosyringone (AS): 0.392 g of AS is dissolved in 10 mL of dimethyl sulfoxide (DMSO). This solution is diluted 1:1 with

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ddH_2O and filter-sterilized. Aliquots (0.5 mL) of stock solution (100 mM) are stored at –20°C for up to 6 months (see Note 11).

8. Cysteine: 500 mg of L-cysteine (Sigma) is dissolved in 5 mL of ddH_2O. The stock solution (100 mg/mL) is filter sterilized and added the same day to autoclaved, cooled cocultivation medium for a final concentration of 300 mg/L. Any unused stock solution is discarded.
9. Silver Nitrate: 0.85 g of silver nitrate is dissolved in 100 mL of ddH_2O. The stock solution (50 mM) is filter sterilized and stored in a foil-wrapped duran at 4°C for up to 1 year.
10. Cefotaxime: 1.0 g of cefotaxime (Phytotechnology Laboratories, Overland Park, KS, USA) is dissolved in 5 mL ddH_2O. The stock solution (200 mg/mL) is filter sterilized, aliquoted (0.250 mL) and stored at –20°C for up to 1 month.
11. Vancomycin: 1.0 g of vancomycin hydrochloride (Phytotechnology Laboratories) is dissolved in 5 mL ddH_2O. The stock solution (200 mg/mL) is filter sterilized, aliquoted (0.250 mL), and stored at –20°C for up to 1 month.
12. Carbenicillin: 1.0 g of carbenicillin (Phytotechnology Laboratories) is dissolved in 10 mL ddH_2O. The stock solution (100 mg/mL) is filter sterilized, aliquoted (1.25 mL) and stored at –20°C for up to 3 months (see Note 12).

2.3.2. Media for Agrobacterium-Mediated Transformation of Hi II

Media 1–5 are after Zhao et al. (14) with the addition of cysteine (300 mg/L) to cocultivation medium and the use of cefotaxime and vancomycin instead of carbenicillin for counter-selection of *Agrobacterium* after cocultivation. Solid media (Media 2–5) use 100×25 mL Petri plates and are stored at room temperature.

1. Infection (liquid): 4 g/L N6 salts (38), 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, pH 5.2. This medium is filter sterilized and stored at 4°C. AS (100 µM) is added prior to use.
2. Cocultivation (see Note 13): 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 (bioWorld PlantMedia, Dublin, OH, USA), pH 5.8. Filter sterilized N6 vitamin stock (1 mL/L), silver nitrate (5 µM), AS (100 µM), and L-cysteine (300 mg/L) are added after autoclaving.
3. Resting: 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)-ethanesulfonic acid (MES), and 8 g/L purified agar (Sigma), pH 5.8. Filter sterilized N6 vitamin stock (1 mL/L), cefotaxime (100 mg/L), vancomycin (100 mg/L), and silver nitrate (5 µM) are added after autoclaving (see Notes 14 and 15).

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4. Selection 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, pH 5.8. Filter sterilized N6 vitamin stock (1 mL/L), cefotaxime (100 mg/L), vancomycin (100 mg/L), silver nitrate (5 μM), and bialaphos (1.5 mg/L) are added after autoclaving.
5. Selection II: The same as Selection I except that bialaphos concentration is increased to 3 mg/L.
6. Pre-regeneration medium (see Note 16): 4.3 g/L MS Salts (39), 1 mL/L (1,000×) MS vitamin stock (modified), 100 mg/L myo-inositol, 0.25 mL/L 2,4-D, 30 g/L sucrose, 3 g/L gelrite, pH 5.8. Filter-sterilized bialaphos (2 mg/L) and cefotaxime (100 mg/L) are added after autoclaving. Use 100×15 Petri plates.

2.3.3. Media for Agrobacterium-Mediated Transformation of B104 Inbred Line

All solid media described below use 100×25-mm Petri plates and are stored at room temperature. Media is modified from Carvalho et al. (22) and L-cysteine (300 mg/L) is added to the cocultivation medium.

1. Infection (liquid): 4.3 g/L MS salts, 1 mL/L modified MS vitamin stock, 0.5 mL/L dicamba, 0.7 g/L L-proline, 68.4 g/L sucrose, and 36 g/L glucose, pH 5.2. This medium is filter sterilized and stored at 4°C. AS (100 μM) is added prior to use (see Note 17).
2. Cocultivation: 4.3 g/L MS salts, 0.5 mL/L dicamba, 0.7 g/L L-proline, 100 mg/L casein hydrolysate, 100 mg/L myo-inositol, 30 g/L sucrose, and 2.3 g/L Gelrite, pH 5.8. Filter sterilized modified MS vitamin stock (1 mL/L), silver nitrate (88 μM), AS (100 μM), and L-cysteine (300 mg/L) are added after autoclaving (see Note 18).
3. Resting: 4.3 g/L MS salts, 0.5 mL/L dicamba, 0.7 g/L L-proline, 0.5 g/L MES, 100 mg/L casein hydrolysate, 100 mg/L myo-inositol, 30 g/L sucrose, and 2.3 g/L Gelrite, pH 5.8. Filter sterilized modified MS vitamin stock (1 mL/L), silver nitrate (88 μM), and carbenicillin (250 mg/L) are added after autoclaving.
4. B104 Selection I: 4.3 g/L MS salts, 0.5 mL/L dicamba, 0.7 g/L L-proline, 0.5 g/L MES, 100 mg/L casein hydrolysate, 100 mg/L myo-inositol, 30 g/L sucrose, and 2.3 g/L Gelrite, pH 5.8. Filter sterilized modified MS vitamin stock (1 mL/L), silver nitrate (88 μM), bialaphos (2 mg/L), and carbenicillin (250 mg/L) are added after autoclaving (see Note 19).
5. B104 Selection II: The same as B104 Selection I medium except that the bialaphos concentration is increased to 6 mg/L.

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2.4. Culture Media for Regeneration of Hi II and B104

Regeneration media, after McCain et al. (40), uses 100×25 mL Petri plates and is stored at room temperature.

1. Regeneration I: 4.3 g/L MS salts, 1 mL/L modified MS vitamin stock, 100 mg/L myo-inositol, 60 g/L sucrose, 3 g/L Gelrite, pH 5.8. Filter-sterilized glufosinate ammonia (6 mg/L) and cefotaxime (100 mg/L) are added after autoclaving.
2. Regeneration II: The same as Regeneration I with the sucrose concentration reduced to 30 g/L and no glufosinate or cefotaxime is added.

2.5. Equipment

1. Horizontal laminar flow benches (The Baker Company, Sanford, ME, USA)
2. Dark biological incubator (I36NL, Percival Scientific, Perry, IA, USA)
3. Light biological incubator (Cu36L5, Percival Scientific)
4. Steriguard 350 bead sterilizers (Inotech Biosystems International, Rockville, MD, USA).
5. Vortex Genie (Fisher Scientific, USA)

3. Methods

3.1. Agrobacterium-Mediated Transformation of Immature Zygotic Embryos

3.1.1. Agrobacterium Preparation

1. The vector system, pTF101.1 in strain EHA101, is stored as a glycerol stock at –80°C.
2. Every 4 weeks, a "mother" plate is re-initiated from this long-term glycerol stock by streaking the bacteria to YEP (with antibiotics) and growing it for 2 days at 28°C.
3. The "mother" plate is then kept in the refrigerator (4°C) and used as a source plate for plating *Agrobacteria* cells (at 19°C for 3 days) in preparation for twice-weekly experiments (see Note 20).

3.1.2. Embryo Dissection

1. Dehusk the ear, break off the tip of the cob and insert a pair of numbered forceps. This labels the ear while acting as a "handle" for aseptic manipulation during dissection. In a laminar flow bench, place up to 15 prepared ears in a sterile, 4 L beaker. Do not use any ears exhibiting extreme tip rot or discolored kernels (see Note 21).
2. Add ~2 L of sterilizing solution (50% commercial bleach (6% hypochlorite) in ddH_2O + 1 drop of surfactant Tween 20 per liter) to completely submerge the ears while leaving the forceps handles protruding (see Note 22).

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3. During the 20-min disinfection, occasionally grasp forceps and swirl the ears in an effort to dislodge air bubbles. Pour off the bleach solution and rinse the ears three times using at least 2 L of sterile ddH_2O at each rinse. The final rinse is drained off and the beaker of ears is left (covered) in the bench until dissections begin.
4. Using aseptic technique, and working in a laminar flow bench, hold onto the end of the forceps, prop the surface-sterilized ear on a large (150×15 mm) sterile Petri-plate, and cut off the top 1–2 mm of the kernel crowns with a sharp scalpel blade. Steriguard 350 bead sterilizers are used for sterilization of utensils throughout this protocol.
5. To excise an embryo, insert the end of a sharpened spatula between the endosperm and pericarp at the basipetal side of the kernel and pop the endosperm out of the seed coat. The embryo axis side of the untouched embryo will be visible and the scutellum side will be nested in the endosperm. Gently coax the IZE onto the spatula tip and transfer it directly to liquid infection medium (see Note 23).

3.1.3. Agrobacterium Infection

1. Grow *Agrobacterium* cultures for 3 days at 19°C (or 2 days at 28°C) on solid YEP medium amended with antibiotics.
2. To begin an experiment, scrape one full loop (3 mm) of bacteria culture from the plate and suspend it in 5 mL infection medium supplemented with 100 µM AS in a 50 mL Falcon tube. Affix the tube horizontally to a Vortex Genie (Fisher) platform head using lab tape and shake on lowest setting for 2 h at room temperature. Using liquid infection medium (with AS), adjust to between OD_{550}=0.30 and 0.40 just prior to use.
3. Once this 2 h *Agrobacterium* pre-culture step is complete, dissect up to 100 IZEs directly into a 2-mL Eppendorf tube filled with *Agrobacterium*-free infection medium (with 100 µM AS). These wash tubes are prepared 2 h ahead of time and stored at 4°C until dissection begins.
4. Remove this first wash then wash the embryos a second time with 1 mL of the same medium. After removing the final wash, add 1 mL of *Agrobacterium* suspension (OD_{550}=0.30–0.40).
5. To infect the embryos, gently invert the tube 20 times before resting it on its side (in the dark) for 5 min with embryos submerged in the *Agrobacterium* suspension (see Note 24). These and all subsequent tissue culture steps are carried out in a laminar flow bench using aseptic technique.

3.1.4. Co-Cultivation

1. After the 5 min infection, use a 1-mL Pipetman equipped with a wide-bore pipet tip (see Note 25) to gradually transfer the embryos, along with a minimum amount of *Agrobacterium* suspension, out of the Eppendorf tube and onto the surface of the cocultivation medium. Embryos are collected, a few at

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a time, with minimal liquid uptake at each transfer to avoid adhesion of the embryos to the inside of the pipet tip.

2. When embryo transfer is complete, use a 1-mL tip to remove excess *Agrobacterium* suspension from the surface of the co-cultivation medium and the area surrounding each embryo. Collect the used bacterial suspension in a disposable Petri-dish (see Notes 26 and 27).
3. Leave the lid of the cocultivation plate ajar for up to 1 h to let the medium and embryo surfaces dry further before orienting each embryo scutellum side up with the aid of a stereo microscope.
4. Wrap plates with vent tape (air permeable adhesive tape) and incubate at 20°C (dark) for 3 days in a biological incubator.

3.1.5. Resting

1. After 3 days cocultivation, transfer all embryos to resting medium at 28°C (dark) for 7 days.
2. Continue to transfer all embryos throughout the following selection steps. Do not discard any embryos prematurely. Tissue culture plates are incubated in a biological chamber throughout resting and selection steps.

3.2. Selection for Stable Transformation Events

3.2.1. Hi II Events

1. After 7 days on resting medium (see Note 28), use sterile forceps to transfer embryos to Selection I medium (35 IZEs per plate) containing 1.5 mg/L bialaphos, for 2 weeks followed by two more 2-week passages on Selection II medium (3 mg/L bialaphos). Plates are wrapped with Parafilm® and incubated at 28°C in the dark. All Hi II selection steps are done without the aid of a stereo microscope.
2. As early as five and as late as 10 weeks after infection, putative Type II callus events are visible (with the naked eye) emerging from a subset of embryos.
3. Putative events are transferred away from the original experiment plate to a fresh plate of Selection II medium for an additional, 2-week subculture to verify that they are bialaphos resistant. We refer to this latter step as "picking" putative events.
4. If a putative callus event continues to grow rapidly, it is assigned an ID number. Callus events containing stalked somatic embryos (prescreened with the aid of a stereo microscope) are subcultured, one event per plate, to Pre-regeneration medium.
5. If the diameter of the callus clump is greater than 2 cm at this stage, it is divided into smaller pieces (1 cm) at transfer. Petri plates are wrapped with Parafilm® and incubated in the dark (25°C) for 10–14 days.
6. Average TF for Hi II using this protocol is 8%, or 8 independent, bialaphos resistant Type II callus per 100 infected (and selected) IZEs.

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3.2.2. B104 Events

1. After 7 days on B104 resting medium (see Note 29), transfer all embryos to B104 Selection I medium (35 IZEs per plate) containing 2 mg/L bialaphos, for 2 weeks followed by two more 2-week passages on B104 Selection II medium containing 6 mg/L bialaphos. Plates are wrapped with Parafilm® and incubated in the dark (28°C).
2. As early as six, and as late as 12 weeks after infection, putative Type I callus events are visible emerging from a subset of selected IZEs (see Note 30).
3. Putative events are transferred away from the original experiment plate to a fresh plate of B104 Selection II medium for 2 additional weeks. Continued vigorous proliferation after this "picking" step verifies that the event is bialaphos resistant.
4. Unlike the corresponding Hi II step in which the clump of Type II callus representing one putative event is kept intact, when a B104 putative event is picked, embryogenic Type I callus is separated from non-embryogenic callus lobes and differentiating leaf or root portions of the callus clump with the aid of a stereo microscope. Only the embryogenic callus is retained and broken into 0.25 cm pieces on the surface of a fresh plate of B104 Selection II medium.
5. After 2 weeks, the Type I embryogenic callus proliferating from some or all of these pieces is regrouped and subcultured in 0.5 cm pieces, again using the stereo microscope, to the surface of B104 Selection II medium in preparation for naming and regeneration.
6. Average TF for B104 using this protocol is 3%, or 3 independent, bialaphos-resistant Type I calluses per 100 infected (and selected) IZEs.

3.3. Regeneration of Transgenic Plants

3.3.1. Hi II

1. With the aid of a stereo microscope, use sterile scalpels or needle nose forceps to transfer 12–15 small pieces (4 mm) of somatic embryo-enriched callus from Pre-regeneration medium to Regeneration I medium. Wrap plates with vent tape and incubate at 25°C (dark, see Note 31).
2. After 2 weeks on Regeneration I medium, somatic embryos appear swollen, opaque and white. In some cases, the coleoptile is already visible emerging from these germinating, somatic embryos.
3. Use a stereo microscope to transfer ~12 individual, mature somatic embryos from Regeneration I medium to the surface of Regeneration II medium for germination in a lighted biological incubator (25°C, 80–100 $\mu E/m^2/s$ light intensity, 16:8 photoperiod).
4. Wrap Petri plates with vent tape. Hi II plantlets sprout leaves and roots on this medium within 1 week and are ready for

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transfer directly to soil about 3 days later (10 days after transfer to Regeneration II medium).

3.3.2. B104 (see Note 32)

1. Using a stereo microscope, transfer 15–20, 5 mm embryogenic Type I callus pieces (pried apart, not cut) from the surface of B104 Selection II medium to the surface of Regeneration I medium. Multiple somatic embryos may be fused together in one piece of callus. Wrap Petri plates with vent tape and incubate in the dark (25°C).
2. After 3 weeks, the majority of callus pieces will produce one or more mature somatic embryos. Like the corresponding Hi II regeneration step, the B104 mature somatic embryos will appear opaque and white, but unlike Hi II, they will form at lower frequency and will, in many cases, be fused together.
3. Using a stereo microscope, pry these mature somatic embryos apart from any unhardened callus and from each other where possible without damaging embryo integrity.
4. Transfer these pieces (fused or not), 15 per plate, to Regeneration II medium for germination in the light (25°C, 80–100 $\mu E/m^2/s$ light intensity, 16:8 photoperiod). Germinated B104 plantlets with roots and shoots are ready for transfer to soil from between 7 and 14 days later (see Notes 33 and 34).

3.4. Growth Chamber and Greenhouse Plant Care

1. A detailed protocol for growing immature embryo donor plants from seed, and for growing regenerated transgenic plantlets to maturity, can be found in the ISU Plant Transformation Facility greenhouse protocol at: http://www.agron.iastate.edu/ptf/protocol/Greenhouse%20Protocol.pdf. Our greenhouse is located in Ames, IA, USA.
2. While this protocol provides helpful guidelines for growing greenhouse maize, it should be noted that conditions for success will vary depending on location and greenhouse conditions.

4. Notes

1. These two parents may differ in vigor. Multiple plantings of both parents ensure constant availability of parent A silks × parent B pollen for the F_1 cross.
2. In general, greenhouse derived Hi II IZEs transform at higher rates (using *Agrobacterium*-mediated methods) than do field embryos using this protocol, although transgenic events have been recovered from both sources.
3. We generally recover at least 120 IZEs from one greenhouse-grown Hi II ear.

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4. In general, greenhouse derived IZEs of B104 transform at higher rates using *Agrobacterium*-mediated methods than do field embryos using this protocol, although transgenic events have been recovered from both.
5. Post-infection ECIF for 1.2 mm B104 embryos is lower than for 1.5 mm B104 embryos or 1.2 mm Hi II embryos.
6. We generally recover ~150 IZEs from one greenhouse-grown B104 ear.
7. We have also used strains LBA4404 and GV3101 with this vector with varying degrees of success.
8. Spectinomycin may come out of solution in the freezer and must be resuspended after thawing and before using.
9. Modified MS vitamins (13) contain higher thiamine HCl and lower nicotinic acid concentrations compared to MS vitamins.
10. Use low heat. Do not boil the 2,4-D while dissolving it in KOH.
11. AS will sometimes precipitate after freezer storage and is re-dissolved by vortexing for 15 min.
12. Carbenicillin efficacy may vary by lot number.
13. Cocultivation medium is either 1 or 4 days old when used.
14. Resting medium is made in small batches to ensure that it is as fresh as possible at use (<3 weeks old).
15. Vancomycin and cefotaxime will form a precipitate if mixed together. Add each to media separately and stir well after adding.
16. This medium is used to slow Hi II callus growth and encourage somatic embryo formation.
17. This is our current liquid infection medium for B104 and is modified from Frame et al. (13).
18. This cocultivation media is also used at 1 or 4 days old and when solidified is hazy compared to Hi II cocultivation medium. Stir well before pouring.
19. This is our current selection scheme for B104 and is modified from Frame et al. (13).
20. We compared the effect of using refrigerator-stored (4°C) *Agrobacterium* mother plates, or –80°C stored glycerol stocks to initiate the 19°C/3 day bacteria plate used for infection experiments. The average TF for embryos infected with the vector pTF102 (12) in EHA101 initiated from a 4°C mother plate was 6.4%. For embryos infected with *Agrobacteria* initiated from glycerol stock (–80°C), TF was 5.6%.
21. Pink kernels in particular may be an indication of bacterial contamination.
22. We reuse this bleach once and store it in the dark between uses.

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23. A skilled technician can dissect at least 150 IZEs per ½ h. Do not damage embryos at dissection or dig around the ear for embryos that are not easily retrieved, as this increases the probability of introducing contamination into your experiment tube.
24. We do not leave embryos in the wash for extended periods of time. Washing, infection and plating to cocultivation medium steps are all carried out without interruption.
25. One mL filtered pipet tips are trimmed using scissors to make a 3 mm bore hole and re-autoclaved before using.
26. This waste, along with all tissue culture plates, medium (liquid or solid), or plant tissues exposed to *Agrobacterium* and the genetically modified DNA it contains are autoclaved as biohazard waste before disposal.
27. If infecting multiple constructs on the same day, be sure to discard the *Agrobacterium*-liquid disposal dish between constructs so that no back splashing occurs; this may result in cross contamination between constructs.
28. After 1 week on resting medium, ECIF for *Agrobacterium*-infected Hi II embryos cocultivated on medium containing 300 mg/L cysteine is ~85%.
29. After 1 week on resting medium, ECIF for *Agrobacterium*-infected B104 embryos cocultivated on medium containing 300 mg/L cysteine is ~70% (13).
30. B104 Type I putative callus events grow vigorously and often appear to "dig into" the medium surface.
31. Do not overfill the plate and keep pieces small and enriched with stalked embryos – the key to this regeneration method is to induce differentiation of the preformed somatic embryos through desiccation and slowed growth.
32. The regeneration method and media described here were reported in Frame et al. (13) and take 35 days to recover transgenic plants. Regeneration protocol comparisons carried out since 2006 using non transgenic B104 callus indicate that 5 plantlets per plate can be regenerated within 15 days using a regeneration protocol modified from Zhao et al. (14) in which the media includes 6% sucrose and zeatin, and for which all regeneration steps are carried out in the light. To date, we have not compared these regeneration protocols using transgenic B104 callus.
33. Plantlet recovery for B104 may require in vitro pruning. Subculture sprouting plantlets to a fresh plate of Regeneration II medium after 10 days in the light to encourage maximum plantlet recovery.
34. To confirm that the *bar* gene is expressed in B104 regenerated plants, 2–3 weeks after being taken to soil, plantlets are sprayed with 500 mg/L glufosinate prepared from the herbicide Liberty® (Bayer Crop Sciences, USA) and 0.1% Tween 20 (v/v).

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