Tissue culture protocols for gene transfer and editing in maize (Zea mays L.)

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Abstract  Efficient methods for gene transfer to maize were developed in the 1990s, first mediated by particle bombardment and then by Agrobacterium tumefaciens. Both methods can efficiently create high-quality events. Genetically modified varieties were commercialized in 1996 and are now planted in more than 90% of the US corn field. Tissue culture protocols for both methods have been well developed and widely employed. Thus, various factors, including handling before gene delivery, techniques to protect cells during gene delivery, and culture media, have been well optimized for various genotypes. Typical protocols for both methods are herein presented to show major outputs from the studies conducted since the early 1990s. As the bombardment protocols tended to be optimized specifically for limited genotypes, the one for B104, a new public inbred with favorable agronomic characteristics, is shown. The Agrobacterium protocol is suitable for various inbred lines, including B104. These protocols are also useful starting points in the optimization of tissue culture for gene editing. The rate-limiting step in both transformation and gene editing is in tissue culture and plant regeneration from modified cells in elite germplasm. Despite the prolonged efforts, large varietal differences in tissue culture responses remain a serious issue in maize. Recently, protocols using morphogenic regulator genes, such as Bbm and Wus2, have been developed that show a strong potential of efficiently transforming recalcitrant varieties.

Key words: gene editing, tissue culture protocol, transformation, Zea mays L.

Introduction

Maize (Zea mays L.) is one of the three most important crops, besides wheat and rice, to provide the staple food for the world population. While wheat is ranked first in the production acreage, and rice is the crop most extensively studied in molecular biology, maize is the leading cereal in biotechnology, an essential component of the efforts to sustain and elevate grain production to underpin the rapidly growing human population. Commercialization of genetically modified maize varieties started in 1996, and currently, more than 90% of the US corn field is planted with such varieties (https://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us). Thus, advancement in the technologies for gene transfer and editing is especially important in maize.

Efficient methods for gene transfer to maize were developed in the 1990s, first mediated by particle bombardment (Gordon-Kamm et al. 1990). Thus, early commercial varieties of genetically modified maize were transformed by this method, which is purely a physical process for delivering DNA, by gold, tungsten, or other types of particles coated with DNA to plant cells. Standard, simple, high copy number vectors are satisfactory for cloning and propagation of the genes of interest to be transferred to maize. Non-target DNA, such as vector backbones, is easily removed by enzymatic digestion before the delivery. Particle bombardment is also a convenient tool in the studies of transient expression of foreign genes in plant cells and tissues. However, plants transformed by particle bombardment tend to have high copy numbers of transgenes often rearranged in a complex manner (Pawlowski and Somers 1996).

Several years later, an efficient transformation protocol mediated by the soil bacterium Agrobacterium tumefaciens was reported (Ishida et al. 1996). The revelation that A. tumefaciens could transfer relatively large DNA segments with defined ends to plant cells with few rearrangements (Hooykaas and Schilperoort 1992), meant gene transfer by A. tumefaciens quickly became a standard method. The more recently commercialized transgenic maize varieties are mostly transformed by A. tumefaciens. However, compared with particle bombardment, Agrobacterium-mediated gene transfer is a complicated biological process. Dedicated transformation vectors, which tend to be large,
are needed. Some plant species and varieties may be sensitive to infection with the bacteria. These advantages and disadvantages of the two methods have been comprehensively discussed by Wang et al. (2009).

In this review, progress in maize tissue culture technology, which is a core component of gene transfer and editing processes, for both particle bombardment and Agrobacterium-mediated methods is overviewed, and typical protocols for creation of transgenic plants are presented for both of the methods to depict an entire picture of the procedures refined by years of cumulative efforts by a number of dedicated laboratories. In addition, tissue culture protocols for gene editing, and recent attempts to exploit morphogenetic regulator genes for tissue culture of recalcitrant genotypes are discussed.

**Progress in tissue culture for gene transfer to maize**

Monocotyledonous plants were considered to be outside the host range of crown gall disease caused by *A. tumefaciens*. Due to a general belief that maize could not be transformed by the bacteria, direct gene transfer to maize cells was extensively studied. The Bio-Rad PDS-1000/He biolistic gun has been popularly employed since the late 1980s (Klein et al. 1987) to the present date. Tissue culture techniques were well developed for various target cells/tissues, including protoplasts, calli, cell suspension cultures, and immature embryos. Eventually, immature embryos, which are the best source of actively growing, undifferentiated cells capable of regenerating plants, became the most preferred tissue for particle bombardment (Wang et al. 2009). In a sense, studies of particle bombardment paved the way for success for Agrobacterium-mediated transformation in maize because the hurdle for Agrobacterium-mediated transformation was overcome by the power of tissue culture and the choice of the starting tissue, immature embryos (Ishida et al. 1996).

The quality of the starting cells/tissues is the most important factor for tissue culture. Achieving highly efficient maize transformation relies heavily on the quality of the immature embryos (Ishida et al. 2007). Good embryos are collected only from healthy plants grown in a well-conditioned greenhouse. Air-conditioning and supplemental lights are needed to ensure a year-round supply of good embryos. Inevitably, immature embryos are quite expensive materials, as dedicated facilities and teams of greenhouse/tissue culture technicians are minimal requirements. Another prerequisite is selecting immature embryos at the right developmental stage. The developmental stage can be conveniently monitored by the size of the embryos, although the time to reach a certain stage is influenced by many factors.

Notable progress has been made in both particle bombardment and Agrobacterium-mediated transformation since the 1990s, and the two protocols described below are outputs from cumulative efforts for optimization. A brief comparison of the protocols reveals that while the gene delivery steps are quite distinctive, many steps, especially those involved in the immature embryo preparation and the selection of transformed cells, for which phosphinothricin is the most effective selective agent for maize, but also the later procedures, are common to both protocols. Several modifications were devised to overcome specific problems in each method, and for general improvement in the viability of and plant regeneration from the cells. For example, osmotic treatments (Vain et al. 1993) and pre-culture of immature embryos (Songstad et al. 1996) before particle bombardment greatly improved survival and transformation efficiency, and a reduction in the size of gold particles minimized damage to targeted cells (Frame et al. 2000). For Agrobacterium-mediated transformation, transformation efficiency varied considerably depending on the combination of the bacterial strains and transformation vectors (Ishida et al. 1996). Meanwhile, adding silver nitrate to the culture medium for selection of transformed cells and using carbenicillin instead of cefotaxime to eliminate bacteria after infection proved effective in the transformation of an inbred sensitive to infection with *A. tumefaciens* (Ishida et al. 2003). In subsequent research on Agrobacterium-mediated transformation, Ishida et al. (2007) noted that adding copper sulfate to the medium when regenerating transformed plant material, improved gene introduction efficiency and transformation efficiency, as well as promoted the growth of transformed plants. Besides, the copper-enriched medium was also useful for particle bombardment (unpublished results). Hiei et al. (2006) improved the transformation frequency several-fold by pretreating immature embryos with centrifugation and heat before infection with *A. tumefaciens*. Although the mechanisms behind the improvement are not yet understood, it appeared that the normal development of shoots and roots from the embryos was suppressed, and undifferentiated growth of the cells was promoted. The centrifugation treatment was also effective in particle bombardment (unpublished results).

Despite the progress, varietal differences in transformation efficiency remain a major issue in both methods. Especially, commercially grown crops of so-called elite genotypes are quite recalcitrant in tissue culture and transformation. In the earlier phase of research, only genotypes, such as A188 and Hi-II, were transformable. Subsequent and continuous painstaking efforts have been made to overcome this issue for both methods. The protocol described here for Agrobacterium-mediated transformation is suitable,
at least for some inbred lines. Modifications favorable for the transformation of a certain genotype mediated by *Agrobacterium* tended to be beneficial for other genotypes too. Conversely, adjustments of various parameters for particle bombardment, including type and size of the particles, pre-culture conditions, helium pressure, and media composition, seemed to be specific to limited genotypes (O’Kennedy et al. 2001; Petrillo et al. 2008; Raji et al. 2018; Shiva Prakash et al. 2008; Wright et al. 2001). Thus, the protocol below for particle bombardment was proven successful only in B104, which is a relatively new, public inbred with reasonably desirable agronomic characteristics (Iowa State University Digital Repository: http://lib.dr.iastate.edu/agron_pubs/235). B104 may be efficiently transformed by *A. tumefaciens* by the protocol below, as well.

**Typical Protocol 1: *Agrobacterium*-mediated gene transfer**

The essence of the following protocol was reported by Ishida et al. (2007). The protocol is useful for a range of genotypes, including A188, A634, H99, B104, and W117.

**Strain and vector**

*Agrobacterium tumefaciens* strain LBA4404(pSB131) is employed (Ishida et al. 1996). pSB131 has a phosphinothricin-resistance gene (*bar*) and a β-glucuronidase (GUS) gene that carries an intron in the coding sequence (*intron-gus*) in the T-DNA and *virB* and *virG* from pTiBo542, which give higher efficiency of transformation, in the vector backbone.

**Media**

All media are listed in Table 1.

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**Table 1. Medium formulations.**

| Medium | Use | Composition |
|--------|-----|-------------|
| YP | Growth of *Agrobacterium tumefaciens* | 5 g l⁻¹ yeast extract, 10 g l⁻¹ peptone, and 5 g l⁻¹ NaCl, pH to 6.8, 15 g l⁻¹ agar |
| LS-inf | Preparation of immature embryos | MS inorganic salts, modified LS vitamins (100 mg l⁻¹ myo-inositol, 1 mg l⁻¹ thiamine hydrochloride, 0.5 mg l⁻¹ pyridoxine hydrochloride, 0.5 mg l⁻¹ nicotinic acid), 1.5 mg l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), 68.46 g l⁻¹ sucrose, 36.64 g l⁻¹ glucose, 1.6 g l⁻¹ casamino acids, pH to 5.2, sterilize by filtering through a 0.22-µm cellulose–acetate filter |
| LS-inf-AS | Infection | Add 1 ml of 100 mM acetosyringone to 1 ml of LS-inf medium |
| LS–AS | Co-cultivation | MS inorganic salts, modified LS vitamins, 1.5 mg l⁻¹ dicamba, 5 µM CuSO₄·7H₂O, 20 g l⁻¹ sucrose, 10 g l⁻¹ glucose, 0.7 g l⁻¹ proline, 0.5 g l⁻¹ MES, pH 5.8, 8 g l⁻¹ agarose. Autoclave at 121°C for 15 min and cool to 50°C, and add 100 µM acetosyringone and 5 µM AgNO₃ poured in 90 mm Petri dishes |
| LSD1.5A | First selection of transformed cells | MS inorganic salts, modified LS vitamins, 1.5 mg l⁻¹ 2,4-D, 20 g l⁻¹ sucrose, 0.7 g l⁻¹ proline, 0.5 g l⁻¹ MES, pH 5.8, 8 g l⁻¹ agarose. Autoclave at 121°C for 15 min and cool to 50°C, and add 250 mg l⁻¹ carbenicillin (only for Protocol 1), 100 mg l⁻¹ cefotaxime (only for Protocol 1), 10 µM AgNO₃, 5 mg l⁻¹ phosphinothricin (Gold Biotechnology, Inc. P-165-250) for bar selection poured in 90 mm Petri dishes |
| LSD1.5B | Second and third selection of transformed cells | Identical to LSD1.5A medium, except for the amount of selective agent—10 mg l⁻¹ phosphinothricin for bar selection poured in 90 mm Petri dishes |
| LSZ | Regeneration of transformed plants | MS inorganic salts, modified LS vitamins, 5 mg l⁻¹ zeatin, 10 µM CuSO₄·7H₂O, 20 g l⁻¹ sucrose, 0.5 g l⁻¹ MES, pH 5.8, 8 g l⁻¹ agar. Autoclave at 121°C for 15 min. Cool to 50°C, and add 250 mg l⁻¹ carbenicillin (only for Protocol 1), 100 mg l⁻¹ cefotaxime (only for Protocol 1), 5 mg l⁻¹ phosphinothricin for bar selection poured in 90 mm Petri dishes |
| LSF | Rooting of transformed plants | MS inorganic salts, modified LS vitamins, 0.2 mg l⁻¹ indole-3-butyric acid, 15 g l⁻¹ sucrose, 0.5 g l⁻¹ MES, pH 5.8, 3 g l⁻¹ gellan gum. Autoclave at 121°C for 15 min. Cool to 50°C, and add 250 mg l⁻¹ carbenicillin (only for Protocol 1), 100 mg l⁻¹ cefotaxime (only for Protocol 1), 5 mg l⁻¹ phosphinothricin for bar selection poured in megenta boxes |
| Buffer P | GUS histochemical staining | 50 mM phosphate buffer, pH 6.8. Sterilize using a 0.22-µm cellulose–acetate filter and store at room temperature. Mix 9.9 ml of this buffer and 0.1 ml of Triton X-100 before use |
| Buffer X | GUS histochemical staining | Mix 8 ml Buffer P, 0.1 ml of 100 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide acid (X-Gluc) and 2 ml methanol just before use |
| MST | Pre-culture of immature embryos | MS inorganic salts, MS vitamins, 100 mg l⁻¹ vitamin assay casamino acid, 2 mg l⁻¹ 2,4-D, 30 g l⁻¹ sucrose, 0.7 g l⁻¹ proline, pH 5.8, 2.5 g l⁻¹ gellan gum. Autoclave and cool to 50°C, and add 5 µM AgNO₃ |
| MSTosm | Pre- and post-bombardment osmotic treatment | MS inorganic salts, MS vitamins, 100 mg l⁻¹ vitamin assay casamino acid, 2 mg l⁻¹ 2,4-D, 30 g l⁻¹ sucrose, 37 g l⁻¹ sorbitol, 37 g l⁻¹ mannitol, 0.7 g l⁻¹ proline, pH 5.8, 2.5 g l⁻¹ gellan gum. Autoclave and cool to 50°C, and add 5 µM AgNO₃ |
| MOT | Resting culture | MS inorganic salts, modified LS vitamins, 1.5 mg l⁻¹ dicamba, 5 µM CuSO₄·7H₂O, 30 g l⁻¹ sucrose, 0.7 g l⁻¹ proline, 0.5 g l⁻¹ MES, pH 5.8, 8 g l⁻¹ agar. Autoclave at 121°C for 15 min |
**Preparation of immature embryos**

1-1. Grow maize plants in 270-mm pots in a greenhouse. Keep day temperature between 30 and 35°C and night temperature between 20 and 25°C. A light intensity >1,000 µmol m⁻² s⁻¹ is desired. Usually, more than 150 kernels can be collected from a single ear of A188.

1-2. Harvest and husk an ear with immature embryos in the right developmental stage from the plants between 8 and 15 days after pollination (DAP). Immature embryos ranging between 1.0 and 1.2 mm in length along the axis are the best for transformation. Time (DAP) for embryos to reach the best stage differs, depending on the genotype and the season.

1-3. Detach kernels from the cob by cutting the base part of the kernel with a scalpel. Insert a scalpel into the detached kernels and remove the immature embryos from the kernel. Steps from 1-3 to 1-29 are performed aseptically.

1-4. Immerse the embryos in 2.0 ml LS-inf medium in a 2.2-ml microtube (Eppendorf Safe-Lock Tubes, 2.0 ml) at room temperature until the remaining embryos have been collected.

1-5. Shake the microtube at maximum speed by Vortex® for 5 s and remove the medium.

1-6. Add 2.0 ml of fresh LS-inf medium and shake as above.

**Pre-treatment with heat and centrifuging**

1-7. Incubate the microtube in a water bath at 46°C for 3 min.

1-8. Cool the microtube on ice for 1 min.

1-9. Remove the medium and add 2.0 ml of fresh LS-inf medium.

1-10. Centrifuge the microtube using a fixed-angle rotor with a maximum radius of 83 mm, at 15,000 rpm, 4°C for 10 min.

**Preparation of inoculum**

1-11. Culture LBA4404(pSB131) on a YP plate containing 50 mg l⁻¹ spectinomycin, in the dark at 28°C for 2 days.

1-12. Collect the bacteria with a loop and suspend in 1.0 ml of LS-inf-AS medium at a density of 1×10⁹ CFU ml⁻¹.

**Inoculation and co-cultivation**

1-13. Remove the medium from the microtube in step 1-10 and add 1.0 ml of the bacterial suspension prepared in step 1-12.

1-14. Shake the microtube at maximum speed by Vortex® for 30 s.

1-15. Let stand for 5 min at room temperature.

1-16. Transfer the suspension of the embryos and bacteria to a Petri dish (60×15 mm).

1-17. Remove 0.7 ml of the liquid from the suspension.

1-18. Place the embryos on LS–AS medium with the scutellum face up and seal the Petri dishes with Parafilm. Up to 200 embryos may be placed on a single plate.

1-19. Incubate at 25°C for 7 days in the dark (co-cultivation).

**Selection of transformed calli**

1-20. Transfer the embryos to LSD1.5A medium and seal the Petri dishes with surgical tape. Up to 25 embryos may be placed on a single plate.

1-21. Incubate at 25°C for 10 days in the dark (first selection).

1-22. Transfer the embryos to LSD1.5B medium and seal the Petri dishes with surgical tape. Up to 25 embryos may be placed on a single plate.

1-23. Incubate at 25°C for 21 days in the dark (second selection).

1-24. Cut type I calli, which are compact and non-friable cell clusters, proliferated from the scutellum into pieces of between 3 and 5 mm in diameter under a stereoscopic microscope, transfer to LSD1.5B medium, and seal the Petri dishes with surgical tape. Up to 25 pieces may be placed on a single plate.

1-25. Incubate at 25°C for 21 days in the dark (third selection).

**Regeneration of transformed plants**

1-26. Cut type I calli further proliferated into pieces of between 2 and 3 mm in diameter under a stereoscopic microscope, transfer to LSZ medium, and seal the petri dishes with Paraflm. Up to 25 pieces may be placed on a single plate.

1-27. Incubate under continuous illumination (50 µmol m⁻² s⁻¹) at 25°C for 14 days. Typically, green shoots of about 10 mm appear.

1-28. Transfer a regenerated shoot to a tube of LSF medium and cover with a polypropylene cap.

1-29. Incubate under continuous illumination (50 µmol m⁻² s⁻¹) at 25°C for 14 days. Typically, rooted plants grew to between 40 and 50 mm.

1-30. Transfer a plant to soil in a 230-mm pot.

1-31. Grow transgenic plants in a greenhouse, as in Step 1-1, and harvest T1 seeds.

**Examination of transient expression of GUS after co-cultivation (optional for a strain that carries a GUS gene)**

1-32. Interrupt Step 1-19 after 3 days of inoculation, immerse the embryos in 0.5 ml of Buffer P in a microtube and incubate at 37°C for 1 h.

1-33. Remove the buffer and add 0.3 ml of Buffer X.

1-34. Place under mild vacuum (half an atmosphere) for 5 min.

1-35. Incubate at 37°C for 18 h.

1-36. Examine embryos for change in the color under
Typical Protocol 2: Gene transfer by particle bombardment

The steps of the bombardment of the following protocol were developed for genotype B104 by Raji et al. (2018). The steps of preparation of immature embryos and culture after the bombardment were modified by the authors.

Media
All media are listed in Table 1.

Preparation of immature embryos and pre-treatment with centrifugation
2-1. Perform Steps from 1-1 to 1-6, and Step 1-10 of Protocol 1, except for the use of immature embryos of B104 that are between 1.5 and 2.0 mm in length collected from an ear between 12 and 15 DAP.

Pre-culture of immature embryos
2-2. Pour the liquid medium with embryos into a Petri dish (60×15 mm). Steps from 2-2 to 2-33 are performed aseptically.
2-3. Remove 1.7 ml of the liquid from the suspension.
2-4. Place the embryos on MST medium with the scutellum-side up and seal the Petri dishes with Parafilm. Up to 200 embryos may be placed on a single plate.
2-5. Incubate at 25°C for 3 days in the dark (pre-culture).

Gold particle preparation
2-6. Wash 30 mg of Bio-Rad 0.6-µm gold particles with 1 ml of 70% ethanol in a microtube.
2-7. Sonoicate (25 W, 40 kHz) the microtube in a water bath for 90 s.
2-8. Centrifuge the microtube at 5,000 rpm for 5 s and remove the ethanol supernatant.
2-9. Add 1 ml of sterile water and shake at maximum speed by Vortex® for 20 s. Pellet the microparticles by brief centrifugation. Remove the liquid. Repeat this wash step two more times.
2-10. Add 2 ml of sterile 50% glycerol to bring microparticle concentration to 15 mg ml⁻¹ (no loss is assumed during the preparation).

Pre-bombardment embryo treatment
2-11. After Step 2-5, a raised ridge is visible at the base of the swollen immature embryos, which are ready for bombardment.
2-12. Draw double circles of 20 mm and 9 mm in diameter on the bottom-center of each MSTosm medium plate with a permanent marker. The area between these two circles is the target area, where the embryos are placed for bombardment.
2-13. At 2–4 h before bombardment, transfer the embryos to the target area of MSTosm medium with the scutellum-side up by using forceps.

Coat the gold particles with DNA
2-14. Add DNA solutions and sterile water to a microtube to obtain a volume of 39.2 µl for each shot of bombardment. Typically, 100–500 ng of DNA per molecule is used for a single shot. Usually, two or more molecules are mixed at equal molarity. In this protocol, it is assumed that a DNA molecule with the bar gene and another with the GUS gene are mixed.
2-15. Sonoicate (25 W, 40 kHz) the suspension of gold particles in the microtube of Step 2-10 in a water bath for 10 s and mix at maximum speed by Vortex® for 5 s. Transfer the gold suspension of 10 µl per bombardment shot to an empty microtube.
2-16. Add TransIT®-2020 transfection reagent (TaKaRa) of 0.8 µl per shot to the microtube containing the gold suspension. Mix at maximum speed by Vortex® for 5 s.
2-17. Add 10.8 µl of the mixture of the gold suspension and TransIT to the microtube containing the DNA solution of Step 2-14. Tap the microtube gently to mix.
2-18. Cool the microtube at 4°C for 10 min.
2-19. Centrifug the microtube using a fixed-angle rotor with a maximum radius of 83 mm, at 10,000 rpm, 4°C for 1 min and remove the supernatant.
2-20. Add 140 µl of 99.5% ethanol to wash the DNA–gold pellet. Mix at maximum speed by Vortex® for 2 s. Centrifug the microtube for 5 s and remove the ethanol supernatant.
2-21. Add 10 µl of 99.5% ethanol to the DNA–gold pellet.

Loading the macrocarriers
2-22. Soak a DNA macrocarrier and a stainless-steel macrocarrier holder in 70% ethanol for 10 s, then air-dry for 1–2 h in a laminar flow bench, and assemble by fitting the macrocarrier into the holder in a sterile dish.
2-23. Sonoicate (25 W, 40 kHz) the microtube of the DNA–gold mixture of Step 2-21 in a water bath for 5–10 s. Pipette 10 µl of the DNA–gold mixture from the tube, immediately after the tube is shaken at maximum speed by Vortex® for 5 s, onto the center of the macrocarrier.
2-24. Let the macrocarrier air-dry for 5–10 min in the laminar flow bench before bombardment.

Microparticle bombardment
2-25. Place a rupture disk (450 psi) in the retaining cap and hand screw the cap back in place inside the chamber of the Bio-Rad PDS 1000/He biolistic gun. Tighten securely using the torque wrench.
2-26. Prepare a launch assembly by first laying in place a stopping screen, followed by the inverted, pre-loaded macrocarrier holder. Screw on the launch assembly lid to hold these parts in place.

2-27. Place the launch assembly in the gun chamber by sliding it into place immediately below the He nozzle and set the gap distance (6 mm).

2-28. Place the Petri dish of Step 2-14 uncovered onto the shelf at a distance of 6 cm from the stopping screen.

2-29. Close the gun chamber door and activate the vacuum switch until it reaches 711 mmHg (displayed as 28 inHg by Bio-Rad PDS 1000/He). Press the fire switch until the disk ruptures with a pop sound in the chamber.

2-30. Press the vacuum release switch and remove the plate containing the bombarded tissues.

Post-bombardment treatment

2-31. Seal the MSTosm plate containing bombarded embryos with Parafilm and incubate at 25°C in the dark for recovery.

2-32. At 20–24 h post-bombardment, transfer the embryos onto MOT resting medium with the scutellum-side up. Seal the plates with Parafilm. Up to 80 embryos may be placed on a single plate.

2-33. Incubate at 25°C for 7 days in the dark.

Selection of transformed calli and regeneration of transformed plants

2-34. Perform Steps from 1-20 to 1-31.

Examination of transient expression of GUS after bombardment (optional for transfer of a GUS gene)

2-35. Interrupt Step 2-33 after 3 days of incubation, immerse the embryos in 0.5 ml of Buffer P in a microtube and incubate at 37°C for 1 h.

2-36. Perform Steps from 1-33 to 1-36.

Tissue culture for gene editing

Remarkable progress was made in the development of site-specific endonucleases, which cause double-strand DNA breaks in living cells, including plant cells. Designed mutations may be induced upon repair of the breaks by non-homologous end-joining or insertion of or replacement with template DNA molecules, which is rapidly emerging as a new technology of crop “gene editing”. Novel editing tools, such as the CRISPR/Cas9 system, which simplify and improve the efficiency of the editing process, are now being extensively studied (Yin et al. 2017). Once proper tools are developed and introduced into plants, it would be reasonable to consider gene editing complete. However, in reality, just like that in crop genetic transformation, the rate-limiting step in crop gene editing is the tissue culture of and plant regeneration from modified cells in elite germplasm. Therefore, all of the discussions and the protocols above for maize transformation are relevant to gene editing.

DNA editing tools may be introduced into maize either by particle bombardment (Svitashev et al. 2015) or by A. tumefaciens (Char et al. 2017; Chen et al. 2018; Feng et al. 2018; Qi et al. 2016; Zong et al. 2017). Particle bombardment may also deliver RNA or protein components of the tools, allowing to create plants edited by a process free from external DNA molecules, conferring the product more readily acceptable by the public (Svitashev et al. 2016). A simple editing experiment is essentially delivery of editing tools in maize transformation by particle bombardment or A. tumefaciens and screening the transformants for edited events. For many experimental applications of gene editing, such edited transformants may be convenient and satisfactory. However, for commercialization of the resultant varieties, edited plants free from foreign DNA are highly desired. Such plants can be obtained from transgenic regenerants because it is possible that the edited loci are segregated from the transgenes in the subsequent generations. Alternatively, and preferably, gene editing by tools transiently expressed may be quite feasible because, in both particle bombardment and Agrobacterium-mediated gene transfer, a vast majority of cells into which DNA is initially introduced, may transiently express the genes on the DNA but would eventually lose the DNA without stable integration to chromosomes. To achieve such “transient gene editing”, protocols need to be modified so that editing tools are introduced into maize, stable transformants are excluded, and edited plants free from the foreign DNA are selected. Procedures have not been optimized in this way, but the protocols discussed here serve as useful starting points.

Tissue culture aided by morphogenic regulator genes

Varietal differences in tissue culture responses are quite large in maize and, especially, elite genotypes were impossible to culture and transform, and remain highly challenging. The idea of using genes to help tissue culture sounds attractive if such genes are available. The search for quantitative trait loci (QTLs) controlling tissue culture response may be a feasible approach to finding genes useful in tissue culture and transformation. While several promising QTLs have been found (Ma et al. 2018; Salvo et al. 2018), progress has been slow, and the outputs have not yet been widely used.

Then, Lowe et al. (2016) found certain transcription factor genes, Baby boom (Bbm) and Wuschel2 (Wus2), improved maize transformation. Bbm is a gene strongly expressed in early embryogenesis, and Wus2 is involved in maintenance of shoot apical meristems.
Lowe et al. (2016) collectively called these genes “morphogenic regulators”, as ectopic expression of these genes vigorously promotes cell divisions. By using these genes, active cell divisions were induced not only from immature embryos but also from other tissues, such as mature embryos and leaf segments, even in genotypes strongly recalcitrant in tissue culture and transformation (Lowe et al. 2016; Mookkan et al. 2017, 2018). As uncontrolled overexpression of these genes tends to suppress plant regeneration or cause morphological abnormalities in regenerants, methods were developed for proper regulation of these genes by specific promoters (Lowe et al. 2018) or removal of these genes by recombinase systems, such as FLP/FRT (Anand et al. 2019) or CRE/lox (Mookkan et al. 2017, 2018). It was even possible to induce somatic embryos on scutella of immature embryos by the optimized combination of promoters and the genes and to drastically shorten the time to create transgenic plants (Lowe et al. 2018). The approaches using these genes were reviewed by Anand and Jones (2018) and Anand et al. (2019), and an efficient maize transformation protocol based on these genes was reported (Jones et al. 2019). For many experimental applications of maize transformation, plants into which these genes are integrated may be convenient and satisfactory, providing normal morphology is exhibited at least in the progeny populations. However, for commercialization of the resultant varieties, transgenic plants free from these genes, preferably in the first generation, are desired. If these genes are properly employed, regeneration of edited plants of highly recalcitrant maize inbred lines may be possible. It is reasonable to suggest that through the active and extensive research of the use of these genes and related genes, efficient protocols for any desired applications will soon be fully established.

Conclusion

Maize tissue culture protocols for transformation mediated by both particle bombardment and Agrobacterium tumefaciens have been well developed and widely employed. Various factors, including pre-culture and handling before gene delivery, techniques to protect maize cells during the gene delivery phase, and various culture media, have been well optimized for various genotypes. Typical protocols for both methods are herein presented to show major outputs from the studies conducted since the early 1990s. While the particle bombardment protocol is for B104, a new public inbred with favorable agronomic characteristics, the Agrobacterium protocol is good for various inbred lines, including B104. These protocols are also useful to transfer tools for gene editing, and as starting points for optimization of tissue culture for gene editing. Large varietal differences in tissue culture responses remain a major issue in maize. Recently, protocols using morphogenic regulator genes, such as Bbm and Wus2, are being developed that show promising potential for efficient transformation of recalcitrant varieties.

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