Morphogenic Regulator-Mediated Transformation of Maize Inbred B73

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Maize B73 is a reference genome and has long been a major resource for genetics and molecular biology research. We have developed an efficient B73 transformation protocol by enabling somatic embryogenesis through differential co-expression of maize morphogenic regulators BBM and WUS2. We describe a successful protocol that utilizes Agrobacterium tumefaciens strain AGL1 harboring binary vector PHP78891 that comprises a BBM and WUS2 expression cassette as well as a green fluorescent protein (GFP) reporter cassette. The PHP78891 vector also contains, within the T-DNA region, a CRE/lox recombination system flanking the CRE/BBM/WUS2 co-expression cassette driven by the desiccation inducible RAB17 promoter that allows removal of the BBM/WUS2 cassette. Introduction and co-expression of BBM and WUS2 induced direct somatic embryogenesis (SE) in non-regenerable maize B73 from immature embryo explants. Removal of the CRE/BBM/WUS2 cassette is essential to allow regeneration to fertile plants. The GFP expression cassette outside the lox excision sites is retained in the transgenic plant genome, allowing subsequent phenotypic analysis of calli and regenerated transgenic events. This transformation system enables a selectable marker-free transformation process by taking advantage of BBM/WUS2-induced SE as a developmental selection system. © 2018 by John Wiley & Sons, Inc.

Keywords: Agrobacterium • B73 • maize • morphogenic regulator • transformation

INTRODUCTION

Maize (Zea mays L.) is the most important food, feed, and biofuel crop in the world. Maize genetic engineering and gene editing play unique roles in maize improvement and elucidation of basic genetic and biological studies. Agrobacterium-mediated maize transformation is considered superior to other DNA delivery systems. (Dai et al., 2001; Komari et al., 1998). However, no successful Agrobacterium-mediated transformation of B73 using somatic embryogenesis has been reported prior to Mookkan et al., 2017).
MORPHOREGONIC-REGULATOR BBM/WUS2-MEDIATED TRANSFORMATION OF MAIZE B73 VIA AGROBACTERIUM TUMEFACIENS

This protocol utilizes the binary vector PHP78891 (Fig. 1). This vector carries the maize BBM and WUS2 transcriptional factor genes (Lowe et al., 2016). The relative weakly expressed nos promoter drives expression of maize WUS2 cDNA whereas the strongly expressed promoter Ubi drives maize BBM cDNA. The three cassettes are flanked by two loxP sites and the recombinase Cre gene (Odell et al., 1990) driven by maize RAB17 drought inducible promoter. To enhance transformation this protocol utilizes super-binary vector pTOK233 (Hiei et al., 1994). The activity of WUS2 and BBM induce somatic embryogenesis from non-regenerable explants or genotypes (Lowe et al., 2016). After induction of somatic embryogenesis, activation of the drought inducible promoter (RAB17) is accomplished by placing the calli on dry filter paper to activate CRE expression, causing excision of BBM/WUS2 cassettes at the recombination sites (loxP) from the recipient genome (Mookkan et al., 2017).

The following protocol describes the production of transgenic maize B73 events using the morphogenic regulator genes of maize BBM and WUS2 via Agrobacterium tumefaciens. This protocol is fast and efficient, allowing transgenic B73 plants to produce within 8 to 10 weeks.

This B73 transformation protocol includes 7 steps: (1) initiation and preparation of Agrobacterium culture; (2) sterilization and isolation of immature embryos; (3) infection, co-cultivation, and resting; (4) desiccation and regeneration; (5) rooting and hardening; (6) transgene GFP confirmation; and (7) Support Protocol for the B73 stock, as well as transgenic plant growth under the greenhouse conditions.

Materials

*Agrobacterium tumefaciens* AGL1 carrying PHP78891 binary vector and a super-binary vector
YEP agar plate (see recipe)
Zm-1 liquid medium for infection (see the recipe)
Maize B73 ears
Commercial bleach
Tween-20
Deionized distilled water
Zm-2 co-cultivation medium (see recipe)
Zm-3 Resting medium (see recipe)
Zm-4 somatic embryo development medium (see recipe)
Zm-5 Desiccation medium (see recipe)
Zm-6 Regeneration medium (see recipe)
Zm-7 Rooting medium (see recipe)
Promix BX soil  
*Optional:* Peters 20-20-20 (Hummert International, cat. no. 07-5400-1)

Parafilm  
Culture incubators with temperature, light, and air circulation  
Sterile stainless-steel loop  
50- and 15-ml Falcon tubes  
Spectrophotometer and cuvettes  
Benchtop small shaker  
2-ml microcentrifuge tubes  
Sterile stainless-steel forceps  
1-litter wide-mouth glass bottle for ear sterilization  
150× 15-mm and 100× 15-mm sterile petri dishes  
No. 11, 15 razor blades (Feather surgical blade, stainless steel)  
Micro spatula  
1-ml pipette tips  
Parafilm  
Whatman filter paper  
Magenta GA-7 boxes (Sigma-Aldrich, cat.no. V8505)  
Plastic trays  
Small Jiffy pots for hardening seedling plants (Hummert International)  
Polyethylene bags  
3-gallon pots  
GFP fluorescent microscope (Leica M205 FA)  
3 M porous tape  
Regular dissecting microscope  
Two, 1-liter conical flasks  
500-ml, 1-liter glass bottles  
Laminar airflow cabinet  
Black color permanent marker for labeling use  
Plant growth chambers with 25°C, 16 hr./light, 8 hr/dark  
Autoclave machine

**Initiation and preparation of Agrobacterium culture**

1. Streak *Agrobacterium tumefaciens* strain AGL1 carrying PHP78891 vector and a super binary vector from −80°C glycerol stock on YEP agar plate containing 100 mg/liter spectinomycin, 30 mg/liter rifampicin, and 2.5 mg/liter tetracycline antibiotics, as the “master” plate. Seal the master plate with Parafilm and incubate 3 days at 28°C dark.  

   *The “master” plate can be used for up to 1 month when stored a 4°C.*

2. Pick a single colony from the “master” plate and streak onto a fresh YEP agar plate containing 100 mg/liter spectinomycin, 30 mg/liter rifampicin, and 2.5 mg/liter tetracycline antibiotics using a sterile wood applicator. Seal with Parafilm and incubate in the dark for 2 days at 28°C.

3. Add 5 ml Zm-1 medium (inoculation medium) to a 15-ml Falcon tube.

4. Use two full loops of *Agrobacterium* culture from the YEP plate to inoculate the tube prepared in step 3. Shake the tube manually to suspend bacterial cells thoroughly.

5. Pipette 1 ml of this suspension and place in a spectrophotometer cuvette to check the optical density at 550 nm (OD$_{550}$). Adjust the suspension culture to 0.35 at OD$_{550}$ (0.5 × 10$^9$ cfu/ml) at 24°C by either diluting the culture with more Zm-1 or adding more *Agrobacterium* cells.
6. Shake the culture in a benchop shaker for 3 to 4 hr at 100 rpm at 24°C.

7. Pipette 1 ml ZM-1 liquid medium into 2-ml sterile microcentrifuge tubes for collection of embryos.

Sterilization and isolation of immature embryos

8. Harvest the (10 to 12 days post-pollination) ear (Fig. 2A) and remove the husks and silks. Insert the metal forceps into the bottom end of the ear as shown in (Fig. 2B).

9. Prepare 0.5 to 0.8 liters of 30% commercial bleach with a few drops of Tween-20 in a sterile 1-liter wide-mouth glass bottle. Put the B73 ear into the bottle and the ear should be fully covered with the bleach solution for 20 min (Fig. 2C).

10. Rinse the ears three to four times with 500 ml sterile distilled water each time.

11. Place the ear on a sterile 150 × 15–mm petri dish.

12. Remove the top half of the kernels with a sterile no. 11 razor blade on a scalpel (Fig. 2D).

Remove the top half of only two rows at a time to prevent the loss of moisture and maintain the vigor of the embryos in the remaining rows.

13. Isolate immature 1.5 to 2.0 mm immature embryos, two rows at a time, (Fig. 2E, F) from the sterile ear with a sterile micro-spatula (Fig. 2G) and transfer 50 embryos/2-ml microcentrifuge tube, which is filled with Zm-1 liquid medium.

14. Wash the embryos three times, each time with 1 ml Zm-1 liquid medium to remove debris and starch.

Agrobacterium infection, co-cultivation, and resting

15. Add immediately 1.5 ml of Agrobacterium suspension to the 2-ml microcentrifuge tube (Fig. 3A) containing the immature embryos, allow the tube to stand 5 min,
Figure 3  *Agrobacterium* infection, co-cultivation, and resting. (A) Immature embryos infection in 2-ml microcentrifuge tube, (B) immature embryos in co-cultivation plate, and (C) resting after 7 days.

Figure 4  Desiccation and regeneration. (A) Desiccation stage, and (B) somatic embryos in regeneration medium.

16. Use a pipette with a 1-ml tip to remove all the *Agrobacterium* suspension from the petri dish, and then place the embryos with scutellum face up in the same petri plate (Fig. 3B).

17. Seal the plate with Parafilm and incubate for 3 days in the dark at 20°C.

18. Transfer the embryos to Zm-3 resting medium with a no. 15 razor blade on a scalpel. (Gently lift the bottom side of the embryo, without any damage).

19. Seal the plate with Parafilm and incubate for 7 days in the dark at 28°C (Fig. 3C).

**Somatic embryo induction**

20. After 7 days, remove the radical portion, and then transfer somatic embryos to Zm-4 induction medium (place 20 to 25 embryos/plate).

21. Seal the plate with Parafilm and incubate for 14 days in the dark at 28°C.

22. Transfer the embryo cultures to the same induction medium, seal the plate with Parafilm, and incubate for 10 to 14 days in the dark at 28°C.

**Desiccation and regeneration**

23. Place three layers of Whatman sterile filter paper into the empty sterile 15 × 100-mm petri dish (Fig. 4A), and then transfer somatic embryos carefully on the filter paper. Seal the plate with Parafilm and incubate for 3 days in the dark at 25°C.
Figure 5  Rooting and Hardening. (A) Rooting stage in Magenta box, (B) hardening stage plantlet covered with a polythene bag, and (C) hardened young plant in small Jiffy pot and ready to move greenhouse.

24. After 3 days, transfer desiccated embryos to Zm-6 regeneration medium in the light with 18-hr photoperiod at 25°C for 20 to 25 days with two subcultures to the same medium.

25. The shoot bud will emerge (Fig. 4B) within 4 to 7 days from the tiny somatic embryos.

*Do not damage the somatic embryos before desiccation or after transfer to the regeneration medium, because usually the somatic embryos are tiny and easily separate.*

**Rooting and hardening**

26. Transfer the regenerated shoot to Zm-7 rooting medium in the Magenta box.

27. Allow the shoot to stay 10 to 14 days for rooting (Fig. 5A).

28. Use a plastic tray with Promix BX soil, spray a limited amount of water and mix well to moisturize the soil, and then to fill 3 × 3-in. small Jiffy pots.

29. Identify the well-rooted and healthy plants, remove gently from agar medium and wash the roots with room temperature tap water, and make sure to remove the entire agar from the roots.

*Do not damage the roots or shoots during these steps.*

30. Make a hole on the center of the soil in the pot, then insert the roots into the hole, and then cover the hole with soil. Completely wet the soil.

31. Use suitable polythene bag and cover the plantlet for one week (Fig. 5B).

32. Remove the polythene bag and allow them another 1 to 2 weeks. During this time watch the plantlets every day and apply water as needed.

33. If plantlets are weak such as showing pale color leaves, apply Peters 20-20-20 fertilizer.

34. After 2 to 3 weeks, plants should be transferred to the greenhouse conditions.

35. Transfer the hardened plant (Fig. 5C) with the soil from the small Jiffy pot to a 3-gallon pot (for more details see the Support Protocol for Greenhouse Management).
Figure 6  Somatic embryo formation and confirmation. (A) Non-infected immature embryo with 0% somatic embryo on the scutulum, (B) infected with morphogenic maize *BBM, WUS2* genes and somatic embryo formation, and (C) confirmed with *GFP* expression.

**GFP confirmation (optional)**

36. GFP expression was observed on transient and stable transformants using a Leica M205 FA stereomicroscope supplied with a DFC 7000T camera with GFP filter. Any other equivalent stereomicroscope should work as well.

37. It is recommended to use the empty control construct carried by strain AGL1 side-by-side with the gene construct of interest to transform immature embryos and follow through various culture stages to help experimental trouble shooting (Fig. 6A).

38. Transfer desiccated somatic embryos to the regeneration medium. To confirm the early somatic embryo development, examine the bright light negative control (Fig. 6B).

*GFP expression is shown in Figure 6D.*

**GROWING MAIZE B73 IN THE GREENHOUSE CONDITIONS**

This protocol is to enhance the transformation rate by taking care of both stock and transgenic plants under the greenhouse conditions.

**Materials**

- Soil mixture (Promix BX)
- Fertilizer: Osmocote 19-6-12 (Hummert International, cat no. 07-6330-1)
- Iron sulfate (Hummert International, cat. no. 07-5400-1)
- Peters 20-20-20 (Hummert International, Catalog No. 07-5400-1)
- Regular water source
- Shoot bags (Lawson Bags, cat. no. 217; [https://www.lawsonbags.com](https://www.lawsonbags.com))
- Seeds of genotype B73 (Maize Genetic Stock center, [https://maizecoop.cropsci.uiuc.edu](https://maizecoop.cropsci.uiuc.edu))
Greenhouse

Light intensity source: Using combined daylight and supplemental lighting from metal halide and high-pressure sodium light and white light bulbs mixed

Pots, 3-gallon sizes (Hummert International)

Paper towels

Measuring spoon 1 oz. (Hummert International cat no. 060092-1)

Black color permanent marker

Sharp blades

Tassel bags (Lawson Bags, cat. no. 402; https://www.lawsonbags.com)

Autoclave

Tags for labeling pots (Hummert International)

Flat tray (Hummert International)

Polythene bag for covering hardened young plants

Greenhouse temperature and light conditions

1. Greenhouse room temperature set up is 28°C for daytime and 20°C for nighttime with a photoperiod of 16-hr light and 8-hr darkness with a mixture of daylight plus 50% metal halide and 50% high-pressure sodium light as supplemental lighting.

Planting

2. Prepare well-cleaned and dried 3-gallon pots.

3. Use a single paper towel to cover the holes at the bottom of the pot (this is to avoid the soil leaking from the holes).

4. Fill the pot with Promix BX soil with fertilizer (Osmocote 19-6-12) at approximately 1 oz. per 3-gallon pot.

5. Move pots to the greenhouse and arrange them in neat rows and then water them three times until the soil is completely wet.

6. Label each pot with a tag with genotype name B73, appropriate date, and experimenter initials using a black color permanent marker.

7. Plant good and healthy maize seeds and insert each seed into the soil to roughly a 2.0-cm depth in the center of the pot and cover the seed with soil (one seed per pot).

8. Avoid watering the pots during the first 3 weeks after planting, to allow roots to develop well.

Fertilization

9. When the maize plant is at the 6-leaf stage, apply a full measuring spoon (one-third of a measuring spoon 1 oz.) per pot of iron sulfate fertilizer.

10. Water the plants after the fertilization.

11. After one week, apply a full measuring spoon (1 oz.) per/pot of Osmocote 19-6-12 fertilizer.

Watering schedule

12. Check the pot, surface of the soil, and plants daily. Water the plants only as needed.

Making a good judgment of whether conditions, surface of the soil color, weight of the pot and plant morphology. During the wintertime, mostly plants need less water, and in summer time they need more water. When the soil surface is dark and the pot is heavy, watering is not needed. When it is dry and light-colored and the pot also is low weight, watering is needed.
13. When the plants reach the six or more leaf stage, provide sufficient water to saturate the soil each time.

*Make sure plants do not suffer from drought conditions, but at the same time do not over-water the plants. If one over-waters the plants, the hairy roots will be damaged and the plants will become wilted despite suppling sufficient water later.*

14. Once a plant reaches 67 to 72 days when the ear and tassel with pollen grains are ready, check each plant daily to prepare for self-pollination.

15. Cover the young ear with Lawson shooting bag.

*Cover the earshot before silk emergence.*

16. When the silk emerges, remove the top of the young ear (along with the silk) with a sharp blade and immediately cover the young ear with the same bag.

*Cut silks do not accept pollen.*

17. Next day, check the top of the young ear to make sure that silks have grown out uniformly (the silk has to be fresh green, indicative of viable and fresh silk). Collect the pollen with the tassel bag (Lawson Bags, cat. no. 402) in the morning about 9–10 AM.

*Make sure to check the pollen quality (light fresh yellow color indicates good pollen. In contrast, dark pollen or clumps of pollen are dead or wet and will not pollinate well).*

18. After pollination, allow the plants to grow another 10 to 14 days, harvest the ear with 1.5–2.0 mm sized immature embryos.

19. Once harvested, autoclave the transgenic plants and dispose properly.

*Wash the pots properly for reuse.*

**Pollination**

20. Details for pollinating maize can be found in standard texts or on several Web sites, including [https://archive.maizegdb.org.IMP.WEB/pollen.htm](https://archive.maizegdb.org.IMP.WEB/pollen.htm).

**REAGENTS AND SOLUTIONS**

**2,4-dichlorophenoxyacetic acid (2,4-D 1 mg/ml stock concentration)**

Weigh 50 mg of 2,4-D (Sigma-Aldrich, cat. no. D6679) into a 50-ml glass beaker

Add 5 ml of 1 N NaOH solution to dissolve 2,4-D completely

Slowly add deionized distilled water with stirring, to 50 ml final volume

Store up to 6 months at $-20^\circ$C

**Acetosyringone, 100 mM**

Dissolve 0.196 g acetosyringone in 5 ml methanol

Add 5 ml distilled deionized water to make a final volume of 10 ml

Divide into 1.5-ml microcentrifuge tubes

Store up to 6 months at $-20^\circ$C

**B5 microsalts, 100× stock**

0.3 g/liter Boric acid

1 g/liter Manganese sulfate

0.2 g/liter Zinc sulfate

75 mg/liter Potassium iodide

25 mg/liter Sodium molybdate

2.5 mg/liter Cupric sulfate
2.5 mg/liter Cobalt chloride
Store in the dark up to 3 months at 4°C

*Eriksson’s Vitamin, 1000 × stock*

- 0.2 g/liter Glycine
- 50 mg/liter Nicotinic Acid
- 50 mg/liter Pyridoxine HCl
- 50 mg/liter Thiamine HCl

Filter sterilize using a 0.22-μm filter
Store in the dark up to 3 months at 4°C

*Indole-3-butyric acid (IBA), 1 mg/ml stock concentration*

Dissolve 50 mg IBA (Sigma Aldrich, cat. no. 15386) in 1 ml of 1 N NaOH
Add deionized distilled water to 50 ml
Divide into 1.5-ml microcentrifuge tubes
Store up to 6 months at −20°C

*KOH, 1 M*

Dissolve 5.6 g KOH in 100 ml deionized distilled water
Store up to 6 months at room temperature

*Murashige and Skoog (1962) (MS) vitamins, 1000 × stock*

- 2 g/liter Glycine
- 0.5 g/liter nicotinic acid
- 0.1 g/liter thiamine HCl
- 0.5 g/liter pyridoxine HCl

Add sterile deionized distilled water to 1000 ml
Filter sterilize using a 0.22-μm filter
Store up to 3 months at 4°C

*N6 Macrosalts, 100 × stock*

- 28.3 g/liter Potassium nitrate
- 4.62 g/liter Ammonium sulfate
- 1.25 g/liter Calcium chloride
- 1.86 g/liter Magnesium sulfate
- 4 g/liter Sodium phosphate

Store in the dark up to 3 months at 4°C

*NaOH, 1 M*

Dissolve 3.99 g NaOH in 100 ml deionized distilled water
Store up to 6 months at room temperature

*Rifampicin stock solution*

Prepare 30 mg/ml rifampicin (Sigma-Aldrich, cat. no. R3501) in dimethyl sulfoxide (DMSO)
Transfer into 1.5-ml microcentrifuge tubes
Store up to 6 months at −20°C

*Spectinomycin stock solution*

Prepare 100 mg/ml of spectinomycin (Sigma-Aldrich, cat. no. S4014) in sterile deionized distilled water
Filter sterilize using a 0.22-μm filter
Transfer into 1.5-ml microcentrifuge tubes
Store up to 6 months at −20°C
**Tetracycline stock solution**
Prepare 2.5 mg/ml of tetracycline (Sigma-Aldrich, cat. no. T7660) in 50% ethanol
Transfer into 1.5 ml microcentrifuge tubes
Store up to 6 months at −20°C

**YEP solid medium**
10 g/liter peptone
7.5 g/liter yeast extract
10 g/liter Bacto agar
5 g/liter NaCl
Adjust to 1 liter with deionized distilled water
Adjust pH to 7.0 using 1 N NaOH solution
Autoclave
Cool to 55°C
Add 100 mg/liter spectinomycin
30 mg/liter rifampin
2.5 mg/liter tetracycline
Pour into 100 × 15-mm petri dishes
Store in the dark up to 1 month at 4°C

**Zm-1 Agrobacterium infection medium**
4 g/liter N6 salts (Chu et al., 1975) (Sigma-Aldrich cat. no.)
68.5 g/liter sucrose
36 g/liter glucose
0.7 g/liter L-proline
0.5 g/liter 2-(N-morpholino) ethanesulfonic acid (MES)
1.5 mg/liter 2,4-D (see recipe)
1 ml/liter of 100× Eriksson’s vitamins (see recipe)
1 mg/liter Thiamine HCl
Adjust the pH to 5.2 using 1 M KOH
Filter sterilize using a 0.22-μm filter
Just before use, add 1000× acetosyringone stock (see recipe) to 1× final concentration
Store the liquid medium up to 2 months at −20°C

**Zm-2 Co-cultivation medium**
4 g/liter N6 salts
20 g/liter sucrose
10 g/liter glucose
0.7 g/liter L-proline
0.5 g/liter 2-(N-morpholino) ethanesulfonic acid (MES)
1.5 mg/liter 2,4-D (see recipe)
Adjust the pH to 5.7 using 1 M KOH
3 g/liter Gelrite
Autoclave for 20 min at 121°C
Allow to cool to 55°C
Prepare the solution in separate container, one by one
1 ml/liter of 100× Eriksson’s vitamins
100 mg/liter Myo-inositol
1 ml/liter of 1000× acetosyringone (see recipe)
Dissolved in 10 ml distilled deionized H₂O
Filter sterilize the solution using a 0.22-μm filter
Then mix into the autoclave medium
Pour into 15 × 100-mm sterile petri plates
Store the solid medium up to 2 weeks at 4°C

**Zm-3 Resting medium**

- 4 g/liter N6 salts
- 30 g/liter sucrose
- 0.7 g/liter L-proline
- 0.5 g/liter 2-(N-morpholino) ethanesulfonic acid (MES)
- 1.5 mg/liter 2,4-D (see recipe)
- Adjust the pH to 5.7 using 1 M KOH
- 3 g/liter Gelrite
- Autoclave for 20 min at 121°C
- Allow to cool to 55°C
- Prepare the solution in separate container, one by one
  - 1 ml/liter of 100 × Eriksson’s vitamins
  - 1 ml/liter of 100 × SH vitamins
  - 100 mg/liter myo-inositol
  - 250 mg/liter cefotaxime
- Dissolved in 10 ml distilled deionized H$_2$O
- Filter sterilize the solution using a 0.22-μm filter
- Then mix into the autoclave medium
- Pour into 15 × 100-mm sterile petri plates
- Store the solid medium up to 2 weeks when at 4°C

**Zm-4 Somatic embryo induction medium**

- 4 g/liter MS salts Murashige and Skoog (1962) (Sigma-Aldrich cat. no.)
- 6 ml/liter of 10 × N6 macronutrients
- 6 ml/liter of 10 × B5 micronutrients (Gamborg, Miller, & Ojima, 1968)
- 30 g/liter sucrose
- 0.7 g/liter L-proline
- 0.5 g/liter 2-(N-morpholino) ethanesulfonic acid (MES)
- 0.5 mg/liter 2,4-D (see recipe)
- Adjust the pH to 5.7 using 1 M KOH
- 3 g/liter Gelrite
- Autoclave for 20 min at 121°C
- Allow to cool to 55°C
- Prepare the solution in separate container, one by one
  - 1 ml/liter of 100 × Eriksson’s vitamins
  - 1 ml/liter of 100 × SH vitamins
  - 100 mg/liter myo-inositol
  - 250 mg/liter cefotaxime
- Dissolved in 10 ml distilled deionized H$_2$O
- Filter sterilize the solution using a 0.22-μm filter
- Then mix into the autoclave medium
- Pour into 15 × 100-mm sterile petri dishes
- Store the solid medium up to 2 weeks at 4°C

**Zm-5 Desiccation treatment**

- Sterile 15 × 100-mm Petri plate
- Two- to three-layer Whatman # 70 mm filter paper
- Incubate for 3 days in the dark at 25°C
**Zm-6 Regeneration medium**

- 4.3 g/liter MS salts (Sigma-Aldrich cat. no.)
- 40 g/liter sucrose
- Adjust the pH to 5.7 using 1 M KOH
- 8 g/liter agar
- Autoclave for 20 min at 121°C
- Allow to cool to 55°C
- Prepare the solution in separate container, one by one
- 5 ml/liter of 1000× MS vitamins
- 100 mg/liter myo-inositol
- 250 mg/liter cefotaxime
- Dissolved in 10 ml distilled deionized H₂O
- Filter sterilize the solution using a 0.22-μm filter
- Then mix into the autoclave medium
- Pour into 15 × 100-mm sterile petri plates
- Store the solid medium up to 2 weeks at 4°C

**Zm-7 Rooting medium**

- 4.3 g/liter MS salts (Sigma-Aldrich cat. no.)
- 30 g/liter sucrose
- Adjust the pH to 5.7 using 1 M KOH
- 8 g/liter agar
- Autoclave for 20 min at 121°C
- Allow to cool to 55°C
- Prepare the solution in separate container, one by one
- 5 ml/liter of 1000× MS vitamins
- 0.5 mg/liter IBA (see the recipe)
- 100 mg/liter myo-inositol
- 250 mg/liter cefotaxime
- Dissolved in 10 ml distilled deionized H₂O
- Filter sterilize the solution using a 0.22-μm filter
- Then mix into the autoclave medium
- Pour into Magenta
- Store the solid medium up to 2 weeks at 4°C

**COMMENTARY**

**Background Information**

*Agrobacterium tumefaciens* is a naturally widespread, Gram-negative soil-born bacterium that has the ability to introduce new genetic material into plant cells (Gelvin, 2003). *Agrobacterium*-mediated transformation has been used for more than 30 years. Major challenges for implementation of *Agrobacterium*-mediated transformation include the host range of *Agrobacterium* on plant genotypes, source of explants, influence of transformation construct, plant genotype, plant media components, and *Agrobacterium* strain dependence (Altpeter et al., 2016).

Numerous key factors must be considered for the establishment of new *Agrobacterium*-mediated transformation of monocots, including the plant genotype, age and type of the explant, *Agrobacterium* strain, types of vectors, antioxidants, co-cultivation duration and temperature, selectable marker and agent, and protocols for plant regeneration. Many studies suggest that immature embryo explants are the most common and suitable for transformation (Hiei et al., 1994). Embryogenic cultures are good target tissues for transformation via either *Agrobacterium*-mediated or particle bombardment, because methods as such cultures provide a high level of cell exposure. Successful *Agrobacterium*-mediated transformation of maize inbred lines has been reported using immature embryos (Frame et al., 2006; Hiei et al., 2006; Huang & Wei, 2005; Huang et al., 2004; Ishida et al., 1996; Luppoto et al., 1999; Negrotto et al., 2000; Vega et al., 2008). Nevertheless,
no success has been reported in transforming maize B73 using Agrobacterium-mediated transformation through somatic embryogenesis until our study (Mookkan et al., 2017).

**BABY BOOM (BBM)** encodes an AP2/ERF transcription factor involved with root, seed, and basal embry, shoot meristem development, and was identified first via subtractive hybridization in *Brassica napus* embryogenic microspore-derived cultures (Boutilier et al., 2002). That study showed that overexpression of BBM in transgenic *Arabidopsis thaliana* resulted in the development of ectopic somatic embryos. BBM overexpression in *Populus tomentosa* (Deng et al., 2009) and *Theobroma cacao* (Florez et al., 2015) has also been shown to promote somatic embryogenesis. **WUSCHEL (WUS)** is a homeodomain-containing transcription factor gene that is involved in flower and shoot meristem development, as well as stem cell specification in plants (Laux et al., 1996; Mayer et al., 1998). Gallois et al. (2002, 2004) showed that when overexpressed, WUS gene promoted somatic embryogenesis and organogenesis in shoot and root tip in *A. thaliana*. The expression of WUS is restricted to a subset of meristematic cells subtending the stem cells during all stages of embryogenesis. This expression pattern may indicate that the WUS protein is involved with maintenance in cell fate by a diffusion gradient acting in a non-cell-autonomous fashion. Ectopic expression of WUS may facilitate morphogenic alterations in plant development (Zuo et al., 2002). Recently, the group at Corteva AgriSciences published breakthrough technology using differential expression of the maize BBM and WUS2 genes to successfully develop direct somatic embryogenesis from immature embryo and seedling leaf tissues of inbred maize and other monocot crops including sorghum, rice and sugarcane (Lowe et al., 2016).

This protocol is based on our recent publication (Mookkan et al., 2017), and may be suitable for other recalcitrant maize inbred lines and monocots.

**Critical Parameters**

(1) Usually, B73 immature embryo sizes are not uniform. Isolate only the middle portion of ear embryos to obtain uniform size of immature embryos. Do not use immature embryos from the top or bottom portions of an ear.

(2) Fresh ears and immature embryo size are critical: a 1.5 to 2.0 mm size is usually optimal, will be suitable for transformation, and will increase the efficiency. Sizes below 1.5 mm are not reliable for *Agrobacterium* transformation. Smaller embryos show a lower survival rate during the co-cultivation.

(3) The desiccation stage is very critical: when the embryogenic cultures are transferred before or after desiccation, care is taken not to damage the small somatic embryos. Somatic embryos are also easily separated and fall off.

(4) Regeneration is not uniform, so subcultures must be conducted regularly in 2-week intervals.

**Troubleshooting**

(1) Growing healthy B73 stock plants in the greenhouse is critical. During plant growth under greenhouse conditions, the stem may not grow straight, with top leaves tangled together. To avoid this problem, do not apply too much fertilizer. Once the above measures are taken, the ear production will be normal.

(2) If a low frequency of somatic embryo formation on the immature embryo during the induction stage is observed, it is likely that the cultures suffer from poor *Agrobacterium* infection. To avoid this problem, always start the *Agrobacterium* culture from a −80°C freezer stock culture.

(3) It is recommendable to use empty vector (no BBM/WUS2 co-expression) as a negative control to confirm the transgenic somatic embryos are induced by maize morphogenic regulator BBM and WUS2 in the construct.

(4) Usually, the somatic embryos are small, so the use of a dissecting microscope to observe the somatic embryos is recommended.

(5) Without the desiccation step, somatic embryos will not regenerate well, but instead, will develop abnormally large roots.

**Anticipated Results**

(1) Approximately 10 to 14 days after co-cultivation, transgenic somatic embryos are developed on the scutellum.

(2) After the resting stage (the culture will be about 10 days old) transient (GFP expression) efficiency should be over 95%.

(3) Approximately 35 to 40 days after the *Agrobacterium* infection, regeneration should start directly from the mature embryos.

**Time Considerations**

(1) When B73 is grown in the greenhouse, the stock plants are ready for pollination in ~68 to 72 days, and immature embryos are ready for experimental use 80–84 days from seed germination.
(2) The overall period from wild type seed germination to transgenic B73 dry seed collection is 8.5 to 10 months.

Acknowledgements
We thank Corteva Agriscience™ Agriculture Division of DowDuPont™ for the PHP78891 construct. The vectors used in the protocol presented here can be requested through Corteva Agriscience™, Agriculture Division of DowDuPont™, who first published on this approach. Send requests to Bill Gordon-Kamm (william-gordon-kamm@pioneer.com). This project has been supported by the National Science Foundation Plant Genome Research Program (NSF-PGR #1444478).

Literatures Cited


**Key References**

Lowe et al. 2016. See above

A groundbreaking milestone work establishing that plant morphogenic regulator genes BBM and WUS enable somatic embryogenesis of many Corteva Agriscience™ maize inbred lines and certain sorghum varieties, leading to efficient Agrobacterium-mediated transformation of these monocots.

Mookkan et al. 2017. See above

A key milestone work demonstrating that plant morphogenic regulator genes BBM and WUS enabled highly efficient somatic embryogenesis of highly recalcitrant maize inbred B73 and sorghum public genotype P898012, leading to efficient Agrobacterium-mediated transformation of these monocot species.