# *Agrobacterium*-mediated Maize Transformation

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**Plant Material**

Immature embryos are used as the preferred explant for maize transformation. Maize cv. Hi II (A188X B73; A X B) is widely considered as the model system. $F_1$ seeds are used for the production of donor plants supplying Hi II ears used as the source for the immature embryos. The plants can be grown under appropriate greenhouse conditions. Proper lighting, soil, containers and watering regimes are essential to plant donor health. Plants can also be grown in the field but is, of course, seasonally dependent. Upon flowering and initiation of pollen shedding, the plants are sib-pollinated. Ears should then be harvested between 9 to 12 d post-pollination, depending upon the size of the embryos found on each ear. Embryo size is more significant than days post pollination. Ears are harvested for transformation experiments when the immature embryos are between 0.9 and 1.8 mm in length. Embryo size can be sampled easily prior to harvest as a check.

**Genotypes**: Maize cv. Hi II (A188X B73; A X B)
others are possible at low frequencies

**Laboratory Supplies and Stock Solutions** (See Appendix 1)

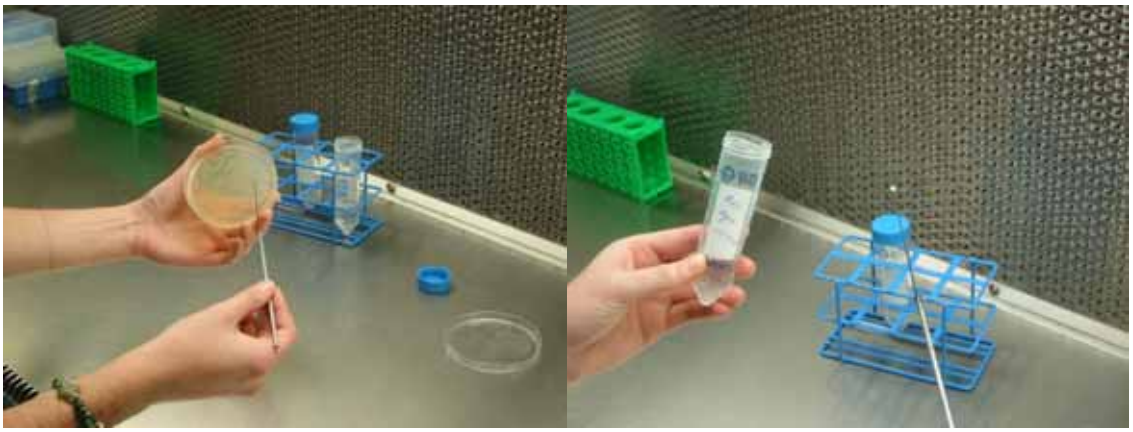
## Initiation of *Agrobacterium tumefaciens* culture:

1. Streak the *Agrobacterium* culture for colony isolation from an -80°C glycerol stock onto a YEP medium plate containing appropriate antibiotics. Incubate the plate at 28°C for 3 days. This plate is the "master" plate, and should be stored at 4°C and can be reused for 30 days. A new master plate needs to be initiated after this period.

2. To begin a maize transformation experiment, streak a single colony onto a fresh YEP plate containing the appropriate antibiotics. Incubate this plate for 3 days at 20°C or 2 days at 25°C.

3. Add 15-25 mL (depending on the size of the experiment) each of sterile infection medium into two sterile 50 mL Falcon tubes. One tube will be used to make the infection with the *Agrobacterium*, the other, will be used to adjust the concentration of bacteria after the OD is read (see below description step 7) and make tubes used in the embryo isolation prior to infection. Add acetosyringone (100 mM/mL stock solution) to the each of the tubes containing infection media for a final concentration of 100 µM/mL acetosyringone.

4. Take about 2.5 full loops from YEP plate and suspend in one of the 50 mL Falcon tubes (about 1 full loopful/10 mL of infection medium.



5. Using the other 50 ml Falcon tube containing the infection media + acetosyringone, and a sterile tip, transfer ~ 1.8 mL of the infection medium + acetosyringone (without *Agrobacterium*) to 10 sterile 2 mL microcentrifuge tubes. These will be used to transfer the isolated embryos prior to infection. This can be done while the *Agrobacterium* suspension is shaking (see Step 6).

6. Shake the *Agrobacterium* suspension at 25-28°C at 175 rpm for 1 hour to re-suspend fully.

7. After 1 hr, transfer an appropriate portion of the *Agrobacterium* suspension to a spectrophotometer curette and measure the $OD_{600}$. The final suspension should be adjusted to = 0.20-0.45. Note; The concentration of *Agrobacterium* may affect duration of co-cultivation and efficiency of transformation

8. Transfer ~ 1.8 mL of the *Agrobacterium* co-cultivation medium (containing acetosyringone) to 10 sterile 2 mL microcentrifuge tubes with caps.

9. Continue shaking the re-suspended culture in a shaker at 175 rpm at room temperature until infection.

## Surface Sterilization of Cobs and Embryo Isolation

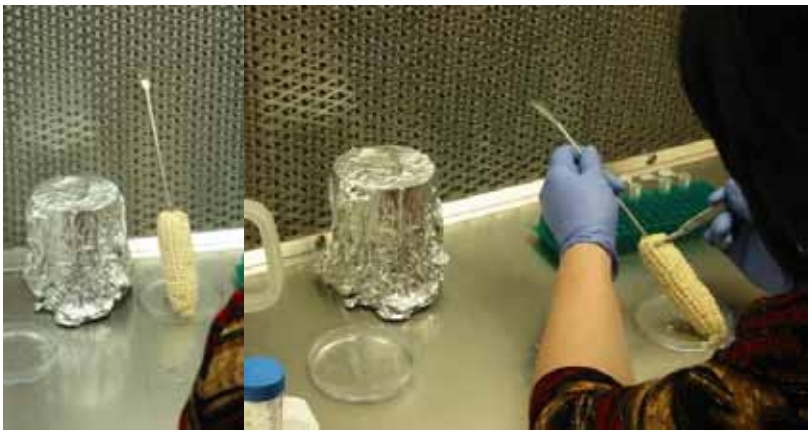
***Note: This procedure is best accomplished by a 2 person team, with one person conducting the embryo isolating and another person rinsing, infecting and plating the embryos***

1. Greenhouse (or field-grown, seasonally dependent) maize variety cv Hi II (A188 X B73; A X B) is used for transformation as described above (see Plant Material).

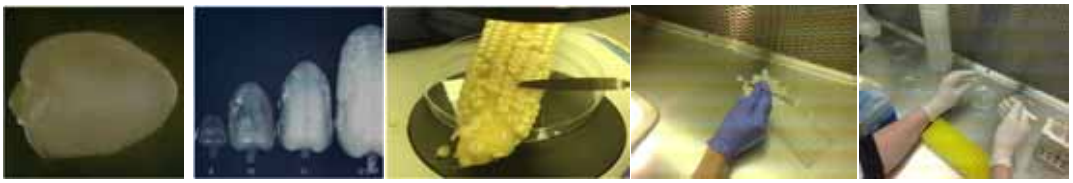
2. Immature embryos are sized on the plant by excision at about 9-12 days post-pollination.

3. Once ears carrying appropriately-sized embryos (0.9-1.8 mm) are selected, they are surface-sterilized by soaking in 50% bleach solution. Prepare a 50% bleach solution by mixing 2 L of bleach with 2 L of sterile dd$H_2O$ in an autoclaved 5 L pitcher. Insert a sterilized stainless steel spatula into the cob of each husked ear to act as a handle, and then place the ears into the bleach solution for 30 min. (Note: The ears may have to held down into the solution to release trapped air and ensure the ears remain submerged in the bleach solution.)

4. After 30 min., rinse the ears with sterile dd$H_2O$ (3) times, leaving the final rinse water in the pitcher. It is important to maintain aseptic technique throughout. Embryos can now be isolated for infection.



5. To isolate the embryos, place a sterile Petri dish on the stage of a dissecting scope. Use a sterile scalpel to shave away about 1/3 off the top of each kernel for 3 – 5 rows. The immature embryos are carefully removed by a twisting motion using the tapered end of a stainless steel spatula. The immature embryo is located on the side of the caryopsis that faces the tip of the ear. The embryo size should be 0.9 - 1.8 mm.



6. Place embryos into a sterile 2.0 mL micro-centrifuge tube containing infection medium supplemented with 100 µM acetosyringone. Continue to collect embryos into micro-centrifuge tube for no longer than 20 minutes to prevent hypoxia of the embryos.

## Infection

1. After 20 minutes, remove the infection medium from the micro-centrifuge tube and replace with ~1.8 mL fresh infection medium + acetosyringone to rinse the embryos Residual endosperm cells may inhibit proper tissue culture response). Remove the rinse, and add 1.8 mL of the *Agrobacterium* suspension. Let stand for 5 minutes for inoculation.

2. After 5 minutes, transfer embryos to a plate containing Co-cultivation medium. Remove the excess suspension and orient the embryos scutellum side up. Allow the plates to sit ajar with no lid for up to 30 min to dry in the laminar flow hood. Wrap the plate in micropore tape (3M 1530-1).

## Co-Cultivation

1. Incubate @ 20°C in darkness for 3 days. *Note that the concentration and /or Agro strain may influence optimum time for co-cultivation.*

2. After the co-cultivation period, transfer the embryos to Resting Medium and incubated for 7 days at 28°C. The orientation of the embryos with scutellum side up must be maintained.

## Selection for Transgenic Events

1. After the resting phase, transfer embryos to Selection I medium with half-strength (1.5 mg/mL) bialaphos selection. The orientation of the embryos with scutellum side up must be maintained. Incubate for 14 d at 28°C in darkness.

2. After 14 d, transfer the embryos to full strength Selection II medium (3.0 mg/mL). After two 14 d rounds of selection on 3.0 mg/mL bialaphos, putatively transformed calli will be distinguishable from non-transformed tissue. An additional subculture for 14 d will allow for identification of all putative transformants.

3. Nearly all maize transformed events using the cv Hi II genotype will be a pale yellow color and have a distinct type II tissue culture response with abundant somatic embryos. There are very few escapes using bialaphos as a selectable marker for the presence of the bar gene. Non-transformed material will not have a type II response, will clearly be stressed, and dying. Non-

transformed material will not have a type II response, will clearly be stressed, and dying.

**Regeneration Step 1**

1. Once a given callus has grown to roughly the area of a circle with a diameter of 2.5 cm, that callus can be given an event ID number and ~ ¾ of the callus is then sub-cultured to Regeneration I medium. The remainder of the callus is sub-cultured to Selection II and maintained as a backup until regeneration and transplantation to soil of the putative event is successful.

2. Putative transgenic calli on Regeneration I medium are cultured for 14 - 21 d to allow the somatic embryos to mature. They will take on an ivory-white appearance, and are ready for subculture to Regeneration II medium after this period of time.

**Regeneration Step 2**

1. Transfer small clusters of mature somatic embryos to Regeneration II medium in 100 × 15–mm Petri dishes. It is easier to isolate well-formed individual plants if the embryos are not densely packed onto the surface of the medium. Use the bottom portion of sterile Petri dishes as lids for these dishes to form a "double tall" container for each event. Incubate at 25°C under 16-hr photoperiod for 2 to 3 weeks until a number of well formed plants are present.

2. Transfer 4 individual well formed plants to a Plant Con® (MP Biomedicals cat. No 26-722-06) containing ~ 80 mL of Regeneration II medium and continue to incubate under 16-hr photoperiod at 25°C until the plantlets reach the top of the Plant Con®. Because individual $T_0$ transgenic plants will often exhibit reproductive abnormalities (e.g., no tassel formation or pollen shed), it is advisable to make 3 Plant Con® containers as described above per putative transgenic event.

3. Once the plantlets have reached the top of the Plant Con® container, they are ready for transplantation to soil.

**Transplantation to Soil**

1. Carefully remove the individual plantlets from the Plant Con. It can be helpful to remove the medium as solid block from the container and then gently break up the medium with gloved hands. It is absolutely necessary to rinse the roots free of any medium particles, and best results are obtained when plants with well developed root systems are chosen.

2. Transfer the plants to 6" plastic pots with moist soil mixture (e.g., MetroMix 510 potting soil). Place the individual pots into a standard greenhouse tray (8 per tray).

3. Each tray should be covered with a "humidome" (Hummert International, cat no. 143850-1). It is absolutely critical that a very humid environment is maintained at the start of transplantation. If necessary, tape the humidomes to the trays. The plants should be maintained at 25°C with a 16 hr photoperiod. Condensation should form within the humidome for the first

48 hrs, and the humidity should only be reduced after it is apparent that the plantlets have survived transplantation (~ 72 hrs.).

4. At this point, gradual venting of the trays is accomplished by either punching a small hole in the humidomes or removing the tape gradually so as to reduce the humidity gradually. This should take a period of one week.

**References:**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## Appendix 1

### Laboratory Supplies

a. 5 L pitcher or beaker (Phytotechnologies Laboratories B265 or similar)
b. Heavy weight, food service aluminum foil (Phytotechnologies Laboratories F041 or similar)
c. Stainless steel spatula/spoon (Phytotechnologies Laboratories S978 or similar)
d. Stainless steel micro spatula(s), 165 mm, with tapered end
e. Stainless steel scalpel handles (Phytotechnologies Laboratories S963 or similar)
f. No. 11 carbon steel scalpels (Phytotechnologies Laboratories S971 or similar)
g. Stainless steel, serrated tip, utility forceps (Phytotechnologies Laboratories F950 or similar)
h. Clear, sterile, polystyrene Petri dishes, 60 × 15 mm (Phytotechnologies Laboratories D940 or similar)
i. Commercial Bleach (e.g., Chlorox)

### Stock Solutions

a. 100 mM Acetosyringone (4-Hydroxy-3',5'-dimethoxyacetophenone)- dissolve 19.6 mg in ~ 500 µL of DMSO (dimethyl sulfoxide); once dissolved, bring the volume 1 mL and filter sterilize into a sterile 1.5 mL microcentrifuge tube. Store frozen at - 20°C for up to 30 d. This can be scaled up for larger batches, but is typically sufficient for 10 + transformation experiments using 5 well pollinated Hi II ears.

b. 100 mM Silver nitrate ($AgNO_3$) – dissolve 850 mg in ~40 mL of $ddH_20$. Once dissolved, bring the volume up to 50 mL and filter sterilize into a sterile 50 mL tube. Wrap the tube with aluminum foil and store at 4 °C for up to 1 year.

c. L-Cysteine (L-Cys) – dissolve 400 mg in ~ 3 mL of $ddH_20$ by affixing the tube to a vortexer/mixer. Shake vigorously until the all the L-Cys is dissolved. Bring the volume up to 4 mL. Filter sterilize the resulting solution (100 mg/mL), and add the appropriate volume to reach a concentration of 400 mg/L co-cultivation medium. Make this fresh for every batch of co-cultivation medium; a transformation experiment using 6 well pollinated ears in our experience requires 12 – 15 co-cultivation plates or 300 – 400 mL total medium, so preparing 200 mg in 2 mL as described above is often the best approach.

d. DL-Dithiothreitol ("DTT" – 1,4- Dithiothreitol) – dissolve 150 mg in ~500 mL $ddH_20$. Once dissolved, bring the volume 1 mL and filter sterilize into a sterile 1.5 mL microcentrifuge tube. Add the appropriate volume to reach a concentration of 150 mg/L co-cultivation medium. As in the case of L-Cysteine above, make this stock fresh for every batch of co-cultivation medium.

**Maize Medium Recipes (1L)**

| Component | Infection | Co-cultivation | Resting | Selection I | Selection II | Regeneration I | Regeneration II |
|---|---|---|---|---|---|---|---|
| $N_6$ salt | 2.0 g | 2.0 g | 4.0 g | 4.0 g | 4.0 g | - | - |
| MS salt | - | - | - | - | - | 4.3 g | 4.3 g |
| MES | 0.5 g | 0.5 g | 0.5 g | 0.5 g | 0.5 g | - | - |
| L-proline | 0.7 g | 0.7 g | 0.7 g | 0.7 g | 0.7 g | - | - |
| Sucrose | 68.5 g | 30 g | 30 g | 30 g | 30 g | 60 g | 30 g |
| Glucose | 36 g | - | - | - | - | - | - |
| 2,4-D (1 mg/mL stock) | 1.5 mL | 1.5 mL | 1.5 mL | 1.5 mL | 1.5 mL | - | - |
| pH | 5.2 | 5.8 | 5.8 | 5.8 | 5.8 | 5.6 | 5.6 |
| Agar (washed)* | - | 8.0 | - | - | - | - | - |
| Agar, Plant cell culture tested** | - | - | 8.0 g | 8.0 g | 8.0 g | - | - |
| Gelrite | - | - | - | - | - | 3.0 g | 3.0 g |
| Autoclave | filtered | 20min | 20min | 20min | 20min | 20min | 20min |
| $N_6$ vitamin stock, 1000× | 1 mL | 1 mL | 1 mL | 1 mL | 1 mL | | |
| MS vitamin, 1000× | | | | | | 1 mL | 1 mL |
| Silver Nitrate | 0.85 mg | 0.85 mg | 0.85 mg | 0.85 mg | 0.85 mg | - | - |
| L-cysteine | - | 0.4 g | - | - | - | - | - |
| DTT | - | 0.15 g | 0.2 g | 0.2 g | 0.2 g | 0.2 g | 0.2 g |
| Acetosyringone (100 mM/mL) | 100 µL | 100 µL | - | - | - | - | - |
| Vancomycin (100 mg/mL) | - | - | 1 mL | - | - | - | - |
| Carbenicillin (200 mg/mL) | - | - | 1 mL | 1 mL | 1 mL* | 1 mL* | - |
| Timentin (150 mg/mL) | - | - | 1 mL | 1 mL | 1 mL* | 1 mL* | - |
| Bialaphos (3 mg/mL stock) | - | - | - | 0.5 mL | 1 mL | 1 mL | - |