

# Particle bombardment of Hi II immature zygotic embryos and recovery of transgenic maize plants

## Materials

### ⌘ Plasmid

A plasmid containing a selectable marker (*bar* gene, Spencer et al., 1990) and a screenable marker (*uidA* or *gus* gene) or gene of interest (GOI). For example, pAHC25 (Christensen and Quail, 1996).

### ⌘ Plant material

Ear of maize Hi II plant (A188xB73 origin, see Armstrong et al., 1991). Greenhouse ears are collected 10-13 days after pollination from greenhouse grown plants when embryos are about 1.2-1.8 mm in size. Immature zygotic embryos are dissected from the ear and used as target tissue for bombardment (Songstad et al., 1996).

### ⌘ Biolistic Gun

PDS 1000/He biolistic gun and all disposables from Bio-Rad (Hercules, CA).

## Media

### ⌘ N6E (callus initiation):

4 g/L N6 salts (Chu et al., 1975), 1 ml/L (1000X) N6 vitamin stock, 2 mg/L 2,4-D, 100 mg/L myo-inositol, 2.76 g/L proline, 30 g/L sucrose, 100 mg/L casein hydrolysate, 2.5 g/L gelrite, pH 5.8 (100x15 mm petri-plates). Silver nitrate (25 µM) added after autoclaving.

### ⌘ N6OSM (osmotic medium):

4 g/L N6 salts, 1 ml/L N6 vitamin stock, 2 mg/L 2,4-D, 100 mg/L myo-inositol, 0.69 g/L proline, 30 g/L sucrose, 100 mg/L casein hydrolysate, 36.4 g/L sorbitol, 36.4 g/L mannitol (Vain et al, 1993), 2.5g/L gelrite, pH 5.8 (100x15 mm petri-plates). Silver nitrate (25 µM) added after autoclaving.

### ⌘ N6S (selection):

4 g/L N6 salts, 1 ml/L N6 vitamin stock, 2 mg/L 2,4-D, 100 mg/L myo-inositol, 30 g/L sucrose, 2.5 g/L gelrite, pH 5.8 (100x15 mm petri-plates). Bialaphos (2 mg/L) and silver nitrate (5 µM) added after autoclaving.

⌘ **Regeneration Medium I:**

4.3 g/L MS Salts (Murashige and Skoog, 1962), 1 ml/L (1000X) modified MS vitamin stock, 100 mg/L myo-inositol, 60 g/L sucrose, 3 g/L gelrite, pH 5.8 (100x25 mm petri-plates). Bialaphos (3 mg/L) or Glufosinate Ammonia (4 mg/L) added after autoclaving.

⌘ **Regeneration Medium II:**

4.3 g/L MS Salts, 1 ml/L modified MS vitamin stock, 100 mg/L myo-inositol, 30 g/L sucrose, 3 g/L gelrite, pH 5.8 (100x25 mm petri-plates).

N6 based media for Hi II is after Songstad et al., 1996.  
Media for regeneration is after Armstrong and Green, 1985.

## Methods

⌘ **Embryo Dissection**

1. Dehusk ear. Cut off and discard top 1 cm of ear and insert a straight nosed forceps into the tip end of the ear. This “handle” facilitates aseptic handling of the cob during embryo dissection. Place impaled ear and forceps into a sterilized mason jar in laminar flow bench. If necessary, sterilize up to 4 ears in one mason jar.
2. Add ~ 700 ml of sterilizing solution (50% commercial bleach (5.25% hypochlorite) in water + 1 drop of surfactant Tween 20) to cover ear. During the 20 minute disinfection, occasionally swirl the ears and tap the mason jar on the surface of the flow bench to dislodge air bubbles for thorough surface sterilization of ear. Holding on to the forceps, pour off bleach solution and rinse the ears three times in generous amounts of sterilized water. The final rinse is drained off and the ears are ready for embryo dissection.
3. In a large (150x15mm) sterile petri-plate, cut off the kernel crowns (the top 1-2 mm) with a sharp scalpel blade. Use sterilizing ovens for intermittent re-sterilizing of utensils throughout this protocol.
4. Excise the embryos by inserting the narrow end of a sharpened spatula between the endosperm and pericarp at the basipetal side of the kernel (towards the bottom of the cob) popping the endosperm out of the seed coat. This exposes the untouched embryo which sits at the top-side of the kernel, close to the kernel base. The embryo is gently coaxed onto the spatula tip and plated with the embryo-axis side down (scutellum side up) onto filter paper overlaying the N6E media in a 2x2 cm grid (30 embryos/plate).
5. Wrap the plate with vent tape and incubate at 28°C in the dark for 2 or 3 days, whichever is most convenient.

⌘ **Gold particle preparation**

1. See protocol “Gold Particle Preparation”.

⌘ **Micro-projectile bombardment**

1. The embryos will be ridged and swollen as Type II callus initiation has begun. This is an appropriate stage for bombardment.

2. Draw a 3.5 cm diameter circle on the bottom of a plate of osmotic medium (N6OSM), Vain, P., et al. 1993. This defines the target area to which callus pieces are loaded for bombardment.
3. Four hours prior to bombardment, use sterile forceps to transfer the embryos and filter paper onto the osmotic medium (N6OSM), (Vain et al., 1993). Center the embryo grid, not the filter paper, on the 3.5 cm diameter circle drawn on the bottom center of the plate. Embryos should be facing scutellum side up at bombardment since it is from this surface that subsequent callus initiation begins from which transformed cells are then selected.
4. Load 650 psi rupture disk.
5. Assemble the macro-carrier launch assembly by first laying in place a stopping screen followed by an inverted, pre-loaded macro-carrier holder (see Gold Particle Preparation Protocol) which is held in place by screwing on the launch assembly lid.
6. Slide the launch assembly into place immediately below the helium nozzle, and set the gap distance (6 mm).
7. Slide a 150 micron mesh screen (McMaster Carr, Elmhurst IL), which is sterilized by autoclaving onto the shelf directly below the launch assembly. This screen is supported on a second plexi-glass stage (like the one that holds the petri plate at bombardment) with a 3.8cm diameter hole cut in the middle of it. Provided the construct being bombarded does not change, we re-use this screen for 8-10 shots before discarding.
8. Slide the opened petri dish containing the target tissue onto the shelf at a selected distance from the stopping screen (6 cm).
9. The vacuum chamber is closed, a vacuum pulled, and the gun fired in time for the rupture disk to break as soon as the vacuum reaches 28 inches of Hg.
10. The chamber is vented, the plate containing the bombarded tissue removed, and the gun prepared for the next bb by replacing the spent rupture disk, macro-carrier and stopping screen (disposables). All plasmid waste is disposed of in biohazard bags for autoclaving.
11. Repeat step 3-9 for each shot.
12. The bombarded embryos (still on filter paper on N6OSM) are gently wrapped with vent tape and incubated in 28°C in the dark. The next day (16-20 hours after bombardment), embryos are transferred off the filter paper on N6OSM to the surface of N6E media with no filter paper to continue callus initiation. Embryos are again oriented scutellum side up and plates are wrapped with vent tape.

⌘ **Selection for stable transformation events**

1. After 10-14 days on initiation medium (N6E) the bombarded embryos are transferred to N6S selection medium (2.0 mg/L bialaphos) to begin the recovery of transformed cells. Plates are wrapped with parafilm throughout selection.
2. Three weeks later, embryos are transferred to fresh N6S. Within 6-8 weeks of bombardment, bialaphos resistant clones emerge from selected embryos.

⌘ **Regeneration of transgenic plants**

1. Regeneration of transgenic Type II callus (friable, stocked somatic embryos present) is accomplished by transferring about 15 small pieces (approximately 4 mm) of highly

embryogenic callus to Regeneration Medium I and incubating for 2-3 weeks at 25°C in the dark. Petri-plates are wrapped with vent tape.

2. After 2-3 weeks, matured somatic embryos are transferred to the light on Regeneration Medium II for germination and again the plates are wrapped with vent tape. Plantlets sprout leaves and roots on this medium.

#### ⌘ Plant Acclimatization

1. Acclimatization of regenerated plants to soil is accomplished as described in Frame et al., 2000. Also see protocol "Greenhouse".

## References

- Armstrong, C.L. and Green, C.E. 1985. Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. *Planta* 164: 207-214.
- Armstrong, C.L., et al. 1991. Development and availability of germplasm with high Type II culture formation response. *Maize Genetics Coop Newsletter* 65: 92-93.
- Christensen, A.H. and Quail, P.H. 1996. Ubiquitin promoter-based vectors from high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Research* 5: 213-218.
- Chu, C.C., et al. 1975. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. *Sci. Sinica* 18: 659-668.
- Frame, B., et al. 2000. Production of transgenic maize from bombarded Type II callus: effect of gold particle size and callus morphology on transformation efficiency. *In Vitro Cell. Dev. Biol.-Plant*. 36:21-29.
- Murashige, T and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*. 15: 473-497.
- Songstad, D.D., et al. 1996. Production of transgenic maize plants and progeny by bombardment of Hi II immature embryos. *In Vitro Cell. Dev. Biol.-Plant*. 32:179-183.
- Spencer, T.M., et al., 1990. Bialaphos selection of stable transformants from maize cell culture. *Theor. Appl. Genet.* 79: 625-631.
- Vain, P., et al. 1993. Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. *Plant Cell Reports* 12: 84-88.