Agrobacterium-mediated transformation of Hi II immature zygotic embryos and recovery of transgenic maize plants

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Materials

⌘ Plasmid

Agrobacterium tumefaciens strain EHA101 (Hood et al., 1986) containing the standard binary vector pTF102 (11.6 kb). pTF102 contains a broad host range origin of replication (pVS1, Hajdukiewicz et al., 1994) and a spectinomycin-resistant marker gene (*aad*A) for bacterial selection. The cauliflower mosaic virus (CaMV) 35S promoter (P35S) was used to drive both the *bar* selectable marker gene and the *gus* reporter gene. A tobacco etch virus (TEV) translational enhancer (Carrington and Freed, 1990) was included in the 5' end of the *bar* gene. The soybean vegetative storage protein terminator (Mason et al., 1993) was cloned to the 3' end of the *bar* gene. The *gus* gene contained a portable intron in its codon region (Vancanneyt et al., 1990) to prevent GUS activity in *Agrobacterium* cells.

⌘ Plant material

F_2 immature zygotic embryos (1.5 to 2.0 mm) of the maize *(Zea mays)* Hi II hybrid genotype (Armstrong et al., 1991) are aseptically dissected from greenhouse-grown ears harvested 10 to 13 days post pollination. Ears are stored up to three days at 4°C before dissection (see Note 1).

Media

Infection, resting, and selection media after Zhao et al (2001) with cefotaxime and vancomycin used instead of carbenicillin. The addition of cysteine to the co-cultivation media is after Olhoft and Somers (2001). Regeneration medium is based on Armstrong and Green (1985).

All media is prepared in 100 x 25 ml plates except for YEP which is in 100x15.

⌘ YEP Medium:

5 g L^{-1} yeast extract, 10 g L^{-1} peptone, 5 g L^{-1} $NaCl_2$, 15 g L^{-1} Bacto-agar. pH to 6.8 with NaOH. Appropriate antibiotics should be added to the medium when cooled to 50°C after autoclaving.

⌘ Infection Medium:

N6 salts and vitamins (Chu et al., 1975), 1.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.7 g L^{-1} L-proline, 68.4 g L^{-1} sucrose, and 36 g L^{-1} glucose (pH 5.2). This medium is filter-sterilized and stored at 4°C. Filter-sterilized acetosyringone (AS, 100 µM) is added prior to use. 100mM AS stocks (dissolved in DMSO to 200mM then diluted 1:1 with water) are stored at –20ºC for use as needed.

⌘ Co-cultivation Medium (make fresh – use within 4 days):

N6 salts and vitamins, 1.5 mg L^{-1} 2,4-D, 0.7 g L^{-1} L-proline, 30 g L^{-1} sucrose, and 3 g L^{-1} gelrite (pH 5.8). Filter sterilized silver nitrate (0.85 mg L^{-1}), AS (100 µM), Cysteine (300 mg L^{-1}), and N6 Vitamins are added to this medium after autoclaving.

⌘ Resting Medium:

N6 salts and vitamins, 1.5 mg L^{-1} 2,4-D, 0.7 g L^{-1} L-proline, 30 g L^{-1} sucrose, 0.5 g L^{-1} 2-(4-morpholino)-ethane sulfonic acid (MES), and 8 g L^{-1} purified agar (pH 5.8). Filter sterilized N6 vitamins, cefotaxime (100 mg L^{-1}) and vancomycin (100 mg L^{-1}), and silver nitrate (0.85 mg L^{-1}) are added to this medium after autoclaving.

⌘ Selection Medium I:

N6 salts and vitamins, 1.5 mg L^{-1} 2,4-D, 0.7 g L^{-1} L-proline, 30 g L^{-1} sucrose, 0.5 g L^{-1} MES, and 8 g L^{-1} purified agar (pH 5.8). Filter sterilized N6 vitamins, cefotaxime (100 mg L^{-1}) and vancomycin (100 mg L^{-1}), silver nitrate (0.85 mg L^{-1}), and Bialaphos (1.5 mg L^{-1}, Shinyo Sangyo, Japan) are added to this medium after autoclaving.

⌘ Selection Medium II:

N6 salts and vitamins, 1.5 mg L^{-1} 2,4-D, 0.7 g L^{-1} L-proline, 30 g L^{-1} sucrose, 0.5 g L^{-1} MES, and 8 g L^{-1} purified agar (pH 5.8). Filter sterilized N6 vitamins, cefotaxime (100 mg L^{-1}) and vancomycin (100 mg L^{-1}), silver nitrate (0.85 mg L^{-1}), and Bialaphos (3 mg L^{-1}) are added to this medium after autoclaving.

⌘ Regeneration Medium I:

MS salts (Murashige and Skoog, 1962) and modified MS vitamins, 60 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol, no hormones and 3 g L^{-1} gelrite (pH 5.8). Filter sterilized cefotaxime (250 mg L^{-1}) and Bialaphos (3 mg L^{-1}) or Glufosinate Ammonia (4 mg L^{-1}) are added to this medium after autoclaving.

⌘ Regeneration Medium II:

MS Salts and modied MS vitamins, 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose, 3 g L^{-1} gelrite, (pH 5.8).

Methods

⌘ *Agrobacterium* Preparation

The vector system, pTF102 in EHA101, is maintained on YEP medium (An et al., 1988) containing 100 mg L^{-1} spectinomycin (for pTF102) and 50 mg L^{-1} kanamycin (for EHA101). Bacteria cultures for weekly experiments are initiated from stock plates that

are stored for up to one month at 4ºC before being refreshed from long-term, –80ºC glycerol stocks.

⌘ Embryo Dissection

1. De-husk ear. Cut off and discard top 1 cm of ear and insert tip end of a numbered straight nosed forceps into this end of the ear. This "handle" facilitates aseptic handling of the cob during embryo dissection.

2. 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 (see Note 2).

3. 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 tap the mason jar containing the ears on the surface of the flow bench to dislodge air bubbles for thorough surface sterilization of ear. Holding on to the forcep ends, 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 (see Note 2).

4. In a laminar flow bench, on a large (150x15mm) sterile petri-plate, cut off the kernel crowns of the ear (the top 1-2 mm) with a new, sharp scalpel blade. Use sterilizing ovens for intermittent re-sterilizing of utensils throughout this protocol.

5. 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 topside of the kernel, close to the kernel base. The embryo is gently coaxed onto the spatula tip and submerged in infection medium (see step 3 in section "*Agrobacterium* Infection").

⌘ *Agrobacterium* Infection

1. *Agrobacterium* cultures are grown for three days at 19°C on YEP medium amended with 100 mg L^{-1} spectinomycin and 50 mg L^{-1} kanamycin.

2. One full loop (3 mm) of bacteria culture is scraped from the three-day old plate and suspended in 5 ml of liquid infection medium (Inf) supplemented with 100 µM AS (Inf + AS) in a 50 ml centrifuge tube. The tube is fixed horizontally to a bench-top shaker or a Vortex Genie platform head and shaken on low speed (~75 rpm) for two to four hours at room temperature. This pre-induction step is carried out for all experiments.

3. For infection, immature zygotic embryos from one ear (1.5 to 2.0 mm) are dissected to bacteria-free Inf + AS medium (2 ml) in 2 ml microcentrifuge tubes (20 to 100 embryos) and washed twice with this medium (see Note 3). The final wash is removed and 1 to 1.5 ml of *Agrobacterium* suspension (OD_{550} = 0.3 to 0.4) is added to the embryos. Embryo infection is accomplished by gently inverting the tube 20 times before resting it upright for five minutes with embryos submerged (see Note 4). Embryos are not vortexed at any time during this procedure.

⌘ Co-cultivation

1. After infection, embryos are transferred to the surface of co-cultivation medium and excess *Agrobacterium* suspension is pipetted off the medium surface. Embryos are oriented with the embryo-axis side in contact with the medium (scutellum side up).

2. Plates are wrapped with vent tape (Vallen Safety Supply, USA) and incubated in the dark at 20ºC for three days.

⌘ Resting

1. After three days for co-cultivation, embryos are transferred to resting medium at 28ºC in dark for 7 days (see Note 5). Plates are wrapped with vent tape. Transfer all embryos from co-cultivation medium to resting and selection media.

⌘ Selection for stable transformation events

1. After seven days on resting medium (28°C, dark), embryos, responding or not, are transferred to selection medium (35 per plate) containing 1.5 mg L^{-1} bialaphos for 2 weeks. They are then sub-cultured for two more 2-week passages on 3 mg L^{-1} bialaphos. Plates are wrapped with parafilm throughout selection.

2. Putatively transformed events are visible as rapidly growing Type II callus as early as five weeks after infection (rate of clone emergence is construct dependent).

⌘ Regeneration of transgenic plants

1. Regeneration of R_0 transgenic plants from Type II embryogenic callus is accomplished by a three-week maturation step on Regeneration Medium I followed by germination in the light on Regeneration Medium II - as described in Frame et al. (2000). Stable transformation efficiency (%) is calculated as the number of bialaphos resistant callus events recovered per 100 embryos infected.

⌘ Plant Acclimatization

1. Acclimatization of regenerated plants to soil is accomplished as described in "Greenhouse protocol".

⌘ Notes

1. Ears are stored in the fridge by necessity. Because of the three-day co-cultivation step, we generally carry out *Agrobacterium*-mediated infection experiments on Mondays and Fridays so that embryos can be taken off co-cultivation medium on Thursdays or Mondays without having to come in on weekends. This means that ears harvested on Tuesday-Thursday are infected on Friday, and ears harvested Friday-Sunday are infected on Monday.

2. For field ears or an excessive number of greenhouse ears at one time, we often sterilize up to 15 ears at once in a 4L pre-autoclaved beaker. This is a far more efficient use of time and resources than sterilizing ears in groups of three.

3. This medium is stored in the fridge between ears.

4. This tube is incubated in the dark for the 5 minutes infection step.

5. When the co-cultivation medium (300 mg/L cysteine) is fresh (1 day old) and your embryos are small (1.0 mm), you may see a delay in embryo response, and in some cases, embryo death after 3 days co-cultivation. Nevertheless, transfer all embryos from co-cultivation medium to resting medium as many of the slow responding embryos will eventually form callus. In our infection experiments, we generally use 1-4 day old co-cultivation medium.

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