

***Agrobacterium*-mediated Rice Transformation from Mature Seeds**

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Plant Material

Mature rice seeds *Oryza sativa* cv. Nipponbare were obtained from USDA Agricultural Research Service; Dale Bumpers National Rice Research Center www.ars.usda.gov/sea/dbnrrc/gsor are used.

Genotypes:

Oryza sativa cv. Nipponbare is considered the model
others are amenable, see literature

Mature Seed Explant Preparation, Callus initiation and Embryogenic Callus culture

Mature Seed Explant Preparation

1. Use mature seeds, stored in seed storage room under proper conditions. Place 300 dehusked seeds in 50 ml Falcon tube. Seeds are manually dehusked using fine grit sand paper being careful not to damage seeds. Select only intact seeds for the sterilization and tissue culture.



Surface Sterilization

2. Sterilize seeds with 50% bleach in a shaking incubator set at 200 rpm for 30 minutes at room temperature.

3. Seeds are then rinsed 3 times with sterile ddH₂O or until there is no odor of bleach. Seeds are carefully transferred to an empty sterile Petri dish.

Callus Initiation

1. Place 20 seeds into Petri dish containing callus induction medium. Seeds are evenly spaced out to allow for growth. Seal the Petri plates with parafilm and incubate at 28°C in dark.

2. After 2 weeks, the germinated roots and shoots are removed to allow for embryogenic callus growth. Seal the Petri plates with parafilm and incubate at 28°C in dark.

3, After 2-3 weeks, growth of embryogenic calli should be visible using a dissecting scope. Embryogenic callus is subcultured using the dissecting microscope onto fresh callus induction media every 2 weeks. Seal the Petri plates with parafilm and incubate at 28°C in dark.

Note: Somaclonal variation increases with the amount of time callus is in culture. For this reason, fresh cultures should be started every 3-4 months.



Initiation of *Agrobacterium tumefaciens* Culture

1. Streak out *Agrobacterium* culture from -80°C glycerol stock onto YEP medium plate containing appropriate antibiotics and incubate the plate at 25°C for 2 days.

2. On day of infection, aliquot 25 ml of sterile infection medium into a 50 ml Falcon tube. Add acetosyringone (100mM) to infection medium.

3. Take about 2.5 full loops from freshly streaked YEP plate and suspend in the tube. (about 1 full loopful/10ml of infection medium).

4. Shake the infection medium + *Agro* at 25°C at 175 rpm for 1 hour to re-suspend fully.

5. Transfer 2 ml of *Agrobacterium* suspension to spectrophotometer cuvette and measure OD₆₀₀ ≈ 0.35-0.50 (1 X 10⁹ cfu/ml).

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



Infection

1. **One week prior to infection:** To make sure that callus is actively growing and dividing, embryogenic callus is subcultured into small pieces (~1 -2 mm.) onto fresh callus induction medium about 50 pieces/plate. Incubate at 25°C in the dark.

2. **On the day before infection:** Transfer the small pieces to co-cultivation media containing acetosyringone. Incubate overnight at 25°C in the dark.

3. **On day of infection:** Dispense 1 drop from P1000 pipette of *Agrobacterium* suspension onto each piece of callus making sure to fully immerse each piece with the suspension. Make sure that every piece is treated.

Allow pieces to dry in the hood ~20 minutes and wrap the plate with parafilm and incubate at 25°C in dark for 3 days for the co-cultivation period.

Note: Make sure that pieces are fully dry before wrapping plates and incubating.

4. Following the 3 day co-cultivation period, callus is transferred to Resting Media containing 200 mg/L Carbenicillin and 150 mg/L Timentin. *Note the antibiotics used are specific to the Agrobacterium strain, check the literature*). Cultures remain on Resting Medium for 7 days to begin killing the *Agrobacterium*. *Care is taken to make sure to not break the pieces apart. The number of pieces transferred to resting media is counted and recorded. This will be the initial number of callus infected for calculating transformation efficiency later.* Incubate calli at 25°C in the dark for one week.

Selection

5. Following the resting phase, each piece of callus is transferred to Selection I medium and incubated at 25°C in dark for 4-6 weeks sub-culturing bi-weekly. *Each piece of callus is kept together and not broken apart.*

Note transformants are selected using 10 mg/L PPT or 3 mg/L Bialaphos selecting for constructs containing the bar gene (right)

Transformants are selected using 50 mg/L Hygromycin selecting for constructs containing the hpt gene



6. Putative transformants (events) can be selected after 8 weeks on selection I medium. *The transformants should be a pale yellow color, friable, and growing. They should look exactly the same as non-transformed callus. If the colonies do not look the same as non-transformed callus, they are probably not transformed.*

7. The putative transformants (events) are sub-cultured onto Regeneration I medium to initiate shoot formation. The transformants should be incubated for one week in the dark at 25°C and then transferred to low light (16 hour light/8 hour dark) for 2 weeks. Shoots should begin to appear after 2-3 weeks. If shoots are not well formed, the transformants can be transferred to fresh Regeneration I media for 2 additional weeks. *Each event is kept separate.*

8. Once shoots are well formed (~2cm), they are transferred individually to Regeneration II medium for root formation. The Regeneration II media should be poured thick into petri dishes. Plantlets are transferred to these thick poured petri dishes and covered with an empty, sterile petri dish bottom. These plates are sealed with parafilm and incubated at 25°C in the light (16 hour light/8 hour dark) for 2 weeks.

9. Once plantlet shoots are elongated and roots are beginning to form, they are transferred to Regeneration II media in plantcons for further growth. The plantcons are incubated at 25°C in the light (16 hour light/8 hour dark) for 2 weeks.

Acclimation to soil

1. Once plants are well formed with shoots and roots, they are acclimated to soil. Up to 10 clones from each event are planted to ensure that seed is obtained.

2. Individual plants are planted into well moistened Metromix 510 in 5 gallon pots. Excess media is carefully washed away by dipping roots into room temperature water to minimize fungal growth. Plants are immediately cover with an empty plantcon top to ensure the high humidity that the plants are accustomed to is maintained. *Plants have a very thin cuticle after tissue culture and care must be taken to prevent desiccation.* Plants are placed in trays, watered thoroughly and placed in the growth chamber under shade cloth at 27°C under sodium halide lighting. After 4-5 days, plantcon tops are propped up slightly to allow plants to begin to develop a thicker cuticle. Plantcon tops can typically be removed after 10 days.

3. Plants in pots are also maintained in trays, watered thoroughly and placed in the growth chamber under shade cloth at 27°C under sodium halide lighting. Transgenic rice can also be grown in a greenhouse with supplemental lighting. Rice is slowly allowed to acclimate to the higher light. *Also, rice uses a lot of water and care must be taken to prevent drought conditions.*



4. Plants are grown to maturity and selfed.

T0 Transgenic Rice in the Greenhouse



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. Acetosyringone (100mM)- dissolve 19.6 mg in ~ 500 μ L of DMSO (dimethyl sulfoxide); once dissolved, bring the volume 1 mL and filter sterilize into a sterile 1.5 mL microcentrifuge tube. This can be scaled up for larger batches, but is typically sufficient for 10 + transformation experiments. can be stored frozen at - 20°C for up to 30

b. 100 mM Silver nitrate (AgNO_3) – dissolve 850 mg in ~40 mL of ddH₂O. 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.

If used:

c. L-Cysteine (L-Cys) – dissolve 400 mg in ~ 3 mL of ddH₂O 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 ddH₂O. 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.