Agrobacterium tumefaciens-mediated transformation of japonica rice

1. **Sterilization of primary seed**: place 50-200 dehusked rice seeds in a 50 ml sterile tube. Add 30-35 ml of a commercial bleach solution (dilute the bleach 1/3 with water and add a few drops of a detergent or Triton-X). Leave seed in bleach solution for 30 minutes, shaking frequently. Rince three times with sterile water.

2. **Seed callus induction**: place 10 seeds/plate on NB medium using sterile forceps. Allow the plates to dry under the flow hood until there is no water remaining around the seed. Parafilm plates and set in the dark, 28°C, for one month.

3. **Multiplication of embryonic units**: place anywhere from 50-100 of the embryonic units (small-medium size whitish calli originating near the zone of shoot emergence) onto fresh NB medium. Picking up the seedlings and rubbing them gently on the medium surface helps to release the embryonic units. Place back into dark, 28°C, for 10-14 days.

4. **Agrobacterium preparation**: four days before the transformation start an overnight liquid culture of the Agro lines (10 ml LB medium + Rif 50 + selective antibiotic). Put on vigorous shaking overnight at 28°C. The next day pipette 200 µl onto AB medium plates (Rif 50 + selective antibiotic) and spread. When dry, loosely parafilm and incubate inverted at 28°C for 3 days.

5. **Transformation**: scrape 1/4 to 1/2 of the Agro off of one of the AB plates, using a sterile spatula, and put into 30 ml R2-CL liquid medium in a sterile 50 ml tube. Vortex vigorously until homogenization is achieved. Measure by flurometer the optical density of a 1 ml aliquot of this solution at 600 nm (use pure R2-CL for blank). Record reading and adjust the Agro solution (either by adding more Agro or R2-CL) to an O.D. between 0.7-1.0.

In a sterile petri dish add 25 ml of Agro (at correct O.D.) and the calli to be transformed (up to 100 calli per dish), incubate with occasional, gentle agitation for 15 min. Remove Agro with a 25 ml sterile pipette, transfer calli with sterile forceps onto a sheet of autoclaved filter paper (Whatman Chromatography paper 1 Chr, catalog #3001 917) and roll across the surface to dry the calli (using forceps to roll the calli). Successively roll the calli across 3-6 sheets of filter paper until completely dry. Attempt to break up clumps of calli to ensure uniform drying. Transfer the dry calli into a sterile petri dish, and under a laminar flow hood place the calli 10 per dish onto R2-CS medium. Let the dishes dry thoroughly under the hood before parafilming. Place the calli into an incubator -- completely dark and at 25°C -- for 3 days.

6. **Selection phase**: move the calli with sterile forceps (sterilize often) 10 per plate, onto R2-S medium. Leave any calli that appear wet and shiny, indicating that they have been overtaken by Agrobacterium growth. After drying thoroughly in the flow hood, parafilm and incubate in the dark at 28°C for two weeks. At the end of two weeks on R2-S, the

co-cultivated callus should have turned brown while the surface should have very small white growths (transformation events).

7. **Proliferation phase**: transfer the calli, 7 per plate, onto NBS medium. Sterilize the forceps often to prevent cross-contamination. Allow plates to dry well, parafilm and place in the dark at 28°C for 2-3 weeks. At the end of week one, open the dishes and gently "spread" the co-cultivated calli on the surface of the NBS medium to detach the transformed calli from the parent callus. Draw lines on the bottom of dishes around each callus to ensure that each "line" of transformed calli remains with the parent callus (potential clones stay together). Re-parafilm and place into in the dark at 28°C for 1-2 additional weeks (depending on speed of callus growth).

8. **Maturation phase**: transfer the yellow-whitish, medium and large sized calli onto PR-AG medium. Divide a single dish of PR-AG into multiple sections by drawing lines on the bottom of the dish. Under each section write a given number to each callus line and place between 7-21 calli per line. Incubate in the dark at 28°C for ideally 7-8 days (do not exceed 10 days).

9. **Regeneration phase**: transfer 7 calli per dish onto RN medium, making sure to keep each dish of the same line labeled with the same number. Incubate the calli for 2 days in the dark at 28°C, and then move the calli under lights in a growth chamber (110-130 mM/mPAR, 12 h light/dark cycle, 28°C) for 4-6 weeks.

10. **Rooting of regenerated shoots**: remove a single, vigorously growing shoot from each callus, cut off the upper 2/3 of the shoot and any root with a sterile scalpel, and place into a tube of P medium. Flame the open top of the tube before placing the cap and parafilming the top. Place the tubes in the growth chamber for 3-4 weeks.

11. **Acclimation to greenhouse**: remove regenerated plants from tubes, cut back shoot and root in the same manner as step 10, and place plantlets into wet, autoclaved Jiffy peat pots. Place the plantlets into a plastic mini-greenhouse with only a small vent open for circulation. Keep plants in Jiffy pots for 15 days, and then do final transfer to pots (provide at least 1L soil per plant).

TIME-TABLE OF RICE TRANSFORMATION

1. Callus induction on NB medium (28ºC, dark, 4 weeks)

2. Multiplication of embryonic units on NB medium (28ºC, dark, 2 weeks)

3. Co-culture of calli with Agrobacterium and placement on R2-CS medium (25ºC, dark, 3 days)

4. Selection of calli on R2-S medium (28ºC, dark, 2 weeks)

5. Proliferation of calli on NBS medium (28ºC, dark, 2-3 weeks), spread calli at 1 week

6. Maturation of calli on PR-AG medium (28ºC, dark, 7-10 days)

7. Regeneration on RN medium (2 days dark at 28ºC, then 12 h light/12 h dark for 4-6 weeks)

8. Plantlet rooting on P medium (28ºC, 12 h light/12 h dark, 3-4 weeks)

9. Acclimation in Jiffy pots (high humidity, 15 days), followed by transfer to 1L or 3L pots

***FROM CALLUS INDUCTION TO ROOTED PLANTLETS = 4.5 - 5.2 MONTHS

***FROM TRANSFORMATION TO ROOTED PLANTLETS = 3 - 4 MONTHS