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Agrobacterium-mediated Transformation of *Japonica* Rice Using Mature Embryos and Regenerated Transgenic Plants

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[Abstract] Identification of novel genes and their functions in rice is a critical step to improve economic traits. *Agrobacterium tumefaciens*-mediated transformation is a proven method in many laboratories and widely adopted for genetic engineering in rice. However, the efficiency of gene transfer by *Agrobacterium* in rice is low, particularly among *japonica* and *indica* varieties. In this protocol, we elucidate a rapid and highly efficient protocol to transform and regenerate transgenic rice plants through important key features of *Agrobacterium* transformation and standard regeneration media, especially enhancing culture conditions, timing, and growth hormones. With this protocol, transformed plantlets from the embryogenetic callus of the *japonica* cultivar 'Taichung 65' may be obtained within 90 days. This protocol may be used with other *japonica* rice varieties.

Keywords: Electroporation, Novel genes, Callus induction, Binary vector, *Oryza Sativa* L.

[Background] Genetic transformation and expression of recombinant proteins in plant cells are major factors for plant genetic engineering. This is also a powerful tool for discovering novel genes and exploring genetically controlled traits. *Agrobacterium tumefaciens*-mediated transformation has been known for its unique ability to transfer a DNA segment from a specialized plasmid into a host plant cell (Gelvin, 2010). This feature allows efficient insertion of stable, unrearranged, single-copy DNA into plant genomes, which may lead to more stable expression than multiple gene copies or scrambled inserts (Iglesias *et al*., 1997). Since the initial reports in the early 1980s using *Agrobacterium* to generate transgenic plants, efforts were made to improve the tool in plant transformation. In most instances, major improvements involved alterations in plant tissue culture transformation and regeneration conditions rather than manipulation of host or bacterial genes. The first effective method for *Agrobacterium*-mediated transformation of *japonica* rice is now a common protocol in many laboratories (Chan *et al*., 1993; Hiei *et al*., 1994; Park *et al*., 1996). Many functional analyses, for example, PCR, *GUS* assay, and southern blot analysis, have confirmed the integration of foreign genes into transgenic rice plants obtained by *Agrobacterium*-mediated transformation (Dai *et al*., 2001); moreover, Mendelian inheritance of the transgenes was also reported (Pawlowski and Somers, 1996; Hiei *et al*., 1997).

The transfer of T-DNA and its integration into the rice genome is influenced by numerous factors, such as genotype, explants, *Agrobacterium* strains, plasmid vectors, addition of *vir*-gene inducing synthetic phenolic compounds, selection marker, and various conditions of tissue culture and regeneration. In tissue culture systems, selection of actively growing regenerable calli is a principal factor for efficient

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plant transformation. Likewise, optimization of culture conditions for co-cultivation of rice calli with *Agrobacterium* (Ozawa and Takaiwa, 2010) and suppression of *Agrobacterium* overgrowth may be applied to improve the transformation efficiency. Scientists have attempted to improve the plasmid constructions and rice transformation by *Agrobacterium*-mediated transformation to investigate the function of seed storage protein gene factors as well as cloning genomic candidate regions of more than 10 kb, which may have low restriction enzymes sites required for cloning. In particular, previous limitations of efficient promoter expression were overcome (Gupta *et al*., 2001; Furtado *et al*., 2008), especially for endosperm-specific expression (Zhou *et al*., 2013).

Several transformation methods have been established by many laboratories using mature *japonica* and *indica* seeds. Taichung 65 is a *japonica* rice variety, selected from a cross between the two Japanese varieties, Shinriki and Kameji, in 1923 (Iso, 1957). Taichung 65 is considered an important cultivar for studying rice genetics and breeding. Several point mutations were induced to the genetic stock of Taichung 65 using the methylnitrosourea (MNU) mutagen and have been maintained at the institute of plant genetic resources of Kyushu university. Taichung 65 has been extensively used as a model cultivar for rice biology, breeding research, and genomic studies initiated in many laboratories. However, transformation efficiency is still low in most *indica* and many *japonica* varieties due to the difficult regeneration; consequently, the procedure to obtain transgenic plants takes an average of 5 months (Nishimura *et al*., 2006). Thus, additional improvements in the transformation potentiality are still possible.

Here, we describe a protocol for *Agrobacterium*-mediated transformation in rice using mature embryos. This protocol is based on the method described by Toki (Toki, 1997), with several modifications; in particular, meropenem is used for plant regeneration (Ogawa and Mii, 2007). Meropenem exhibits the highest antibacterial activity and also achieves a high shoot formation rate in rice and tomato (Ogawa and Mii, 2007). Using this protocol, we succeeded to produce hundreds of independent transgenic lines and transformed several novel genes over the past decade, including *Endosperm Storage Protein (ESP1),* encoding a eukaryotic chain release factor1 (eRF1) (Elakhdar *et al*., 2019); *Esp2*, encoding the protein disulfate isomerase 1-1 (PDI 1-1) (Satoh-Cruz *et al*., 2010); *Glup3*, encoding a vacuole processing enzyme (VPE) (Kumamaru *et al*., 2010); *Glup4,* encoding the small GTPase Rab5a (Fukuda *et al*., 2011); and *Glup6*, encoding the guanine nucleotide exchange factor (GEF) (Fukuda *et al*., 2013).

In summary, this protocol is a step-by-step approach to obtain stably transformed plants from mature rice embryos by optimizing several stages of *Agrobacterium* transformation and standard regeneration medium components, including culture conditions, timing, and growth hormones. In this method, we use a 13.8 kb construct carrying the *hygromycin phosphotransferase gene (hpt)*, *a firefly luciferase reporter gene (LUC)*, *spectinomycin (Sp)*, an attR1 site, the chloramphenicol resistance gene (Cm^R), the *ccdB* gene, the attR2 site, and the *ZmUbi1* promoter (Figure 1 and Figure 2).

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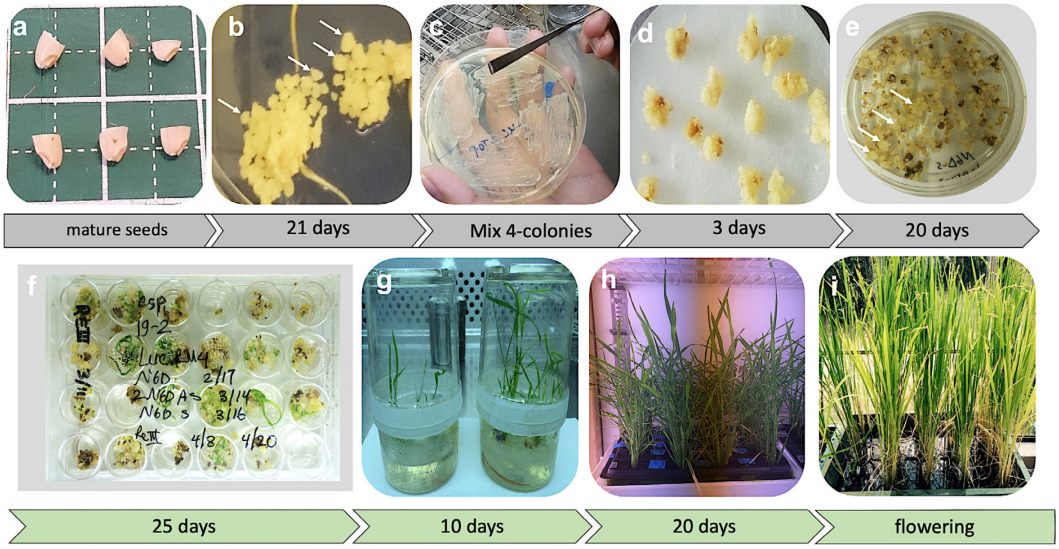

Figure 1. Flow chart for *Agrobacterium*-mediated transformation and regeneration of rice calli using mature embryogenic seed (*cv; Taichung 65*). (a) Seed embryos. (b) Calli pieces with granular structure, yellowish-white. (c) Growth of transformed *Agrobacterium* harboring the pSMAH638OX/Ubilp binary vector. (d) Co-cultivation of infected calli. (e) De-colonization of *Agrobacterium tumefaciens*. (f) Shoot regeneration. (g) Rooting in regenerated shoots. (h) Hardening of rooted plants. (i) Transplanted hardened plants.

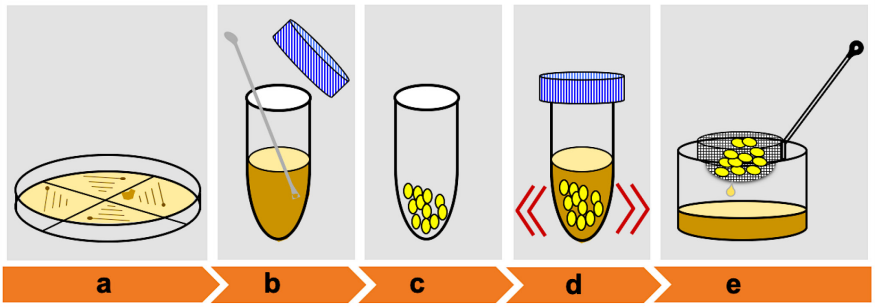

Figure 2. Schematic showing embryo calli infection by *Agrobacterium*. (a) Growth of *Agrobacterium*-mediated harboring pSMAH638OX/Ubilp vector on LB medium. (b) Mix a small portion of the positive colonies. (c) Collect healthy calli for infection. (d) Calli infection. (e) Remove excess *Agrobacterium* suspension.

Materials and Reagents

Plasticware and Glass

1. Petri dish, 90 × 20 mm
2. Petri dish, 90 × 15 mm
3. 24-well multi-well plates
4. Sterile plastic falcon tubes, 50 ml

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5. Filter paper, 70 mm
6. Parafilm tape (Parafilm Pechiney PM996; 125' L X 4"; www.parafilm.com)
7. Sterile syringe filters 0.22 µm
8. Sterile syringe, 30 ml
9. Stainless-steel sieve
10. Microspatula
11. Plant culture pots for rooting (6.5 × 6.5 cm)
12. Plant soil pots (23 × 23 × 19 cm)

Biological materials

1. Mature dry seeds of *japonica cv.* Taichung 65
   The target cultivar of the current transformation method.
2. Binary vector containing gene of interest
   In the following protocol, the binary vector pSMAH638OX/Ubilp (13.8 Kb) construct was derived by the Gateway recombination cloning technology (Invitrogen™, Thermo Fisher Scientific, Inc.), harboring firefly luciferase (*LUC*) reporter gene, the hygromycin phosphotransferase (*hpt*) selectable gene marker, spectinomycin (Sp), an attR1 site, the chloramphenicol resistance gene (CmR), the ccdB gene, and the attR2 site. We used the ZmUbi1 promoter for LUC expression (Hakata *et al*., 2010; Elakhdar *et al*., 2019)
3. *Agrobacterium* tumefaciens strains EHA101 or EH105 (Hood *et al*., 1993).

Reagents and chemicals

1. Ethanol CH_3CH_2OH (Sigma-Aldrich, catalog number: N0640), 70% (v/v) in distilled water, store at room temperature (RT)
2. Sodium hypochlorite 10% NaClO (Sigma-Aldrich, catalog number: 105614), store at RT
3. Sodium chloride, NaCl (Sigma-Aldrich, catalog number: 7647-14-5), store at RT
4. Sodium hydroxide, NaOH (Sigma-Aldrich, catalog number: 1310-73-2), store at RT
5. Tween® 20 EMD, (CALBIOCHEM, catalog number: 655205), store at RT
6. Sucrose $C_{12}H_{22}O_{11}$ (Wako, catalog number: 196-00015), store at RT
7. D-Glucose $C_6H_{12}O_6$ (Sigma-Aldrich, catalog number: 07-0680-5), store at RT
8. D-Sorbitol $C_{16}H_{14}O_6$ (Sigma-Aldrich, catalog number: 28-4770-5), store at RT
9. Casamino acid (Sigma-Alorich, catalog number: 65072-00-6), store at RT in dry condition
10. L-Proline (PEPTIOE, catalog number: 2718), store at RT
11. CHU (N6) Basal salt mixture (Sigma-Aldrich, catalog number: C1416-10L), store at 2-8°C
12. Murashige & Skoog Basal Medium (Sigma-Aldrich, catalog number: M552450L), store at 2-8°C
13. CHU N6-Vitamin solution X1000 (Phyto, catalog number: PHT:C149), store at 2-6°C
14. LB Broth, Miller (Luria-Bertani; BD Difco, catalog number: 244620), store at RT
15. Bacto Peptone (BD Difco, catalog number: 211677), store at RT
16. Bacteriological agar (Bacto Agar; BD Difco, catalog number: 214010), store at RT

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17. 2,4-Dichlorophenoxyacetic acid (Wako, catalog number: 040-18532), store at RT
18. 1-Naphthaleneacetic acid NAA $C_{10}H_7CH_2COOH$ (Wako, catalog number: 86-87-3), store at RT
19. Kinetin $C_{10}H_9N_5O$ (Wako, catalog number: 110-00331), store at 2-10°C
20. Gelrite (0.4%) (Wako, catalog number: 067-04035), store at RT
21. Carbenicillin sodium salt (Sigma-Aldrich, catalog number: C1389-5G), store at 2-8°C
22. Hygromycin B (Wako, catalog number: 085-06153), store at -20°C
23. Acetosyringone (Sigma-Aldrich, catalog number: D134406-5G), store at -20°C
24. Meropenem (Dainippon Sumitomo Pharma, Osaka, Japan), store at -20°C
25. Dimethyl sulfoxide DMSO $(CH_3)_2SO$ (Wako, catalog number: 046-21981), store at RT
26. KOD-FX (TOYOBO, catalog number: KFX-101), store at -20°C
27. Sterilizing solution (see Recipes)
    Solution A
    Solution B
28. Antibiotics (see Recipes)
    Carbenicillin (500 mg/ml)
    Hygromycin (50 mg/ml)
    Kanamycin (50 mg/ml)
    Acetosyringone (100 mg/ml)
    Acetosyringone (10 mg/ml)
    Meropenem (12.5 mg/ml)
    2,4-D (0.2 mg/ml)
    NAA (0.2 mg/ml)
    Kinetin (1 mg/ml)
29. Cultivation medium (see Recipes; Table 1)
    LB sold medium
    LB liquid medium
    YEP medium

Notes:

a. *All liquids and equipment containing Agrobacterium must be appropriately sterilized.*
b. *Essential elements that may influence the effectiveness of transformation are reagents and chemicals. Therefore, we strongly suggest following the methodology, particularly when starting a new protocol. The usage and storage conditions of chemicals are different; therefore, please make sure to follow the recommendations of each company, with particular attention to hazard information on the substances.*

Equipment

1. -86°C Ultra-Low temperature freezer (SANYO, catalog number: MDF-U538)
2. -30°C Ultra-Low temperature freezer (SANYO, catalog number: MDF-792AT)

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3. MicroPluser™ (Bio-Rad, Hercules)
4. Spectrophotometer (Thermo Scientific™, model: NanoDrop 2000)
5. Incubator/ shaker 28°C for Agrobacterium
6. Plant growth chamber (SANYO)
7. Shaker for seed sterilization
8. Laminar flow hood (SANYO)
9. Autoclave
10. pH meter
11. Spinbar® magnetic stir bar (Sigma-Aldrich, catalog number: Z127116)
12. Polycarbonate vacuum desiccator (SANSYO, catalog number: SPD-WVGT240)
13. Thermal cycler (Bio-Rad, model: Tetrad 2 Thermal Cycler (4 × 96-well)
14. Locking system greenhouse
15. Rice seedling raising soil (JA Kumiai King Soil; Agr. Japan Co., Ltd.)

Procedure

A. *Agrobacterium* culture and transformation

1. Transformation of competent *Agrobacterium* cells (EHA101 or EHA105) with binary vector pSMAH638OX/Ubilp (13.8 Kb) constructs (Figure 3).

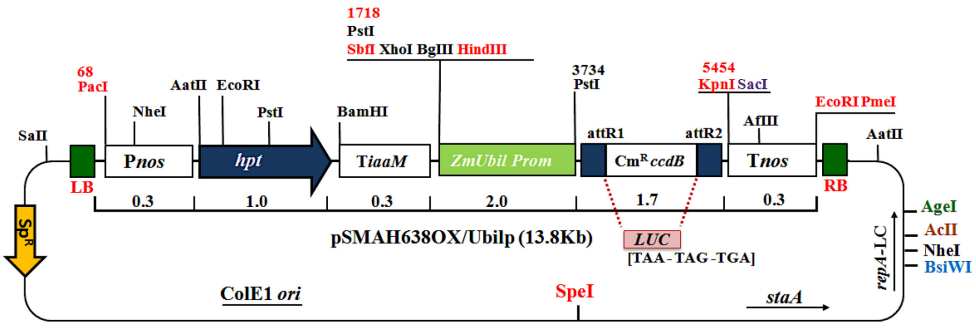

Figure 3. Binary vector pSMAH638OX/Ubilp (13.8 Kb) construct carrying the hygromycin phosphotransferase gene (hpt), firefly luciferase reporter gene (luc), *spectinomycin* (Sp), an attR1 site, the chloramphenicol resistance gene (CmR), the ccdB gene, the attR2 site, and the ZmUbi1 promoter.

a. Thaw 50 µl *Agrobacterium* competent cells (EHA101 or EH105) on ice (Figure 4A).
b. In 1.5 ml tube, mix 2 µl plasmid DNA (100 picogram/µl) with competent cells.
c. Incubate the cell mixture on ice for 10 min.
d. Perform transformation using the MicroPluser™ (Bio-Rad) electroporation method with the "Agr" mode (Figure 4B).
e. After 10 min, move the cell mixture to the cuvette tube and then the cuvette to chamber slide.

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f. Press the Pluse button until a tone sounds indicating that the pulse has been given.
g. Immediately add 250 µl YEP medium to cells.
h. Transfer cells to 25 ml tube containing 3.75 ml YEP medium (Figure 4C).
i. Warp the tube with foil and incubate for 2 h in the dark at 28°C.
j. Centrifuge the mixture at 1,500-3,000 × *g* for 5 min at RT.
k. Discard around 3.5 ml of the supernatant.
l. Mix the remaining cells gently.
m. Plate 20-100 µl of cells on LB agar amended with 50 mg/L hygromycin and 50 mg/L kanamycin.
n. Make sure that cells are completely dry, then wrap the Petri dish with parafilm and incubate 28°C for 2-3 days.
o. Pick four single colonies and verify the transformation by PCR (Figure 4D).
p. Streak out the positive colonies of *Agrobacterium* strain EHA101 that harbor the gene of interest in a 1 × 4 grid pattern on a single LB agar containing 50 mg/L hygromycin and 50 mg/L kanamycin (Figure 4E).
q. Incubate the culture for 2-3 days at 28°C.

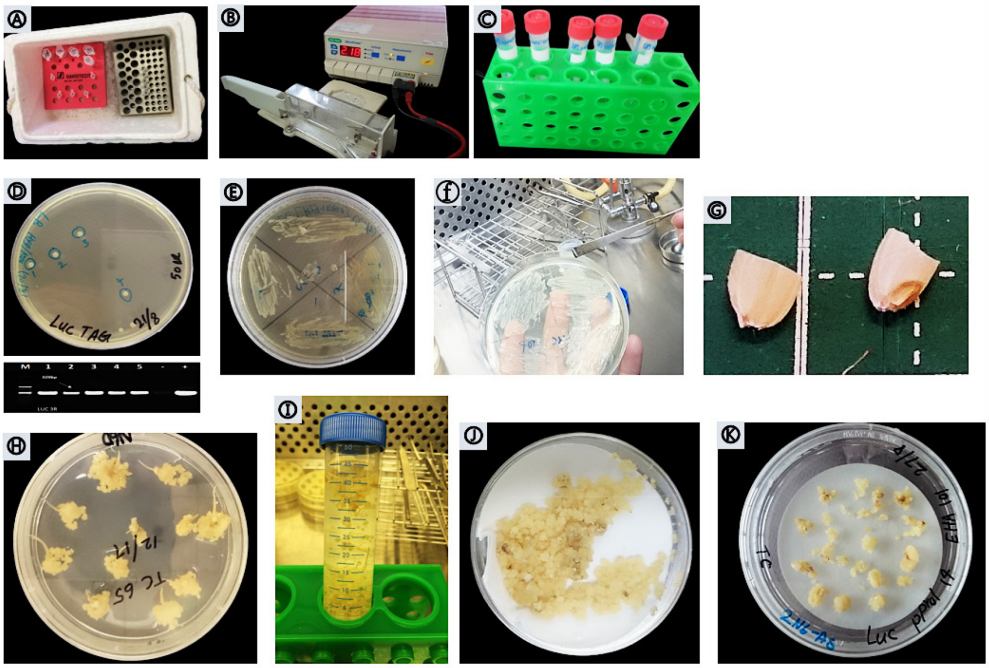

Figure 4. Procedure of *Agrobacterium*-mediated transformation. (A) *Agrobacterium* competent cells. (B) MicroPluser™ (Bio-Rad). (C) YEP medium. (D-F) Positive transformed *Agrobacterium* verified by PCR. (G) Mature embryos of Taichung 65. (H) Callus induction after 23 days on N6D medium. (I) Callus infection. (J) Drying calli on sterilized filter paper. (K) Co-cultivated calli on 2N6AS solid medium.

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B. Preparing mature embryos

1. Seed surface disinfection
   a. Dehusk healthy mature seeds, then hand-cut with a razor. Save the embryo seed halves and discard the endosperm seed halves (Figure 4G).
   b. Disinfect the surface of embryogenic seed parts after soaking and stirring once in 70% ethanol for 10 s in a 50 ml tube (up to 150 seeds).
   c. Rinse the embryo seeds halves in distilled water for 30 s.
   d. Shake the tube vigorously (45 rpm) for 15-20 min in bleach Solution A and then rinse 3-5 times using distilled water.
   e. Wash the seed parts with Solution B under vigorous shaking for 15-20 min, followed by five rinses in distilled water up to 30 s each time.
   f. Place the sterilized embryo seed halves on sterile filter paper over Petri dish (see Note 3).
2. Callus induction (3-4 weeks)
   a. Partially submerge 8-12 disinfected embryo seed halves per dish within N6D callus induction medium.
   b. Seal the dishes with surgical tape, then wrap every five dishes with aluminum foil and incubate at 28°C in the dark for 3-4 weeks (see Note 4).
3. Preparation of calli, *Agrobacterium* suspension, and infection (48 to 72 h)
   a. Mix a small portion of four colonies of *Agrobacterium* strain EH101 on a micro-spatula and resuspend in 40 ml 2N6D-AS liquid medium containing 16 µl acetosyringone (100 mg/ml stock solution) and mix gently (*Agrobacterium* colonies must be analyzed by PCR using specific primers to verify the integration of the construct with the bacterial gDNA) (Figure 4F).
   b. Discard brown calli and seed coleoptile from the N6D medium (at the beginning of week 4). Collect the active embryogenic calli (yellowish-white, relatively dry, 1-3 mm in diameter (Nishimura *et al*., 2006) and globular (Hiei and Komari, 2008) into a 50-ml tube. At this stage, the calli can be used directly for *Agrobacterium* infection (Figure 4H).
   c. Pour the bacterial suspension prepared in Step B3a into the 50-ml tube.
   d. Soak the calli in the *Agrobacterium* suspension and gently mix for 90 s (Figure 4I).
   e. Decant the bacterial suspension into a sterilized stainless-steel sieve (12 mesh) and hold in 500-ml sterile beaker.
   f. Blot the sieve on sterilized filter paper placed in a 90 mm × 20 mm Petri dish to remove excess *Agrobacterium* suspension (Figure 4J).
   g. Place a sterilized filter paper on solid 2N6D-AS medium. Saturate the filter paper by dripping 0.5 ml 2N6D-AS liquid medium and then co-cultivate an appropriate amount of calli (20-25 calli) on the filter paper (Figure 4K).
   h. Seal plates with parafilm tape and wrap with aluminum foil and incubate at 25°C in the dark for 3 days (see Note 5).
4. Wash calli and select transformed cells (3-4 weeks)

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a. Collect infected calli in a 50 ml sterile tube and wash with sterile water 3-5 times to remove *Agrobacterium* (until water is clear). Gently shake the tube and pour out the water.
b. Rinse calli with 40-ml sterile water containing 40 µl carbenicillin 500 mg/ml stock solution to kill *Agrobacterium* cells.
c. Pour the water suspension and blot the calli on sterile filter paper held over Petri dish. Dry the calli well (see Note 6).
d. Transfer calli onto N6D-HC selection medium; 15 to 20 calli can be placed on a single plate.
e. Seal the plate with surgical tape, and incubate the calli at 28°C in the dark [according to the Toki method; 33°C (light for 10 h)/30°C (dark for 14 h); Saika and Toki (2010)].
f. Check the culture regularly for contamination. In case of contamination, transfer uncontaminated calli to fresh medium immediately.
g. Subculture the yellowish-white calli to new fresh N2D-HC medium after 10 to 12 days, seal plates with surgical tape, and incubate at 28°C in the dark. Resistant calli (yellowish-white) can be observed after 20-25 days (Figure 5A).

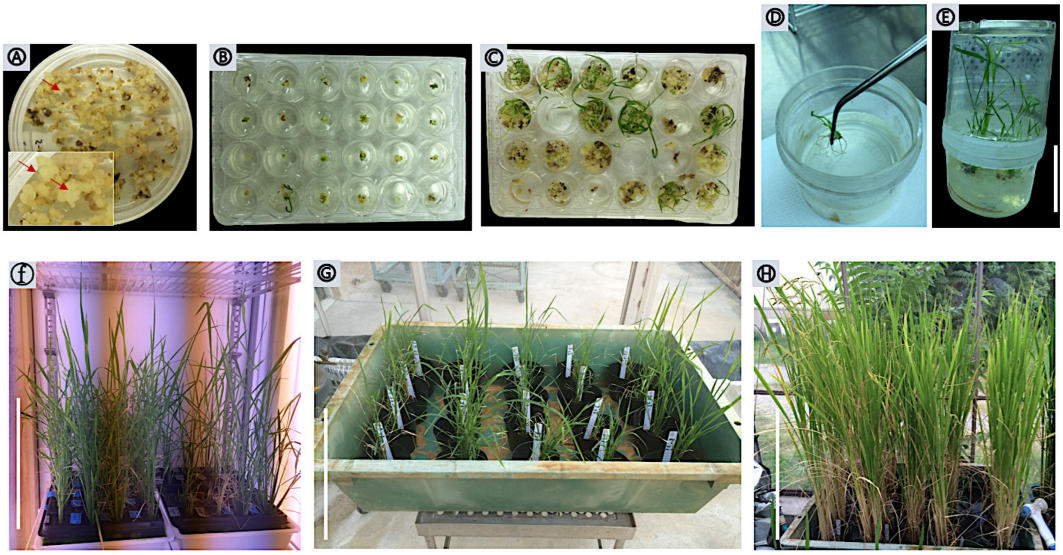

Figure 5. Calli selection and regeneration. (A) Hygromycin-resistant calli on N6D-HC selection medium. Arrows indicate resistant calli after 25 days from the infection. (B) Proliferated calli with greening spots. (C) Subculture green pots on the same fresh medium. (D) Rooting. (E) Regenerated plantlets on MS HF medium. (F) Transgenic plants transplanted to soil in the growth chamber. (G-E) Transgenic plants transferred to locking system greenhouse until harvesting. Scale bars: 5 cm in (E), 30 cm in (F), 30 cm in (G), 30 cm in (H).

5. Regeneration of rice transgenic plants (3-4 weeks)
   a. Transfer resistant calli to REIII regeneration medium containing suitable antibiotics. Using sterilized forceps, subculture a single callus to each well of 24-multi-well plate. Seal the plate with surgical tape and incubate at 30°C under continuous illumination (Figure 5B). Re-

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transfer transgenic calli to fresh REIII medium every 10 days with the same conditions. The transformed shoot begins to differentiate after 3-4 weeks (Figure 5C).

b. Transfer 3-4 cm shoots to plantlet pots containing HF medium with appropriate antibiotics at 28°C under 10 h days light (Figure 5D). Several days later, remove the surgical tape from the top of the pots and then remove the pot cover (Figure 5E) (see Note 7).
c. Transplant transformant rice plants to 6.5 cm pots containing rice seedling raising soil, with one plantlet per pot, in a growth chamber at 28°C (Figure 5F).
d. When the transformants are 15 cm tall, transplant them to 23-cm pots containing paddy field soil, with one plantlet per pot, in a greenhouse at 28°C until harvest (Figure 5G-5H).
e. Conduct PCR with DNA from the transformed rice plants to confirm T-DNA integration into rice plants (Figure 6).

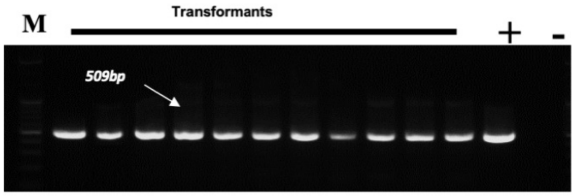

Figure 6. Agarose gel profile for verifying the integration of T-DNA insertion into the plant genome. A 509bp *LUC* gene amplicon size. M, marker; +, construct DNA; -, Taichung 65 gDNA.

Notes

1. Bacto agar dissolves well with autoclaving. Add the hygromycin after cooling to 50-45°C because hot culture media will degrade the antibiotic. For *A. tumefaciens* strain EHA105, add 0.2 ml of 50 mg/ml hygromycin per 200 ml LB medium. For strain EHA 101, add 0.2 ml of 50 mg/ml hygromycin and 0.2 ml 500 mg/ml carbenicillin per 200 ml LB medium.
2. Meropenem (25 mg/L) in the selection medium completely suppresses the overgrowth of *Agrobacterium*, which results in high transformation efficiency (Ogawa and Mii, 2007).
3. Seeds one year after harvesting – rather than seeds taken immediately after harvesting – are preferable for callus induction. Healthy mature seeds are critical as starting material for transformation and high-frequency callus formation. Remove lemma and palea (outer coat) from 40-50 seeds (enough for one transformation). Remove starchy endosperm from the seed to reduce contamination.
4. Callus selection is a key point for efficient transformation. We often check the culture and remove any contaminated seed parts immediately with a sterilized spoon. In such cases, uncontaminated seed parts are transferred to new plates.
5. Select four single *Agrobacterium* colonies. Perform PCR using marker-specific primers for the reporter gene to confirm its incorporation into the plant genome. Bacterial suspension density should be OD_{600} = 0.2 (Ozawa, 2012) or lower (Nishimura *et al.*, 2006), and OD_{600} = 0.05-0.1 is

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recommended. This process is important to prevent excess *Agrobacterium* growth, which can result in damage to calli. Approximately 150 calli can be placed on a single plate (Hiei and Komari, 2008). Seal plates with Parafilm to prevent evaporation.

6. Wash calli after co-cultivation. Resistant calli contain small nodular embryos on the surface. A critical step is to remove *Agrobacterium* from calli by washing without causing much physical damage to the nodular embryo cells, as this would prevent the regeneration of the transgene. Prior the co-cultivation, it is very important to ensure that the selectable-antibiotic is removed appropriately so that it does not affect calli growth. Place the calli on the filter paper to allow the extra water to evaporate.
7. Hardening: The hardening of transgenic plantlets is a crucial step prior to transplanting them into soil. The hardening can be done slowly from low to high light intensity conditions as well as from high to low humidity. The agar medium can be gently removed from the root by rinsing with water.

Recipes

A. Sterilizing solution
   1. Solution A
      Add 50 ml sodium hypochlorite 10% into 50 ml dH_2O
      Store at RT
   2. Solution B
      Add 50 ml Sodium hypochlorite, 50 ml dH_2O, and 50 µl Tween® 20
      Store at RT

B. Antibiotics
   1. Carbenicillin (500 mg/ml)
      Dissolve 5 g carbenicillin powder completely in 10 ml dH_2O.
      Sterilize the solution through 0.22 µm syringe filter.
      Store at -20°C in 1 ml aliquots.
   2. Hygromycin (50 mg/ml)
      Dissolve 500 mg hygromycin B in 10 ml dH_2O.
      Sterilize the solution through 0.22-µm syringe filter.
      Store at -20°C in 1.5 ml portions.
   3. Kanamycin (50 mg/ml)
      Dissolve 500 mg Kanamycin to 10 ml.
      Sterilize the solution through 0.22-µm syringe filter.
      Store at -20°C in 1.5 ml portions.
   4. Acetosyringone (100 mg/ml)
      Dissolve 1 g acetosyringon to 1 ml DMSO.

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Dilute with 10 ml dH_2O.

Sterilize the solution through 0.22-μm syringe filter.

Store at -20°C in 1.5-2 ml aliquots.

5. Acetosyringone (10 mg/ml)

   Dissolve 100 mg acetosyringon to 1 ml DMSO.

   Dilute with 10 ml dH_2O.

   Sterilize the solution through 0.22-μm syringe filter.

   Store at -20°C in 1.5-2 ml aliquots.

6. Meropenem (12.5 mg/ml)

   Dissolved 12.5 g meropenem in 10 ml dH_2O.

   Sterilize the solution through 0.22-μm syringe filter.

   Aliquot in 2 ml portions and store at -20°C.

7. 2,4-D (0.2 mg/ml)

   Dissolve 20 mg of 2,4-D powder in 0.5 ml DMSO.

   Adjust to 100 ml dH_2O.

   Store at -20°C.

8. NAA (0.2 mg/ml)

   Dissolve 20 mg of NAA completely in 1 ml 0.1 N sodium hydroxide solution.

   Adjust volume to 100 ml dH_2O.

   Store at -20°C.

9. Kinetin (1 mg/ml)

   Dissolve 10 mg kinetin in 0.2 ml 1 M sodium hydroxide.

   Adjust the volume to 10 ml with dH_2O.

   Store at -20°C.

C. Prepare cultivation medium (Tanle 1)

   Culture nutritional components: All culture media are prepared fresh in 1,000 ml dH_2O, autoclaved at 120°C for 20 min and cooled to 50-40°C; finally, it is asepticaly distributed to approximately 12 (90 mm × 20 mm) Petri dishes in a laminar flow hood. The poured medium plates containing antibiotics can be stored at 4°C for up to one month.

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Table 1. List of callus induction and regeneration medium components

Components	Medium					
	N6D	2N6-AS	2N6D-AS solution	N6D-HC	REIII	HF
Sucrose	30 g	30 g	30 g	30 g	30 g	30 g
Sorbitol	-	-	-		30 g	
Casamino acids	0.30 g	0.30 g	0.30 g	0.30 g	1g	-
Proline	2.878 g	-	-	2.878 g	-	-
Murashige and Skoog	-	-	-	-	4.4 g	4.4 g
CHU (N6)	3.981 g	3.981g	3.981g	3.981 g	-	-
CHU N6- Vitamin solution	1 ml	1 ml	1 ml	1 ml	-	-
2,4-D (0.2 mg/ml)	10 ml	10 ml	10 ml	10 ml		-
Acetosyringon (10 mg/ml)	-	1 ml	1 ml	-	-	-
NAA (x 5000)	-	-	-		10 µl	
Kinetin (x 500)	-	-	-		1 ml	
Gelrite (0.4%)	4 g	4 g	-	4 g	4 g	4 g
Carbenicillin 500 (mg/ml)	-	-	-		1 ml	-
Hygromycin 500 (mg/ml)	-	-	-		1 ml	1 ml
Meropenem 12.5 (mg/ml)	-	-	-	2 ml		

1. LB solid medium (200 ml)
   LB BROTH 5 g
   Bacto Agar 1.5 g
   Autoclave (120°C for 20 min)
2. LB liquid medium (200 ml)
   LB BROTH 5 g
   Autoclave (120°C for 20 min)
3. YEP medium (200 ml)
   Yeast Extract 2 g
   Bacto Peptone 2 g
   NaCl 1 g
   Autoclave (120°C for 20 min)

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Competing interests

The authors declare that they have no conflict of interests.

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