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Generation of Stable Transgenic Rice (*Oryza sativa L.*) by *Agrobacterium*-Mediated Transformation

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Rice (*Oryza sativa L.*) is the staple food for more than half of the world's population, and has also become an important model monocot. As a result, numerous genetic transformation protocols have been developed to improve and better understand this particular agronomic plant. Here we introduce a convenient transformation method using *Agrobacterium*. The explants used are embryogenic calli derived from mature seeds, which are easily obtained and can be used all year round. After selection and regeneration, transformants are obtained from resistant calli cultured on the regeneration medium. This protocol has been used to generate transgenic rice (*Oryza sativa L.)* in as little as 4 months. © 2016 by John Wiley & Sons, Inc.

Keywords: *Agrobacterium*-mediated transformation • calli • regeneration • rice

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INTRODUCTION

Methods for introducing DNA into plant genomes have been used by plant scientists to explore gene functions. For crops such as rice, transformation also provides an important tool for breed improvement. *Agrobacterium* is able to deliver DNA to plant cells, and *Agrobacterium*-mediated transformation has many advantages: the transformation efficiency is high, low numbers of copies of the transferred DNA (T-DNA) are integrated, the T-DNA segments are relatively large, with defined ends, the procedure is easy to perform and the cost is low. Therefore, *Agrobacterium*-mediated transformation has become one of the most widely used methods for plant genetic modification.

In the following protocol, we describe the procedure for stable transformation of rice calli by *Agrobacterium tumefaciens,* which gives rise to transgenic plants within 4 months.

NOTE: The plastic petri dishes are new and sterile, whereas the glass containers need to be sterilized before use. They are sealed with 16 × 16-cm plastic film and autoclaved for 20 min at 121°C.

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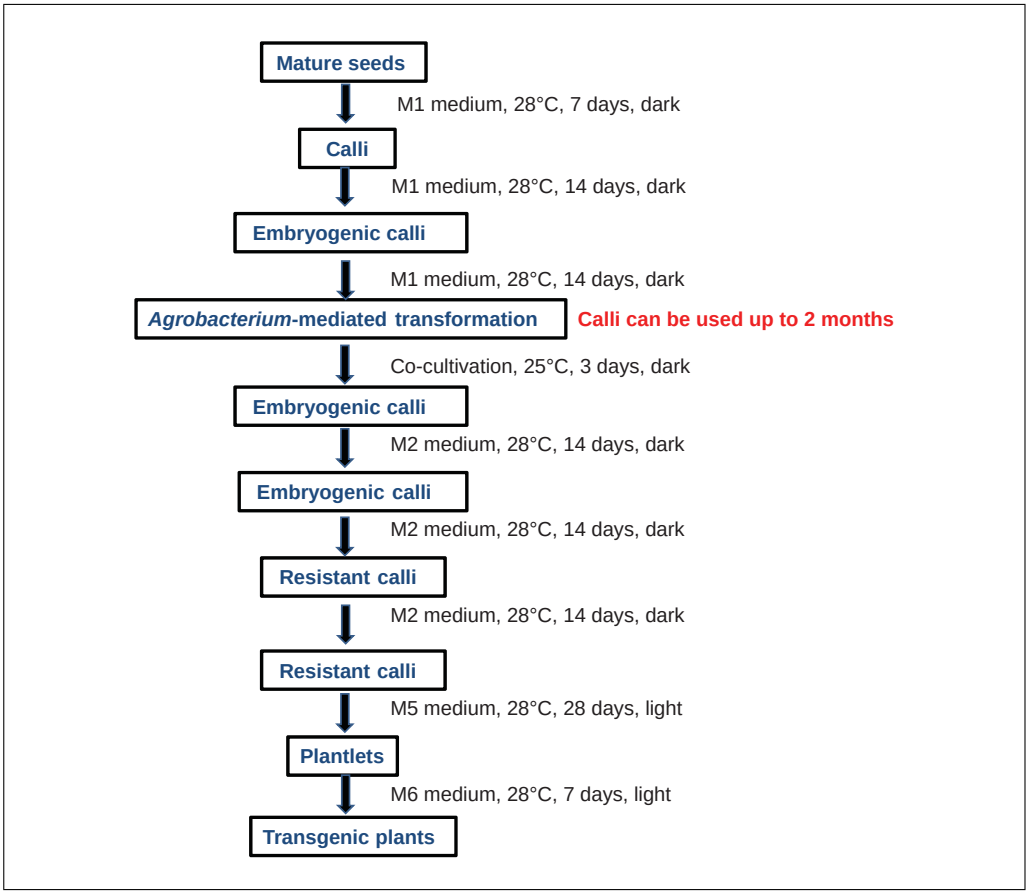

Figure 1 Flow chart illustrating the successive steps and times required for the production of transgenic rice by *Agrobacterium*-mediated calli transformation (d: day).

BASIC PROTOCOL

AGROBACTERIUM–MEDIATED TRANSFORMATION OF RICE CALLI

Although immature embryos are considered to be good explants for transformation, they cannot be obtained easily in the laboratory. Mature seeds can be preserved all year round, and the use of embryogenic calli induced from them makes transformation inexpensive and convenient. Thus, embryogenic calli derived from mature seeds are the material of choice for transformation in most laboratories.

The protocol has five stages: (i) calli induction and subculture; (ii) activation of *Agrobacterium*; (iii) *Agrobacterium*-mediated transformation of calli; (iv) selection of resistant calli, and (v) regeneration of transgenic plants. A flow chart illustrating the steps and times for producing transgenic rice is shown in Figure 1.

Materials

Rice seeds
2% (v/v) sodium hypochloride
Tween 20
M1 calli induction medium (see recipe)
Agrobacterium tumefaciens strain harboring constructs (see the Support Protocol)
LB medium (see recipe)
AAM medium (see recipe)
Sterile water containing 200 mg/liter carbenicillin or cefotaxime
M2 selection medium (see recipe)
M5 regeneration medium (see recipe)
M6 rooting medium (see recipe)

Shaker: setting 250 rpm/min; 28°C (used for *Agrobacterium* multiplication and activation)
50-ml tubes
Sterile filter papers (9-cm diameter)
9-cm diameter plastic petri dishes
Parafilm
Sterile glass containers (18-cm in length)
16 × 16-cm plastic film
Growth chamber: settings 28°C/24 hr dark (used for calli induction, subculture and selection)
Forceps
Stereoscopic microscope
100-ml conical flasks
Sterile glass petri dishes (6- and 9-cm in diameter)
Micropore medical sealing tape
Growth chamber: settings 25°C/24 hr dark (used for co-cultivation)
Growth chamber: settings 28°C/24 hr light (used for calli regeneration and plantlet growth)

Calli induction and subculture

1. Dehusk mature rice seeds manually without damaging the embryos. Wash the dehusked seeds for 30 min with vigorous shaking in 2% sodium hypochloride containing 1 to 2 drops of Tween 20 in a 50-ml tube. Then rinse the seeds with 30 to 40 ml sterilized water about 4 to 6 times.

   After washing the seeds with sodium hypochloride, all the following operations should be conducted in a laminar airflow cabinet to maintain an aseptic environment.

2. Drain the seeds on filter paper and place them in plastic petri dishes containing 30 ml M1 calli induction medium. Seal the petri dishes with Parafilm. Incubate the mature seeds at 28°C in the dark.

   Plastic petri dishes 9-cm in diameter containing medium are used for cultivating seeds and calli. Each petri dish can contain about 15 seeds or calli.

3. After seven days, calli have formed from the mature seeds (Fig. 2A). Separate the seeds and calli carefully with forceps, and put the calli into a plastic 9-cm petri dish containing 30 ml fresh M1 medium. Incubate them at 28°C in the dark.

   The calli are formed between the seeds and the rice buds. Discard the seeds and buds and only subculture the calli.

   After two weeks of culture, embryogenic calli appear on the surface of the non-embryogenic calli. The embryogenic calli tend to be yellow or light yellow and are dry, compact, and globular in appearance, and differ slightly from the non-embryogenic calli (Fig. 2B).

4. Select the embryogenic calli under a stereoscopic microscope and transfer them to a 9-cm plastic petri dish with 30 ml fresh M1 medium. In two weeks, the vigorously dividing calli will grow to 5-mm in diameter.

   The embryogenic calli are very small and are usually scattered about near the initial calli. If you do not obtain enough embryogenic calli in this step, you can preserve the initial calli for 5 to 7 days while waiting for more.

5. Transfer the calli to a 9-cm petri dish with 30 ml fresh M1 medium. After 3 days, they will be used for transformation (Fig. 2C).

   When calli are transferred to fresh medium, they need 3 to 4 days to adapt to the new environment and to be ready to be transformed.

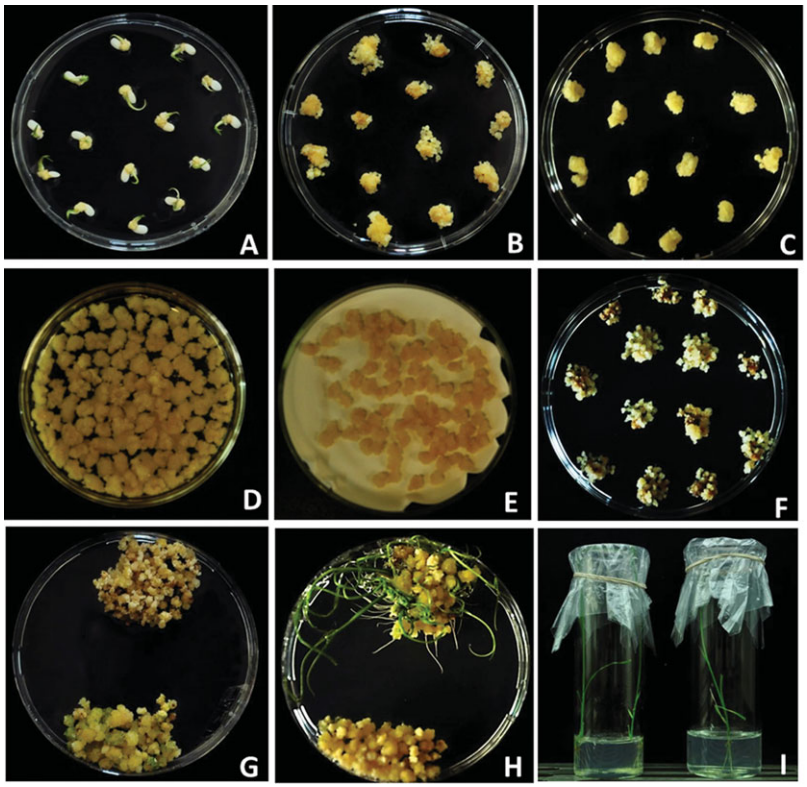

Figure 2 Generation of stable transgenic rice by *Agrobacterium*-mediated calli transformation. (**A**) Calli formed from mature seeds after 7 days. (**B**) Embryogenic calli derived from the non-embryogenic calli. (**C**) Embryogenic calli of appropriate size for transformation. (**D**) *Agrobacterium*-mediated calli transformation. (**E**) Co-cultivation in a sterile glass Petri dish. (**F**) Resistant callus clones growing out from susceptible necrotic calli after two rounds of selection. (**G**) Green tips appearing on the surface of resistant calli. (**H**) Resistant plantlets generated from resistant calli. (**I**) Plantlets that have been transferred to containers with rooting medium for further development.

Embryogenic calli can divide constantly, so large numbers can be obtained by repeated subculture. However, calli more than 2 months old are difficult to regenerate.

Activation of Agrobacterium

6. Inoculate an *Agrobacterium tumefaciens* strain, for example, AGL1, harboring a variety of standard binary plasmids with genes of interest, into a 50-ml tube with 5 ml LB medium containing appropriate antibiotics for selection.

   Usually the LB medium contains two kinds of antibiotic, e.g., Kana^{50}+Rif ^{25}; Rif ^{25} to select for Agrobacterium resistance and Kana^{50} to select for plasmid resistance.

   Agrobacterium strains harboring constructs are stored at −80°C (Support Protocol).

   The ratio of the volume of Agrobacterium tumefaciens suspension to the volume of liquid LB medium should be 1:100, which means adding 50 μl Agrobacterium to 5 ml LB medium. The antibiotics used depend on the resistances of the Agrobacterium tumefaciens strain and plasmid of interest.

7. Incubate the tube overnight on a shaker at 28°C and 250 rpm/min to A_{600} 1.0-2.0.

8. Centrifuge for 5 min at 13,400 × *g*, 25°C, and re-suspend the pellet in a final volume of AAM medium of about 25 ml (A_{600} ~ 0.1).

   Before use, make sure the acetosyringone has been added to the AAM medium at a final concentration of 100 μM.

9. Incubate the *Agrobacterium* in a 100-ml conical glass flask on a shaker at 28°C and 250 rpm/min for 4 hr to activate it for calli transformation.

Agrobacterium-mediated transformation of calli

10. Collect the calli from step 5 into a sterile glass dish. Add the *Agrobacterium* suspension from step 9 to the glass dish until all the calli are submerged (Fig. 2D). Stir for 30 min until the infection process is completed.

    Usually, we use about 100 calli in good condition for each transformation.

    Make sure all the calli are submerged in the Agrobacterium suspension. If some float, use a little force to submerge them.

    The sterile glass petri dish used in this step is of diameter 6 cm, as this step needs a smaller volume of Agrobacterium suspension.

11. Remove excess bacterial suspension after incubation and drain the infected calli onto filter paper. Transfer them with forceps to two pieces of filter paper in a sterile glass petri dish and seal the dish with Micropore medical sealing tape.

    Do not leave the calli too wet, because the presence of too many Agrobacterium during the co-cultivation stage damages the calli.

    The sterile glass petri dish used in this step is 9 cm in diameter, which gives each callus enough space and helps in desiccation.

    Micropore medical sealing tape is used during co-cultivation because of its permeability to air and water vapor.

12. Incubate at 25°C in the dark for 3 days (Fig. 2E).

    We use two pieces of filter paper instead of co-cultivation medium in this step. This is because the co-cultivation medium increases the multiplication of the Agrobacterium, which can kill the calli.

Selection of resistant calli

13. After co-cultivation with *Agrobacterium*, rinse the infected calli 2 to 3 times in sterile water containing 200 mg/liter carbenicillin or cefotaxime until the water is clear after washing.

    This step is intended to remove the Agrobacterium that are on the calli. The number of washes depends on the amount of Agrobacterium on the infected calli.

14. Drain the infected calli on filter paper, allow for sufficient air-drying and then transfer the tissue to a 9-cm plastic petri dish containing 30 ml M2 selection medium. Incubate the infected calli at 28°C in the dark for two weeks.

    Make sure the selective agents and antibiotics have been added to the selection medium. The antibiotics are used to kill the bacteria, and the selective agents are used to kill calli not harboring the plasmid of interest. Two selective agents can be used for rice transformation: Hygromycin B and G418. The M2 selection medium should contain 200 mg/liter carbenicillin or cefotaxime, and 50 mg/liter Hygromycin B or 150 mg/liter G418.

15. After two weeks' selection, many of the infected calli will turn black. Transfer all the calli to fresh M2 selection medium, and incubate at 28°C in the dark for another two weeks.

    Do not discard the necrotic calli after the first round of selection—they might eventually produce resistant calli.

    During the second selection, healthy, yellowish, compact and friable resistant callus clones grow out from the necrotic susceptible calli (Fig. 2F). Select the resistant callus

clones and transfer them to a new plastic petri dish containing M2 selection medium and incubate at 28°C in the dark.

In this step, you can discard the necrotic calli. All the resistant callus clones from a given infected callus should be combined, as they may derive from the same transformation event.

Regeneration of transgenic plants

16. Ten to fourteen days later, the resistant calli have enlarged and can be transferred to M5 regeneration medium. Place the resistant calli in 9-cm petri dishes with 30 ml M5 regeneration medium and incubate at 28°C in the light.

    Before use, make sure the selection agents and antibiotics have been added to the regeneration medium. The concentrations of selective agents in regeneration medium are usually lower than in selection medium, for example 30 mg/liter Hygromycin B or 100 mg/liter G418. That is because regeneration is the limiting step in the transformation procedure, and low concentrations of selective agents may promote calli regeneration.

17. About 3 weeks later, many green tips should have formed on the surface of calli (Fig. 2G), and these will give rise to resistant green plantlets (Fig. 2H).

    When the green tips appear, do not transfer them to fresh medium until plantlets are formed. If green tips are transferred to new M5 medium, they will have difficulty regenerating.

18. Transfer the resistant green plantlets to18-cm length glass containers with about 60 ml M6 rooting medium for further development at 28°C in the light (Fig. 2I).

    Make sure the selective agents and antibiotics have been added to the rooting medium. The concentration of the selective agents in rooting medium is higher than in regeneration medium in order to kill nontransgenic plants: 50 mg/liter for Hygromycin B and 150 mg/liter for G418.

19. When the transgenic plants have grown up, open the lids of the containers and add some water for acclimatization. One week later, transplant the transgenic plants to soil.

SUPPORT PROTOCOL

PREPARATION OF *AGROBACTERIUM* HARBORING CONSTRUCTS

In this protocol, we describe the preparation of *Agrobacterium* harboring a standard binary plasmid with a gene of interest.

Materials

Competent *Agrobacterium tumefaciens* cells (TransGen Biotech)
The construct: a binary plasmid with the gene of interest
Ice
Liquid nitrogen
LB liquid and solid media
Appropriate selective antibiotic
40% glycerin

1.5-ml microcentrifuge tubes
Shaking incubator
Centrifuge

1. Mix 1 μg plasmid DNA and 200 μl competent *Agrobacterium* cells gently in a 1.5-ml microcentrifuge tube, and put the tube on ice for 30 min.

2. Freeze the mixture in liquid nitrogen for 1 min.

3. Melt the tube for 3 min at 37°C.
4. Quickly put the tube on ice for 2 min.
5. Add 800 μl liquid LB medium to the tube and incubate it on a shaker at 28°C for about 4 hr.
6. Centrifuge the *Agrobacterium* for 1 min at 10,000 × *g*, 25°C; discard the supernatant.
7. Resuspend the pellet with 20 μl LB medium in the 1.5-ml microcentrifuge tube and plate the *Agrobacterium* on solid LB medium with appropriate selective antibiotics in a 9-cm diameter plastic petri dish.

   The LB medium contains two antibiotics, e.g., Kana^{50}+Rif^{25}. Rif^{25} selects for Agrobacterium resistance and Kana^{50} for plasmids resistance.

8. Two days later, positive clones should appear; transfer them to 50-ml tubes containing 5 ml liquid LB medium with appropriate selective antibiotics.
9. Grow these clones to A_{600} 1.0-2.0.
10. Transfer 500 μl of each *Agrobacterium* suspension to a sterile 1.5-ml microcentrifuge tube and add 500 μl 40% glycerin. Mix gently and store up to 12 months at −80°C.

REAGENTS AND SOLUTIONS

Use Milli-Q purified water in all recipes and protocol steps.

2, 4-D stock solution

1 g of 2, 4-dichlorophenoxyacetic acid (1 mg/ml final)
Add some 1 N KOH (see recipe) to dissolve
Add sterile distilled H_2O to 1000 ml
Filter sterilize using a 0.22-μm filter
Store up to 3 months at 4°C

AA major salts stock solution (10×) (Toriyama and Hinata, 1985)

29.5 g KCl
2.5 g $MgSO_4 \cdot 7H_2O$
1.7 g NaH_2PO_4
1.5 g $CaCl_2 \cdot 2H_2O$
Add sterile distilled H_2O to 1000 ml
Filter sterilize using a 0.22-μm filter
Store up to 3 months at 4°C

AA minor salts stock solution (1000×) (Toriyama and Hinata, 1985)

3 g H_3BO_3
10 g $MnSO_4 \cdot H_2O$
2 g $ZnSO_4 \cdot 7H_2O$
0.75 g KI
0.25 g Na_2MoO_4
0.025 g $CuSO_4 \cdot 5H_2O$
0.025 g $CoCl_2 \cdot 6H_2O$
Add sterile distilled H_2O to 1000 ml
Filter sterilize using a 0.22-μm filter
Store up to 3 months at 4°C

AA vitamins stock solution (10×) (Toriyama and Hinata, 1985)

8.76 g L-Glutamine
2.66 g L-Aspartic acid
1.74 g L-Arginine
0.075 g L-Glycine
Add sterile distilled H_2O to 1000 ml
Filter sterilize using a 0.22-μm filter
Store up to 3 months at 4°C

AS^{200} stock solution

1 g acetosyringone (200 mM final)
Add 25.45 ml absolute ethyl alcohol
Filter sterilize using a 0.22-μm filter
Store up to 6 months at −20°C

B5 vitamins stock solution (1000×) (Gamborg et al., 1968)

5 g Thiamine
0.5 g Pyridoxine
0.5 g Nicotinic acid
Add sterile distilled H_2O to 500 ml
Filter sterilize using a 0.22-μm filter
Store up to 3 months at 4°C

$Carb^{200}$ stock solution

10 g carbenicillin (200 mg/ml final)
Add sterile distilled H_2O to 50 ml
Filter sterilize using a 0.22-μm filter
Store up to 6 months at −20°C

$Cefo^{200}$ stock solution

10 g cefotaxime (200 mg/ml final)
Add sterile distilled H_2O to 50 ml
Filter sterilize using a 0.22-μm filter
Store up to 6 months at −20°C

G418 stock solution

7.5 g G418 (150 mg/ml final)
Add sterile distilled H_2O to 50 ml
Filter sterilize using a 0.22-μm filter
Store up to 6 months at −20°C

HCl, 1 N

3.6 ml of 98% concentrated HCl
Add sterile distilled H_2O to 100 ml
Store up to 6 months at room temperature

Kinetin stock solution

100 mg kinetin (1 mg/ml final)
Add some 1 N KOH (see recipe) to dissolve
Add sterile distilled H_2O to 100 ml
Filter sterilize using a 0.22-μm filter
Store up to 6 months at −20°C

KOH, 1 N

5.6 g KOH
Add sterile distilled H_2O to 100 ml
Store up to 6 months at room temperature

LB medium

10 g/liter Casein Tryptone
10 g/liter NaCl
5 g/liter Yeast Extract
Adjust pH to 5.8 with 1 N NaOH (see recipe)
For solid medium, add 1.5% (w/v) agar
Autoclave for 20 min at 121°C
After sterilization, add antibiotics such as kanamycin and rifampicin from sterile stock solutions to final concentrations of 50 mg/liter and 25 mg/liter, respectively.

Liquid AAM medium (Hiei and Komari, 2008)

AA major salts (see recipe)
AA minor salts (see recipe)
AA vitamins (see recipe)
MS vitamins (see recipe)
Fe-EDTA
0.5 g/liter casein hydrolysate
0.1 g/liter inositol
68.5 g/liter sucrose
36 g/liter glucose
Add sterile distilled H_2O to 1000 ml
Adjust pH to 5.2 with 1 N NaOH (see recipe)
Autoclave for 20 min at 121°C
Add 100 μM acetosyringone
Store up to 3 months at 4°C

MS vitamins stock solution (1000×) (Murashige and Skoog, 1962)

0.1 g Thiamine
0.5 g Pyridoxine
0.5 g Nicotinic acid
2 g Glycine
Add sterile distilled H_2O to 1000 ml
Filter sterilize using a 0.22-μm filter
Store up to 3 months at 4°C

NAA stock solution

50 mg naphthalene acetic acid (0.5 mg/ml final)
Add some absolute ethyl alcohol to dissolve
Add sterile distilled H_2O to 100 ml
Filter sterilize using a 0.22-μm filter
Store up to 6 months at −20°C

NaOH, 1 N

4 g NaOH
Add sterile distilled H2O to 100 ml
Store up to 6 months at room temperature

Solid M1 medium

4.1 g/liter NB basal medium (Gamborg et al., 1968; Chu, 1978)
0.5 g/liter casein hydrolysate
0.1 g/liter inositol
2.8 g/liter proline
0.5 g/liter L–glutamine
30 g/liter sucrose
2 mg/liter 2,4-D (see recipe)
3.75 g/L phytagel
Adjust pH to 5.8 with 1 N NaOH (see recipe)
Autoclave for 20 min at 121°C

Solid M2 medium

4.1 g/liter NB basal medium (Gamborg et al., 1968; Chu, 1978)
0.5 g/liter casein hydrolysate
0.1 g/liter inositol
2.8 g/liter proline
0.5 g/liter L-glutamine
30 g/liter sucrose
2 mg/liter 2,4-D (see recipe)
3.75 g/liter phytagel
Adjust pH to 5.8 with 1 N NaOH
Autoclave for 20 min at 121°C
After sterilization, add antibiotics such as ampicillin or cefotaxime from sterile stock solutions to final concentrations of 200 mg/liter and selective agents, for example Hygromycin B, from sterile stock solutions to final concentrations of 50 mg/liter.

Solid M5 medium

4.3 g/liter Murashige & Skoog medium (Basal Salt Mixture)
B5 vitamins (see recipe)
Fe-EDTA
0.5 g/liter casein hydrolysate
0.1 g/liter inositol
30 g/liter sucrose
20 g/liter mannitol
2 mg/liter N6-Benzyladenine
0.2 mg/liter Kinetin
4.6 g/liter phytagel
Adjust pH to 5.8 with 1 N NaOH
Autoclave for 20 min at 121°C
0.2 mg/liter NAA (see recipe)
After sterilization, add antibiotics such as ampicillin or cefotaxime from sterile stock solutions to final concentrations of 200 mg/liter and selective agents, for example Hygromycin B, from sterile stock solutions to final concentrations of 30 mg/liter.

Solid M6 medium

2.2 g/liter Murashige & Skoog medium (including vitamins)
0.1 g/liter inositol
30 g/liter sucrose
2.4 g/liter phytagel
Adjust pH to 5.8 with 1 N NaOH

continued

Autoclave for 20 min at 121°C

After sterilization, add antibiotics such as carbenicillin or cefotaxime to final concentrations of 200 mg/liter, and selective agents, for example Hygromycin B, to final concentrations of 50 mg/liter.

COMMENTARY

Background Information

Rice (*Oryza sativa L.)* is a staple cereal that is important in the diet of more than 80% of the world's people. Many breeding programs have been conducted to increase the environmental stress tolerance and pest and disease resistance of the plant. Genetic engineering has also been a prominent tool for improving rice. Since the first report of tobacco transformation in 1983, a number of gene transfer methods have been widely used in plants. In 1989, the first fertile transgenic rice was regenerated from protoplasts transformed by electroporation (Shimamoto et al., 1989) and, following that, transgenic rice was produced by PEG-mediated protoplast transformation (Datta et al., 1990). However, the transformation efficiencies in these systems are low and other laboratories have found it difficult to regenerate the protoplasts. In 1991, workers obtained transgenic rice from immature embryos by particle bombardment (Christou et al., 1991) and in 1994, Hiei and co-workers established an *Agrobacterium*-mediated transformation system *(*Hiei et al., 1994*)*. *Agrobacterium*-mediated transformation has several advantages over particle bombardment, including simplicity, high transformation efficiency, low copy number of integrated T-DNA , transfer of relatively large segments of DNA with defined ends, and low cost. Therefore, *Agrobacterium*-mediated transformation has become the preferred method of transformation in rice in most laboratories.

Critical Parameters

The A_{600} of *Agrobacterium* used for transformation is about 0.1. If the concentration is too low, the transformation efficiency is low, while if it is too high the *Agrobacterium* renders the calli susceptible to necrosis.

The size of the embryogenic calli appears to influence transformation rates; their diameters should be 4 to 6 mm. Calli of diameter >6 mm are more easily contaminated by *Agrobacterium* while calli <4 mm cannot survive after selection.

Theoretically speaking, embryogenic calli can divide continuously, so large number of calli can be obtained by subculture. However, it has been reported that after several successive subcultures embryogenic calli regenerate with low efficiency, and two rounds of subculture give the highest efficiency of calli regeneration (Gao et al., 2004).

Troubleshooting

If calli are always contaminated in the first round of selection, the *Agrobacterium* may be contaminated.

If the calli remain contaminated during selection, use two antibiotics at a total concentration of 200 mg/liter, such as 100 mg/liter carbenicillin and 100 mg/liter cefotaxime.

If no green tips appear after 3 weeks of regeneration, the most likely reason is that the calli are too wet. Drain the water off the calli and keep the container dry.

If plantlets do not grow from the green tips, there may not be enough nutrients in the medium because of prolonged cultivation. So use a bigger container for regeneration, or put only two to three resistant clones in one petri dish.

Anticipated Results

Using the Basic Protocol for *Agrobacterium*-mediated transformation of rice calli, an average of 80 to 85 resistant calli can be obtained from 100 embryogenic calli. After regeneration, about 50 to 70 resistant calli will generate plantlets. By adding 50 mg/liter Hygromycin B or 150 mg/liter G418 to the rooting medium, >90% of the healthy plants generated are transgenic.

Time Considerations

Agrobacterium-mediated transformation of rice calli should be accomplished within 4 months from when the mature seeds were formed. If the embryogenic calli are prepared in advance, transformation can be finished in 3 months.

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