Agrobacterium-mediated transformation of Japonica cv. Nipponbare callus tissue and recovery of transgenic rice plants

Materials

⌘ Plasmid

Agrobacterium tumefaciens strain EHA101 (Hood et al., 1986) containing the standard binary vector pTF101.1. The base vector pTF101.1 is a derivative of the pPZP binary vector (Hajdukiewicz et al., 1994) that includes the right and left T-DNA border fragments from a nopaline strain of *A. tumefaciens*, a broad host origin of replication (pVS1) and a spectinomycin-resistant marker gene (*aadA*) for bacterial selection. The plant selectable marker gene cassette consists of (1) double 35S promoter (2x P35S) of cauliflower mosaic virus (CaMV) (Odell et al. 1985), (2) tobacco etch virus translational enhancer (Carrington and Freed 1990), (3) the phosphinothricin acetyl transferase (*bar)* gene from *Streptomyces hygroscopicus* that confers resistant to herbicide phosphinothricin and its derivatives. pTF101.1 contains a multiple cloning site (MCS) for facilitating subcloning of a gene of interest in between the right border region and the plant selectable marker cassette. The soybean vegetative storage protein terminator (Mason et al., 1993) was cloned to the 3' end of the *bar* gene.

⌘ Plant material

Mature Japonica cv. Nipponbare rice seeds are dehusked, surface sterilized and placed onto induction medium containing 2,4-D. The callus tissue derived from the mature embryo is then used as the starting material for transformation.

Media

Callus induction, co-cultivation, selection and regeneration I media are after Hiei et al. (1994). Selection medium incorporates modifications from Toki (1997) but uses 2 mg L^{-1} bialaphos instead of 5 mg L^{-1} for selection and 500 mg L^{-1} carbenicillin for counter-selection against *Agrobacterium*. Regeneration II medium follows from Armstrong and Green (1985).

All media is prepared in 100 x 15 ml petri plates except for the regeneration media which uses 100x25 ml plates.

⌘ **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. After autoclaving, the appropriate antibiotics are added to the medium when it has cooled to 50°C.

⌘ **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. Acetosyringone (AS, 100 µM) is added just prior to use. [100µM stocks of filter-sterilized AS (dissolved in DMSO to 200mM then diluted 1:1 with water) are stored at –20ºC for use as needed]. This infection medium is published for use in *Agrobacterium*-mediated transformation of maize by Zhao et al., 2001, however, we routinely use this same medium for rice transformation.

⌘ **Callus Induction Medium:**

N6 salts and vitamins, 300 mg L^{-1} casamino acids, 2.8 g L^{-1} L-proline, 30 g L^{-1} sucrose, and 4 g L^{-1} gelrite (pH 5.8). Filter sterilized N6 Vitamins and 2 mg L^{-1} 2,4-D, are added to this medium after autoclaving.

⌘ **Co-cultivation Medium (make fresh):**

N6 salts and vitamins, 300 mg L^{-1} casamino acids, 30 g L^{-1} sucrose, 10 g L^{-1} glucose , and 4 g L^{-1} gelrite (pH 5.8). Filter sterilized N6 vitamins, acetosyringone (AS) 100 µM and 2 mg L^{-1} 2,4-D are added to this medium after autoclaving.

⌘ **Selection Medium:**

N6 salts and vitamins, 300 mg L^{-1} casamino acids, 2.8 g L^{-1} L-proline, 30 g L^{-1} sucrose, and 4 g L^{-1} gelrite (pH 5.8). Filter sterilized N6 vitamins, 2 mg L^{-1} 2,4-D, 2 mg L^{-1} Bialaphos (Shinyo Sangyo, Japan) and 500mg L^{-1} carbenicillin are added to this medium after autoclaving.

⌘ **Regeneration Medium I:**

MS salts and vitamins (Murashige and Skoog, 1962), 2 g L^{-1} casamino acids, 30 g L^{-1} sucrose, 30 g L^{-1} sorbitol, and 4 g L^{-1} gelrite (pH 5.8). Filter sterilized MS vitamins, 100 mg L^{-1} cefotaxime, 100 mg L^{-1} vancomycin, 0.02 mg L^{-1} NAA (naphthaleneacetic acid), 2 mg L^{-1} kinetin (Toki 1997) and 2 mg L^{-1} Bialaphos are added to this medium after autoclaving.

⌘ **Regeneration Medium II:**

MS Salts and 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, pTF101.1 in EHA101, is maintained on YEP medium (An et al., 1988) containing 100 mg L^{-1} spectinomycin (for pTF101.1) and 50 mg L^{-1} kanamycin (for

EHA101). Bacteria cultures for weekly experiments are initiated on fresh YEP plates from stock plates that are stored up to one month at 4ºC before being refreshed from long-term, –80ºC glycerol stocks.

⌘ Callus Induction

1. Dehusk the rice seeds. Pre-rinse 15-20 seeds in 10 ml of 70% Ethanol (50 ml conical tube) by vigorously shaking the tube for one minute. Rinse once with sterile water.

2. Add 10 ml of 50% commercial bleach (5.25 % hypochlorite) and place on a shaker for 30 minutes (low setting). Pour off the bleach solution and rinse the seeds five times with ~10ml of sterilized water each time. With a small portion of the final rinse, pour the seeds onto sterilized filter paper (in a sterile petri plate) and allow them to dry.

3. Using sterile forceps, transfer five seeds to the surface of each petri plate containing callus induction medium. Wrap the plates with vent tape and incubate in the light (16:8 photoperiod) at 29°C. Observe the seeds every few days and discard those showing signs of contamination.

4. After two-three weeks, developing callus is visible on the scutellum of the mature seed. Sub-culture callus to fresh induction medium and allow it to proliferate.

5. Four days prior to infection, cut the callus tissue into 2-4 mm pieces and transfer it to fresh induction medium.

⌘ *Agrobacterium* Infection

1. *Agrobacterium* cultures are grown for three days at 19°C (or 2 days at 28°C) on YEP medium amended with 100 mg L^{-1} spectinomycin and 50 mg L^{-1} kanamycin.

2. A small amount of bacteria culture is scraped from the plate and suspended in ~15 ml of liquid infection medium supplemented with 100 µM AS in a 50 ml conical tube (no pre-induction). Adjust the optical density to <0.1 (OD_{550} =0.06-0.08) before use.

3. For infection, rice calli are first placed into bacteria-free infection medium + AS (50 ml conical). This pre-wash is removed and replaced with 10 ml of the prepared *Agrobacterium* suspension (OD_{550} <0.1). Then, the conical is fastened onto a vortex shaker (low setting) for two minutes.

⌘ Co-cultivation

1. After infection, calli are poured out of the conical onto a stack of sterile filter paper in a 100 x 15 petri dish to blot dry. Then, they are transferred off the filter paper and onto the surface of co-cultivation medium with sterile forceps.

2. Co-cultivation plates are wrapped with vent tape and incubated in the dark at 25ºC for three days.

⌘ Wash

1. After three days of co-cultivation, the calli are washed five times with 5 ml of the liquid infection medium (no AS) supplemented with carbenicillin (500mg L^{-1}) and vancomycin (100mg L^{-1}). Calli are blotted dry on sterile filter paper as before. Individual callus pieces are transferred off the paper and onto selection medium containing 2 mg L^{-1} bialaphos.

2. Selection plates are wrapped with parafilm and placed in the light at 29°C.

⌘ Selection for stable transformation events

1. Every two weeks, the tissue is sub-cultured onto fresh selection medium. This should be done with the aid of a microscope to look for any evidence of *Agrobacterium* overgrowth. If overgrowth is noted, the affected calli should be avoided (do not transfer contaminated calli). The remaining tissue (unaffected by overgrowth) can then be carefully transferred using a newly sterilized forceps for each calli.

2. Putative clones begin to appear after six to eight weeks on selection. A clone is recognized as white, actively growing callus and is distinguishable from the brown, unhealthy non-transformed tissue. Individual transgenic events are identified and the white, actively growing tissue is transferred to their own plate in order to produce enough tissue to take to regeneration.

⌘ Regeneration of transgenic plants

1. Regeneration of R_0 transgenic plants is accomplished by selecting new lobes of growth from the callus tissue and transferring them onto Regeneration Medium I (light, 25°C). After two to three weeks, the maturing tissue is transferred to Regeneration Medium II medium for germination (light, 25°C). Stable transformation efficiency (%) is calculated as the number of bialaphos resistant callus events recovered per number of calli pieces infected.

⌘ Plant Acclimatization

1. Acclimatization of regenerated plants to soil is performed as described in the "Growth Chamber" section of the protocol menu.

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