Plastid transformation of liverwort *Marchantia polymorpha* L. suspension-cultured cell and immature thalli

*6 to 10 transformants by a shot of particle bombardment were obtained for suspension-cultured cell. 10 to 20 transformants by 10 shots of particle bombardment can be expected for immature thalli with this protocol.

Materials

(Plant)

Maintain liverwort suspension-cultured cells in 70 ml of 1M51C medium in a 300 ml flask on rotary shaker (130 rpm) under continuous white light (50-60 μmol photons m^{-2} s^{-1}) at 22°C. Use cells from 7- to 10-day-old cultures for plastid transformation. For immature thalli, use mature sporangia (the authors use F1 sporangia of Takaragaike-1 × Takaragaike-2).

(Transformation vector)

pCS31 vector

- Approximately 1 kb each of the *trnI* and *trnA* sequences were cloned into pBluescript II as homologous sequences*[1]. The *aadA* expression cassette was integrated between the *trnI* and *trnA* sequences. The *aadA* expression cassette consists of the promoter of the ribosomal RNA operon from the tobacco plastid genome, the *rbcL* ribosome binding site, the *aadA* gene, and the tobacco *psbA* 3'-UTR (Fig. 1).
- Plasmid DNA was prepared by QIAGEN Plasmid Midi Kit, and adjusted to 1 μg/μl.
- For transformation of immature thalli, pCS31 was linearized by *Eco*RI and *Sca*I prior to bombardment.

*1: DNA fragments of approx. 1 kb is sufficient for homologous recombination.

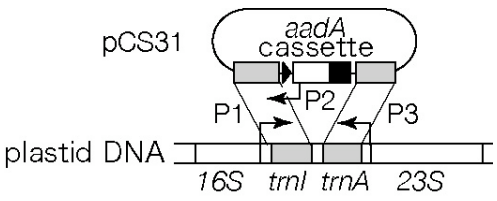

Fig. 1 Schematic illustration of pCS31. Gray boxes represent homologous sequences. Arrows indicate PCR primers for selection of plastid transformants and checking of homoplastomic state.

(Equipment)

- Filter (Nalgene: 134 mm x 230 mm, #300-4050) *[2]
- Filter disks (Whatman: No. 1ϕ55 mm) *[2]
- Cellophane *[1] (washed with boiled water before autoclaving)

- Aspirator
- Particle delivery system (Bio-Rad: PDS-1000/He Particle delivery system)
- 0.6 μm gold particle (Bio-Rad: #165-2262)
- 900-psi Rupture Disk (Bio-Rad: #165-2328)*3
- Macrocarrier (Bio-Rad, #165-2335)*3
- Stopping Screen (Bio-Rad, #165-2336)*3

*2: Autoclaved

*3: Sterilized with 70% ethanol and air dried in clean hood

(Reagents)

- 2.5 M $CaCl_2$
- 0.1 M Spermidine
- Spectinomycin dihydrochloride hexahydrate (SIGMA, #S9007-5G). Stock 50 mg/mL
- DNA extraction buffer

50 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 0.3 M NaCl, 0.5% SDS, 5 M urea, 5% (v/v) phenol (pH 7.0)

- 0M51C/1M51C media (1 L)

10× 0M51C stock solution 100 ml

sucrose 20 g, L-glutamate 0.3 g, Casamino acid 1.0 g, (+ 2,4-dichlorophenoxyacetic acid 1 mg/L for 1M51C media). Adjust to pH 5.5 with 1N KOH. Add 1.2% agar for plate.

- 10 × 0M51C stock solution (4 L)

KNO_3 80 g, NH_4NO_3 16 g, $MgSO_4 \cdot 7H_2O$ 14.8 g, $CaCl_2 \cdot H_2O$ 12 g, KH_2PO_4 11 g, EDTA-NaFe (III) 1.6 g, B5 micro components 40 ml, B5 vitamin 40 ml, 0.75% KI 4 ml

- B5 micro components (100 ml)

$NaMoO_4 \cdot 2H_2O$ 25 mg, $CuSO_4 \cdot 5H_2O$ 2.5 mg, $CoCl_2 \cdot 6H_2O$ 2.5 mg, $ZnSO_4 \cdot 7H_2O$ 200 mg, $MnSO_4 \cdot 7H_2O$ 1 g, H_3BO 300 mg

- B5 vitamin (100 ml)

Inositol 10 g, nicotinic acid 100 mg, pyridoxine hydrochloride 100 mg

thiamine hydrochloride 1 g

Methods

(Preparation of plant material)

For suspension-cultured cell

1: Layer suspension-culture cells onto the center of a filter disk using vacuum filtration (1-2 mm thickness).

2: Place filter disk with the cells onto a 1M51C plate, and incubate over night (22°C, 50-60 μmol photons m^{-2} s^{-1}) (Fig. 2A)

For immature thalli:

1: Suspend sporangia in sterile water (100 μl/sporangium)

2: Drop the spore suspension on the center of 0M51C plate covered with cellophane, and culture spores for a week (22°C, 50-60 μmol photons m^{-2} s^{-1}) (Fig. 2C).

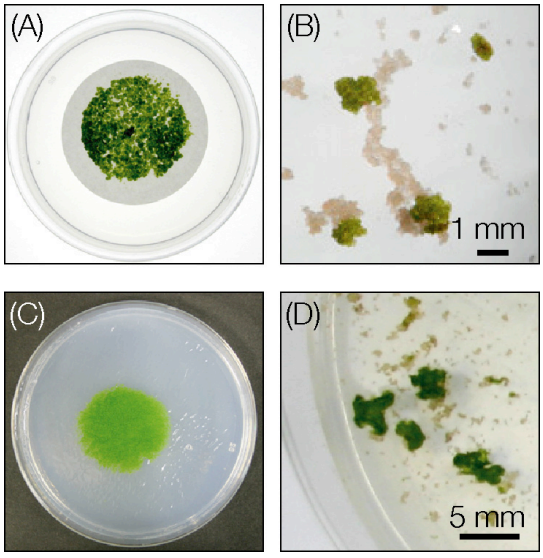

Fig. 2 Preparation of plant materials for particle bombardment and selection for spectinomycin resistant lines. (A) Suspension-cultured cells layered on a filter disk. (B) Spectinomycin-resistant calli on selective medium. (C) Immature thalli grown for 1 week. (D) Spectinomycin-resistant thalli selected on selective medium.

(Particle bombardment)

1: Successively add 250 μl of 2.5 M $CaCl_2$, 25 μL of 1 μg/μl DNA solution, and 50 μl of 0.1 M spermidine into 230 μl of 10 mg/ml 0.6-μm diameter gold particle in 1.5 ml plastic tube.

2: Incubate on ice for 10 min with vigorous mixing for 10 seconds once in a minute.

3: Centrifuge at 2,000 × g for 3 min at 4°C.

4: Discard supernatant.

5: Add 500 μl ethanol and vortex thoroughly.

6: Centrifuge at 2,000 × g for 3 min at 4°C.

7: Repeat steps 4-6.

8: Suspend gold particles in 60 μl ethanol.

9: Use 5.4 μL aliquot of the gold particle suspension for each bombardment.

10: Perform particle bombardment according to manufacturer's instructions. Parameters: vacuum = 28 inHg, the distance between the target stage and the stopping screen = 120 mm.

11: Culture bombarded cells overnight (22°C, 50-60 μmol photons m^{-2} s^{-1}).

12: Spread cells evenly onto four selective 1M51C plates containing 500 mg/L spectinomycin dihydrochloride without sucrose.

13: Transfer spectinomycin-resistant calli to fresh selective media after 4 weeks of culture (Figs. 2B and 2D).

(Establishment of homoplastomic transformant)

1: Transfer ~10 mg of cells or tissue (2 × 2 mm) in 1.5 ml plastic tubes.*[4]

2: Add 100 μl of DNA extraction buffer and disrupt cells by pestle.

3: Add 400 μl of DNA extraction buffer and vigorously mix.

4: Add 500 μl of phenol/chloroform, vigorously mix and centrifuge (10,000 ×g, 5 min).

5: Transfer 200 μl of supernatant and add 500 μl of ethanol.

6: Centrifuge (10,000 ×g, 4°C, 15 min).

7: Add 1 ml of 70% ethanol and centrifuge (10,000 ×g, 4°C, 15 min).

8: Suspend the pellet to 100-200 μl of Tris-HCl (pH 8.0) containing 10 μg/ml RNase A.

9: Select plastid transformants by PCR using primers P1 and P2 for the *aadA* cassette (Fig. 1) .

10: Subculture the candidate plastid transformants on selective media.

11: Check homoplastomic state by PCR using primers P1 and P3 which anneal to the regions outside the homologous sequences (Fig. 1).*[5]

*4: For DNA extraction, QIAGEN DNeasy Plant Mini Kit also works well.

*5: Homoplastomic transformants are readily obtained after the primary selection.

Reference

Chiyoda,S., Linley,P.J., Yamato, K.T., Fukuzawa,H., Yokota,A. and Kohchi,T (2007) Simple and efficient plastid transformation system for the liverwort *Marchantia polymorpha* L. suspension-culture cells. *Transgenic Res.*, **16**, 41-49.