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 EcoRI and ScaI 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)*
● Macrocarrirer (Bio-Rad, #165-2335)*
● Stopping Screen (Bio-Rad, #165-2336)*
*2: Autoclaved
*3: Sterilized with 70% ethanol and air dried in clean hood

(Reagents)
● 2.5 M CaCl₂
● 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)
KNO₃ 80 g, NH₄NO₃ 16 g, MgSO₄·7H₂O 14.8 g, CaCl₂·H₂O 12 g, KH₂PO₄ 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)
NaMoO₄·2H₂O 25 mg, CuSO₄·5H₂O 2.5 mg, CoCl₂·6H₂O 2.5 mg, ZnSO₄·7H₂O 200 mg, MnSO₄·7H₂O 1 g, H₃BO 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 overnight (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).

(Particle bombardment)
1: Successively add 250 µl of 2.5 M CaCl₂, 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 xg, 5 min).
5: Transfer 200 µl of supernatant and add 500 µl of ethanol.
6: Centrifuge (10,000 xg, 4°C, 15 min).
7: Add 1 ml of 70% ethanol and centrifuge (10,000 xg, 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