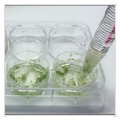

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Marchantia agrobacterium transformation of sporelings in multi-well plates (plus materials info) V.2

Forked from Marchantia agrobacterium transformation of sporelings in multi-well plates

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OpenPlant Project

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ABSTRACT

A modification of the Ishizaki et al 2008 *Agrobacterium* mediated Marchantia sporeling transformation protocol is used.

Sterilised spores are grown for 5 days in 0.5x strength Gamborg plates prior to co-cultivation for 2 days with agrobacterium in liquid media in multiwell plates. Sporelings are then spread on media with the appropriate selective antibiotic. In about 7 days, positive transformants start to emerge.

MATERIALS TEXT

Samples required:

Fertilised spore heads from Cam2 archegonia heads stored at -80ºC or 4 ºC (dried using self-indicating silica gel – Fisher Scientific S/0761/53)
GV2260 Agrobacterium Electro-Competent Cells - included in these cells is the pSoup helper plasmid (Hellens et al, 2000)
Plasmid of interest to transform Marchantia

Overview of Antibiotics Required for selection of Agrobacterium transformants and Marchantia transformants

Agrobacterium strains:
GV2260 strain is resistant to Rifampicin 10µg/ml + Carbenicillin 50µg/ml
GV3103 strain is resistant to Rifampicin 10µg/ml + Gentamycin 25µg/ml (currently not using this strain in the lab).

Plasmid of interest: example Loop pCsA plasmid that confers spectinomycin resistance to Agrobacterium and Hyg resistance to Marchantia plants
- GV2260 transformed with the Loop pCsA plasmid: will be screened in LB plates with Sm 100µg/ml + Rif 10µg/ml + Carb 50µg/ml
- Marchantia sporelings infected by Agrobacterium with a Loop pCsA plasmid will be screened on Gamborg plates with Hyg 20µg/ml + Cefo 100µg/ml

Overview Chemicals and Media required:
- **Sterile Water**
- **Milton Tablets** purchased from Boots (1 tablet in 25ml sterile water = 0.05% of Sodium dichloroisocyanurate active component
- **Acetosyringone** (3',5'-Dimethoxy-4'-hydroxyacetophenone – Sigma D134406) - 100mM stock solution - dissolved in DMSO
- Liquid **LB media**
- **Antibiotics** stocks
- Cefotaxime: 100 mg/ml stock solution, dissolved in water

- **LB plates** (LB agar 1.2%) + **antibiotics** (see ex above)
- **Gamborg:** 0.5x Gamborg B5 with vitamins pH5.8 (Duchefa Biochemie Cat. G0210
- **Gamborg plates** 0.5x Gamborg B5 agar 1.2%, for plating spores
- **Gamborg media plus supplements** = 0.5x Gamborg B5+ 0.1% N-Z amino A (Sigma C7290) + 0.03% L-Gultamine + 2% sucrose
- **Gamborg plates** 0.5x Gamborg B5 **+ antibiotics** (see example above)

Consumables required:
40 µm cell strainer,
2 mm electroporation cuvettes,
50 mL falcon tubes,
empty 1L/500ml bottles,
6-well or 12-well Cell Culture Plates (Corning Costar 3516 and 3513),
1.5 mL Eppendorf tubes

Spores sterilisation and plating

1 **Day 0**

Transfer sporangia to a 1.5 mL Eppendorf tube. Use 2 sporangia per construct, e.g. for 6 transformations, use 12 sporangia.

1.1 Use metal tweezers to crush the sporangia, add 0.5 mL of Milton solution per tube, vortex to get a fine pulp.

1.2 Place a 40µM cell strainer on a 50 mL falcon tube, pour the 0.5 mL of crushed sporangia onto the filter, rinse the eppendorf tube with 1 ml of fresh Milton solution and pour onto the filter. Wash filter with 2.5ml of fresh Milton solution.

1.3 Divide the filtrate solution containing the spores (total volume aprox 4 mL) in 4 new 1.5 mL eppendorf tubes. Spores should be sterilised in Milton for 20-30 min, starting from step 1.2. Let tubes stand for the remaining time.

1.4 Spin tubes at 16000g for 2min in a tabletop centrifuge.

1.5 Discard supernatant and pool spores in a single tube, re-suspend pellets in 25µL per transformation planned.

1.6 Plate spores on 0.5 x Gamborg plates using 50 µL per plate (1 plate is used for 2 transformations). Seal plates with micropore tape and grow for 5 days at 21^{o}C under continuous light (A in Figure).

Agrobacterium preparation

2 **Day 0/1**

Transform Agrobacterium with your plasmid of interest.
On ice, add 50 ng of the plasmid to 50 µL of thawed electro-competent Agrobacterium cells and transfer to a chilled 2 mm gap Electroporation cuvette. Electroporate with a 2.5 kV pulse.

2.1 Recover transformed cells by adding 0.5 mL of LB or SOC to the cuvette and incubate 1-2h at 28°C and 150 rpm

2.2 Plate 50 µL on LB plates with appropriate antibiotics for selection. Incubate plates for 2-3 days at 30°C

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Day 3

Inoculate a single colony of Agrobacterium transformed with the plasmid of interest, into 5 mL LB media plus antibiotics, incubate at 28°C with shaking at 150 rpm for 2 days.

4 **Day 5**

Centrifuge the 5 mL agrobacterium culture for 10 minutes at 3000g. Discard supernatant, re-suspend in 5 mL of 0.5x **Gamborg B5 plus supplements**. Add acetosyringone to a final concentration of 100 µM

Incubate with for 6h at 28°C and 150 rpm.

Agro-mediated sporelings transformation

5 **Day 5**

Prepare a 50 mL Falcon tube with 4 mL per transformation of 0.5 x Gamborg B5 plus supplements media. Add acetosyringone to a final concentration of 100µM.

5.1 Using a sterile scalpel transfer 5 days old Marchantia sporelings from plates to the 50 mL Falcon tube (B and C in Figure) and mix well.

5.2 Using a serological pipette add 4 mL of the spore solution into each well of a 6-well plate (D in Figure).

5.3 Add 100 µL of the Agrobacterium culture to each well.

5.4 Seal the plate with micropore tape and place on a shaker at 120 rpm for 2 days at 21^{o}C under continuous light (E in Figure).

6 **Day 7**

After 2 days, plate the transformed sporelings as follows:

6.1 Using a pipette transfer the sporelings from the first well to a 70 µm cell strainer placed in a 50 mL Falcon tube (F and G in Figure).

6.2 Wash the sporelings by pouring into the strainer 50 mL of sterile water supplemented with 100 µg/ml cefotaxime to remove excessive Agrobacterium.

6.3 Plate sporelings on 0.5 x Gamborg B5 plus vitamins 1.2% agar plates with 100 µg/ml cefotaxime and selective antibiotics (H and I in Figure)

6.4 Repeat steps 6.1 to 6.3 for all wells.

7 **Day 14**

After 5-7 days successful transformants start to be visible on the plate and can be screened for fluorescence (J in Figure)

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