Transformation of the Moss *Physcomitrella patens* Using T-DNA Mutagenesis

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**INTRODUCTION**

In this protocol, the transformation of moss (*Physcomitrella patens*) protoplasts is performed via *Agrobacterium*-mediated transfer of T-DNA. Protoplasts are incubated with *Agrobacterium* and acetoseringone in regeneration medium. They are then washed and plated on antibiotic-containing medium to select for T-DNA insertion in stable transformants. The transformation rate for this protocol is typically $10^{-4}$ (expressed as the frequency of stable transformants among regenerants surviving the transformation procedure).

**RELATED INFORMATION**

For more information about *P. patens* as a model organism, see *The Moss Physcomitrella patens: A Novel Model System for Plant Development and Genomic Studies* (Cove et al. 2009a). A method for isolating protoplasts can be found in *Isolation and Regeneration of Protoplasts of the Moss Physcomitrella patens* (Cove et al. 2009b), and for details on the use of a hemocytometer to estimate protoplast density, see *Estimation of Cell Number by Hemocytometry Counting* (Sambrook and Russell 2006).

**MATERIALS**

**CAUTIONS AND RECIPES:** Please see Appendices for appropriate handling of materials marked with <!>, and recipes for reagents marked with <R>.

**Reagents**

<!> 3′,5′-dimethoxy-4′-hydroxyacetophenone (Acetoseringone) (100 mM, prepared in ethanol)

*Agrobacterium tumefaciens* culture (strain Agl-1)

About 1 mL of a mid-log-phase culture ($OD_{600} = 0.4-0.6$) is needed.

<R> BCD medium, modified (BCDm)

<R> Protoplast regeneration medium for the bottom layer, modified (PRMBm)

<R> Protoplast regeneration medium for the top layer, modified (PRMTm)

<R> Protoplast regeneration medium, liquid (PRML)

<R> Protoplast wash solution for *Agrobacterium* transformation (APW), maintained at 25°C

Prepare 35 mL of APW for each transformation.

Protoplasts, isolated as described in Steps 1-8 of *Isolation and Regeneration of Protoplasts of the Moss Physcomitrella patens* (Cove et al. 2009b)
METHOD

1. Use a hemocytometer to estimate the density of protoplasts (see Estimation of Cell Number by Hemocytometry Counting (Sambrook and Russell 2006)).

2. Centrifuge the protoplasts at 100-200 g for 4 min at room temperature with no braking and discard the supernatant. Resuspend the protoplasts in PRML at a concentration of 5 × 10^5 protoplasts/mL.

3. For each milliliter of protoplast suspension, prepare a Petri dish containing 9 mL of PRML.

4. Add 1 mL of protoplast suspension to each prepared Petri dish.

5. Add 200 µL of an *Agrobacterium* culture with an OD_{600} of 0.4-0.6 to each Petri dish.

6. Add 20 µL of 100 mM acetoseringone to each Petri dish.

7. Wrap each dish with Micropore surgical tape and incubate for 48 h at 25°C under a 16-h light/8-h dark cycle. The protoplasts will tend to adhere to the Petri dish surface during incubation.

8. Use a pipette to carefully resuspend the protoplasts.

9. Centrifuge the protoplasts at 100-200 g for 4 min at room temperature with no braking and discard the supernatant. Resuspend the pellet in 10 mL of APW.

10. Repeat Step 9 twice, but after the last centrifugation step, resuspend the pellet in 2 mL of APW and add 2.4 mL of PRMTm.

11. Dispense 1.1 mL of the protoplast suspension into each of four Petri dishes containing PRMBm and overlaid with sterile cellophane discs. Incubate the dishes for 7 d at 25°C under continuous white light.

12. Transfer the cellophane overlays onto plates containing selection medium and incubate for 7 d at 25°C under a 16-h light/8-h dark cycle.

13. Transfer the cellophane overlays to plates containing BCDm and incubate for 7 d at 25°C under continuous white light.

14. Transfer the cellophane overlays to plates containing selection medium and incubate for 7-10 d at 25°C under a 16-h light/8-h dark cycle.

15. Pick small clumps of tissue from each vigorously growing plant and place them on plates containing BCDm. Incubate the plates for 7 d at 25°C under continuous white light. These should be stable transformants. However, to confirm that these are transformants, test them once more for resistance by growing small clumps of tissue on selection medium for 7-10 d under continuous white light.
REFERENCES
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