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Preparation and Transfection of *Populus tomentosa* Mesophyll Protoplasts

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[Abstract] Mesophyll protoplasts freshly isolated from leaves are a useful research system in plants. However, cell walls in woody plants contain more pectin, making mesophyll protoplasts isolation difficult in *Populus.* This has limited their application in biochemical, molecular, cellular, genetic, genomic, transcriptomic, and proteomic assays. In this protocol, a simple and efficient method to prepare and transfect mesophyll protoplasts of *Populus tomentosa* is presented in detail. Leaves of *P. tomentosa* plants grown in tissue culture media were pre-treated in D-mannitol solution and then digested with an enzyme solution. After washing with W5 and MMg buffers, the protoplasts were incubated in PEG/Ca^{2+} solution with plasmid for transfection. The mesophyll protoplasts isolated were used to express the histone variant H2B fused with green fluorescent protein (GFP) for confocal microscopy imaging. This "*P. tomentosa* mesophyll protoplasts preparation and transfection" system provides a useful tool for studying woody plants using a variety of applications, including gene expression, subcellular localization, protein-protein interaction, chromatin immunoprecipitation, western blot, single-cell sequencing, and genome editing.

Keywords: *Populus*, Mesophyll protoplasts, Transient transfection, Green fluorescent protein, Subcellular localization

[Background] As perennials species, *Populus* is an ideal model system to explore scientific questions that can not be easily addressed in annual plants such as *Arabidopsis* and rice (Jansson and Douglas, 2007). For instance, autumn leaf senescence (Wang *et al.*, 2021), sex determination (Muller *et al.*, 2020; Xue *et al.*, 2020), wood formation (Chen *et al.*, 2019), and seasonal dormancy (Tylewicz *et al.*, 2018), can be more easily studied in *Populus* than in annual plants. As the lifespan of *Populus* plants usually spans decades, centuries, or even millennia (Munné-Bosch, 2008), it is necessary to establish a simple and rapid system to study gene function and signal transduction in woody plants. Mesophyll protoplasts are a powerful and versatile tool for conducting cell-based experiments to study gene function and signaling pathways (Yoo *et al.*, 2007). They provide a good way to study important scientific questions at biochemical, molecular, cellular, genetic, genomic, transcriptomic, proteomic, and single-cell levels. Previously reported protocols for preparation and transfection of mesophyll protoplasts mainly focused on the model plant *Arabidopsis* (Yoo *et al.*, 2007). However, the protocol for isolating *Arabidopsis*

mesophyll protoplasts is not suitable for *Populus* protoplast preparation because of the different cell wall components (Lin *et al.,* 2014). The protocol summarized here is a simple (fewer reagents, only four reagents are required) and efficient (less time, isolation and transfection takes approximately 7 h) procedure (Figure 1) compared with previously reported *Populus* protoplast preparation (Guo *et al.*, 2012; Tan *et al.*, 2013). A histone variant HTB9 fused with GFP is transiently expressed in *P. tomentosa* mesophyll protoplasts (Figure 2). This cellular system provides a helpful tool for studying gene expression in woody plants. Moreover, this protocol can also be applied for other *Populus* species leaves such as *Populus alba*, *Populus trichocarpa*, *Populus alba* × *P. tremula* var. *glandulosa* (84K), and *Populus davidiana* × *P. bolleana*, or leaves of other woody species such as *Eucalyptus urophylla* × *E. grandis* or *Eucalyptus grandis* × *E. urophylla* grown in tissue culture media.

Materials and Reagents

1. 10 ml centrifugal tubes (HOUDIOR)
2. 1.5 ml microfuge tubes (Axygen)
3. Blades (Gillette blue, Super Gillette Blue Blades, 4.3 cm × 2.2 cm)
4. Culture dish (90 mm diameter)
5. Glass-bottom dish (In Vitro Scientific, catalog number: D35-10-1.5-N)
6. Strainers, 400 mesh (BIODEE, catalog number: DE2009)
7. Cellulase “ONOZUKA” R-10 (Yakult, catalog number: L0012-10g)
8. Macerozyme R-10 (Yakult, catalog number: L0021-5g)
9. Pectolase Y-23 (Yakult, product agent (BIODEE) catalog number: AOV0094-1g)
10. MES (Sigma-Aldrich, catalog number: M3671-50G)
11. PEG4000 (Sigma-Aldrich, catalog number: 81240-1KG)
12. D-glucose (Sigma-Aldrich, catalog number: G7021-100G)
13. β-thioglycol (Amresco, catalog number: 0482-100 ml)
14. BSA (Amresco, catalog number: 0332-100g)
15. D-Mannitol (BIODEE, catalog number: BN20023-250g)
16. KCl (BIODEE, catalog number: DE-0395A-250g)
17. NaCl (BIODEE, catalog number: DE0008-500g)
18. $MgCl_2 \cdot 6H_2O$ (BIODEE, catalog number: DE-0288A-500g)
19. $CaCl_2$ (BIODEE, catalog number: DE-0556A-500g)
20. Plasmid (dissolve in double distilled water, 2 μg/μl)
21. Enzymatic solution (see Recipes) (Table 1)
22. W5 solution (see Recipes) (Table 2)
23. MMg solution (see Recipes) (Table 3)
24. PEG/Ca^{2+} solution (see Recipes) (Table 4)
25. D-mannitol solution (see Recipes)

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Equipment

1. 1,000 µl micropipette (Eppendorf)
2. 200 µl micropipette (Eppendorf)
3. 10 ul micropipette (Eppendorf)
4. Cell counting plate (MARIENFELD, model: MARIENFELD-0650030)
5. Erlenmeyer flasks (Bomex, 50 ml, 500 ml)
6. Plant incubator (Percival, model: AR-36L3)
7. Bacterial shaker incubator (Crystal Technology & Industries, model: IS-AX-190L)
8. Centrifuge (Eppendorf, model: 5804 R)
9. Confocal microscope (Carl Zeiss, model: Zeiss LSM780)

Procedure

A. Preparation of *P. tomentosa* materials and solutions

1. One-month-old *P. tomentosa* young plants are grown in tissue culture media. Stem fragments are cultured on shoots propagation medium [Murashige and Skoog (MS) medium containing 0.1 mg/L α-naphthalene acetic acid (NAA), 0.02 mg/l thidiazuron (TDZ)] for 30 days to get plantlets, then the plantlets are transferred to rooting medium (1/2-strength MS containing 0.05 mg/L NAA) with a 16/8 h (light/dark) photoperiod. After 30 days, the plantlets are approximately 10 cm in height (Figure 1).

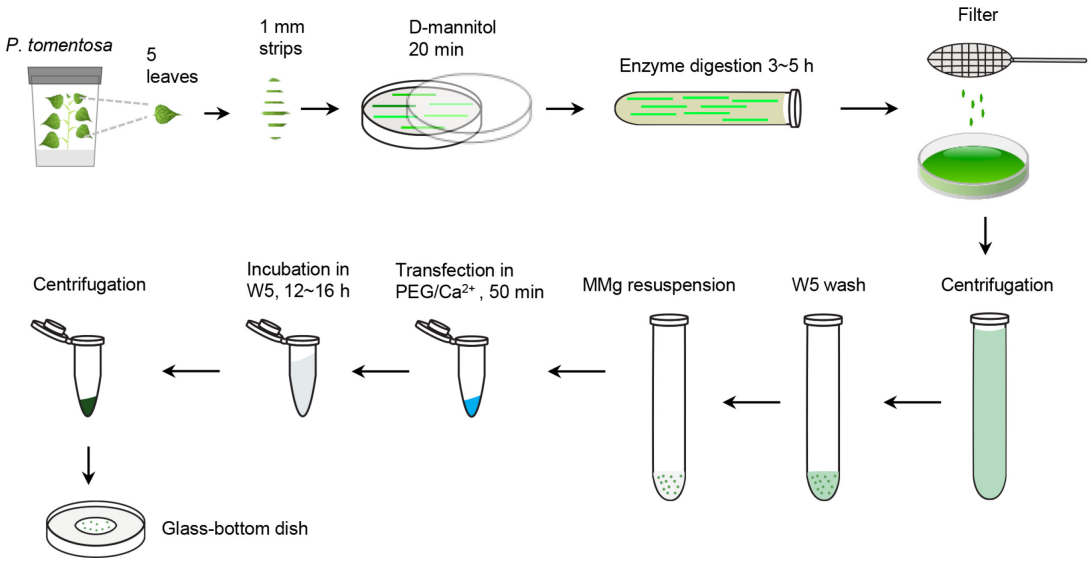

Figure 1. Experimental scheme of the preparation and transfection of *P. tomentosa* mesophyll protoplasts.

Cut the leaves of one-month-old *P. tomentosa* plants grown on tissue culture media into 1 mm strips pre-treated in 0.8 M D-mannitol for 20 min. Digest the leaf strips in enzyme solution for 3-5 h, then filter with 400-mesh cell strainer, centrifuge and wash the protoplasts with W5 solution

twice, followed by wash with MMg buffer twice. The transfection reaction containing 10 µl plasmid, MMg resuspended protoplasts (100 µl), and 40% PEG/Ca^{2+} solution (110 µl) occurs for 50 min. Wash with W5 solution and incubate for 12-16 h. Centrifuge and observe the transfected protoplasts using a glass-bottom dish.

2. Prepare enzymatic, W5, MMg, and PEG/Ca^{2+} solutions. Place the W5 and MMg solutions at 4°C for at least 1 h before use.

B. Isolation of *P. tomentosa* protoplasts

1. Prepare D-mannitol solution (see Recipes).
2. Use a sharp blade to cut 5 *P. tomentosa* young leaves into 1 mm wide strips, completely immerse them in the D-mannitol solution, and place the culture dish on the bench (room temperature, 23°C) for 20 min.
3. Transfer the leaf strips into a 10 ml centrifuge tube with the prepared enzymatic solution, place the tube horizontally in the bacterial shaker incubator in the dark, and shake at 10 rpm for 3-5 h at 23°C until the color of solution changes to green.
4. Filter with a 400-mesh cell strainer, transfer the filtrate into a new 10 ml centrifuge tube, and centrifuge at 100 × *g* for 2 min at 23°C. Remove the supernatant and keep 2 ml of the precipitate.

C. Plasmid transfection

1. Add 2 ml of pre-cooled W5 solution gently along the tube wall to wash the protoplasts, gently invert and mix, centrifuge at 100 × *g* for 1 min at 4°C, and remove the supernatant.
2. Repeat C1 once.
3. Resuspend the protoplasts with 2 ml of pre-cooled W5 solution and place on ice for 30 min.
4. Centrifuge at 100 × *g* for 1 min at 4°C and remove the supernatant.
5. Add 2 ml pre-cooled MMg solution to resuspend the protoplasts, centrifuge at 100 × *g* for 1 min at 4°C, and remove the supernatant.
6. Resuspend the protoplasts to 1 × 10^5-5 × 10^5 per ml (determine their density with the cell counting plate) with pre-cooled MMg solution, and use a cut micropipette tip (0.5-1 cm) to add 100 µl of protoplast suspension for each plasmid of gene transfection.
7. Handle the following steps at 23°C. Add 10 µl of plasmid (2 µg/µl) for a target gene to a 1.5 ml microfuge tubes, and add 100 µl of protoplast suspension liquid and gently mix with micropipette. Add 110 µl (equal volume) of the PEG/Ca^{2+} solution and gently mix with a cut micropipette tip (0.5-1 cm).
8. Incubate in the dark at 23°C for 50 min (transfection step).
9. Add 440 µl (twice volume) of W5 solution, gently mix with a cut micropipette tip (0.5 cm), centrifuge at 100 × *g* for 1 min at 23°C, and remove the supernatant.
10. Add 100 µl of W5 solution, mix with cut micropipette tip (0.5-1 cm), centrifuge at 100 × *g* for 1 min at 23°C, and remove the supernatant.

11. Repeat C10 once.
12. Add 1 ml of W5 solution, mix with the 1,000 µl micropipette, and incubate in the dark at 23°C for 12-16 h.
13. Centrifuge at 100 × *g* for 1 min at 23°C and remove the supernatant. Transfer the precipitate onto a glass-bottom dish for confocal microscopy observation (50-80% transfection efficiency, Figure 1). The results of transfection for PtHTB9 and EgHTB9 are displayed in Figures 2 and 3, respectively.

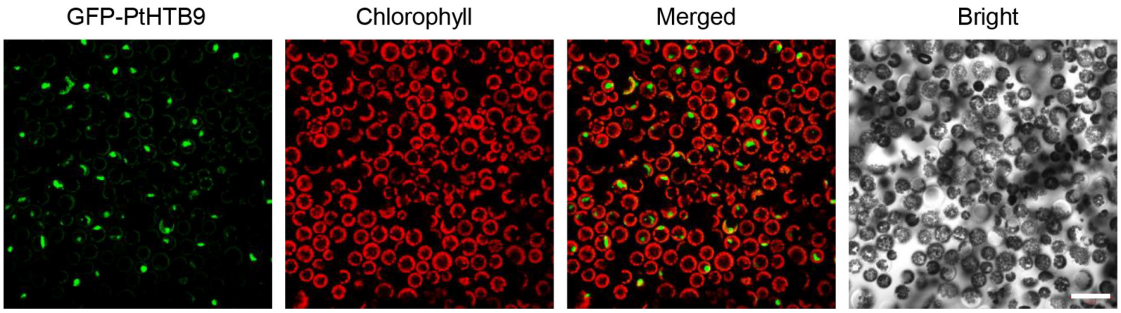

Figure 2. Transfection of GFP-PtHTB9 in *P. tomentosa* mesophyll protoplasts.
The coding sequence (CDS) of the *P. tomentosa* HTB9 gene (PtHTB9, Potri.008G030600) was inserted into a modified pUC19-GFP vector, and 10 µl of GFP-HTB9 plasmid (2 µg/µl) was used for transfection. Images were taken with a Zeiss 780 confocal microscopy. Scale bar = 50 µm.

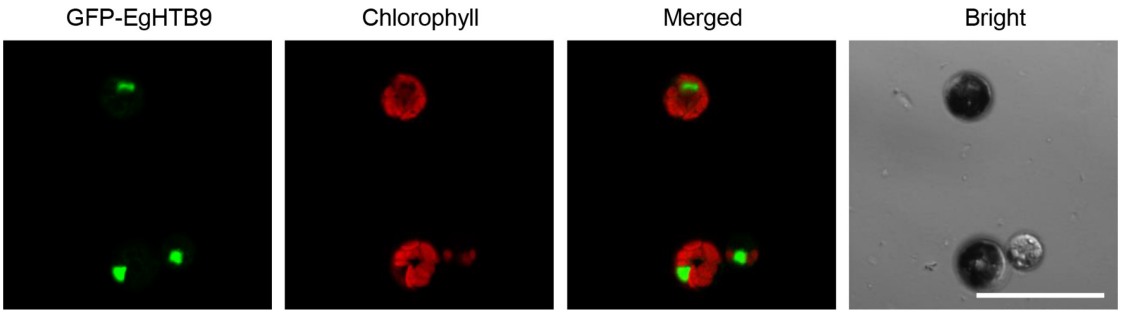

Figure 3. Transfection of GFP-EgHTB9 in *Eucalyptus urophylla* × *E. grandis* mesophyll protoplasts.
The coding sequence (CDS) of the *E. grandis* HTB9 gene (EgHTB9, Eucgr.B03925) was inserted into a modified pUC19-GFP vector. Scale bar = 50 µm.

Notes

1. All the chemicals used in this study are Analytical Reagent (A.R.) standard.
2. For high transfection efficiency, we strongly recommend using PEG4000 (Sigma-Aldrich, catalog number: 81240-1KG) at a concentration of 40%.
3. Use scissors to cut off the tip (0.5-1 cm length) of all 100 µl micropipette tips for C6, C7, C10, and C11 steps, and cut off the tip (0.5 cm length) of 1,000 µl micropipette tips for C9 step.

4. Addition of β-mercaptoethanol to the enzymatic solution (Table 1) is optional.
5. Sterilized and separately stored W5 or MMg solutions can be placed at 4°C for 1 year, while enzymatic and PEG/Ca^{2+} solutions should be freshly prepared before use.
6. Set all the acceleration and deacceleration of centrifugation to 5 (max 10).
7. Leaves digested horizontally in 10 ml centrifuge tubes with 10 ml enzymatic solution provides better isolation. Too much air in the tubes (10 ml enzymatic solution in 15 ml centrifuge tubes) or higher speeds (>60 rpm) in step B3 lead to more broken protoplasts.
8. Smaller protoplast concentrations in the C6 step give higher transfection efficiency, while higher suspension concentration provides more yield (transfected cells).

Recipes

1. Enzymatic solution (Table 1)

Table 1. Recipe for the enzymatic solution

Stock Solutions	Weight or volume	Final Concentration
Cellulase R-10	0.15 g	1.5%
Macerozyme R-10	0.04 g	0.4%
Pectolase Y-23	0.05 g	0.5%
D-mannitol	1.092 g	0.6 M
200 mM KCl	1 ml	20 mM
200 mM MES	1 ml	20 mM
5 ml ddH_2O, vortex, 55°C 10 min, leave to cool at room temperature		
1 M $CaCl_2$	100 µl	10 mM
7.5 mM β-thioglycol	66.67 µl	0.05 mM
1% BSA	1 ml	0.1%
Fill the volume to 10 ml with ddH_2O		

2. W5 solution (Table 2)

Table 2. Recipe for the W5 solution

Stock Solutions	Weight or volume	Final Concentration
2 M NaCl	38 ml	154 mM
1 M $CaCl_2$	62.5 ml	125 mM
200 mM KCl	12.5 ml	5 mM
200 mM MES	5 ml	2 mM
D-glucose	0.45 g	5 mM
Fill the volume to 500 ml with ddH_2O		

3. MMg solution (Table 3)

Table 3. Recipe for the MMg solution

Stock Solutions	Weight or volume	Final Concentration
D-mannitol	7.29 g	0.6 M
150 mM $MgCl_2$	10 ml	15 mM
200 mM MES	2 ml	4 mM
Fill the volume to 100 ml with ddH_2O		

4. PEG/Ca^{2+} solution (Table 4)

Table 4. Recipe for the PEG/Ca^{2+} solution

Stock Solutions	Weight or volume
PEG4000	3 g
ddH_2O	2.25 ml
0.8 M D-mannitol	1.785 ml
1 M $CaCl_2$	0.75 ml

5. D-mannitol solution
   Dissolve 2.912 g mannitol in 20 ml ddH_2O in 90 mm diameter culture dish.

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Competing interests

No financial, personal, or professional interests have influenced the work.

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