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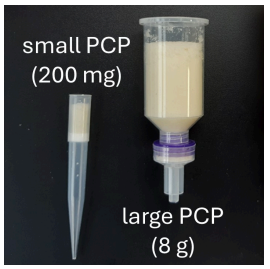
Arabidopsis thaliana plant cell pack (PCP) transformation



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We use this protocol and it's working

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Disclaimer

Abstract

This protocol describes the transient and stable transformation of *Arabidopsis thaliana* suspension cells by agroinfiltration of plant cell packs (PCPs) adapted from Rademacher *et al*., 2019. It was optimized to be performed with standard laboratory equipment and gives the user the choice between a high throughput and a high amount of transformed biomass. The transient expression workflow can be completed in 4 days if suspension cultures are readily available. The stable transformation workflow optimized for large volumes can yield 100 g (fresh weight) of transgenic cells within 2 weeks after transformation.

Guidelines

- This protocol consists of two different workflows that provide a high throughput at a scale of 60-200 mg cells per transformed sample (referred to as "small PCPs") or a lower throughput but a large scale of 8 g cells per transformed sample (referred to as "large PCPs").
- Note that this protocol has been optimized for Col-0 and MM1 cell lines (kindly provided by Laszlo Bako, Umeå University, Sweden). While we recommend using these two cell lines for transient and stable transformation, respectively, other cell lines might also work using this protocol. The cell line YG1 has also been successfully tested but is not recommended due to slower growth in our hands. For other cell lines that are routinely cultured under light conditions, we recommend culturing them under darkness for at least one round of propagation before PCP preparation. This modification was crucial for high transformation efficiency for the cell line MM1. The cell lines T87 and PSB-L could not be transformed using this protocol when cultured under a 16-h photoperiod.
- The *A. tumefaciens* strain GV3101::pMP90 is the only strain that has been tested for both *A. thaliana* cell lines and is therefore recommended. Note that the strains EHA105 and AGL-1 have also been successfully tested for transient expression of Col-0 cells.
- We recommend including the RUBY reporter (He et al., 2020) as a control to visually assess the success of the transformation protocol.

Materials

Biological material:

- *Agrobacterium tumefaciens* strain GV3101::pMP90
- *Arabidopsis thaliana* suspension cell line Col-0 (Alternatively: cell line YG1)
- *Arabidopsis thaliana* suspension cell line MM1

Media and buffers:

- **Col-0 Growth Medium:**

Murashige & Skoog medium including vitamins [M] 4.4 g/L

Sucrose [M] 30 g/L

2,4-Dichlorophenoxyacetic acid (2,4-D) [M] 0.24 µg/mL

Kinetin [M] 0.014 µg/mL

KOH to adjust pH pH 5.7

- for **Col-0 Selection Medium**, add:

Carbenicillin [M] 250 µg/mL

Vancomycin [M] 165 µg/mL

Hygromycin [M] 20 µg/mL

- **MM1 Growth Medium:**

Murashige & Skoog medium (basal salts) [M] 4.3 g/L

Sucrose [M] 30 g/L

Thiamine HCl [M] 0.4 µg/mL

Myo-inositol [M] 0.1 mg/mL

1-naphthaleneacetic acid (NAA) [M] 0.5 µg/mL

Kinetin [M] 0.05 µg/mL

KOH to adjust pH pH 5.7

- for **MM1 Selection Medium**, add:

Carbenicillin [M] 250 µg/mL

Vancomycin [M] 165 µg/mL

Hygromycin [M] 20 µg/mL

- **Paul's Medium (Buschmann 2016)**:

Murashige & Skoog medium (basal salts) [M] 4.3 g/L

Sucrose [M] 10 g/L

KOH to adjust pH [pH] 5.8

- ***Agrobacterium* Infiltration Buffer (adapted from Gengenbach *et al.*, 2020)**:

Murashige & Skoog medium (basal salts) [M] 0.5 g/L

Sucrose [M] 50 g/L

Glucose [M] 10 millimolar (mM)

2-(N-morpholino)ethanesulfonic acid (MES) [M] 15 millimolar (mM)

KOH to adjust pH [pH] 5.6

- **Lysogeny broth (LB) medium:**

Tryptone [M] 10 g/L

Yeast extract [M] 5 g/L

Sodium chloride (NaCl) [M] 10 g/L

- **Lysis Buffer:**

Tris-HCl (pH 8) [M] 100 millimolar (mM)

NaCl [M] 150 millimolar (mM)

$MgCl_2$ [M] 10 millimolar (mM)

NP-40 [M] 2 % volume

Glycerol [M] 5 % volume

β-mercaptoethanol [M] 5 millimolar (mM)

EDTA [M] 5 millimolar (mM)

Protease inhibitor cocktail (Sigma P9599) [M] 0.5 % volume

Equipment:

- For Col-0 cells: Incubator-shaker set to 25°C
- For MM1 cells: Incubator-shaker set to 22°C with illumination.
- Incubator-shaker set to 28°C
- Laminar flow hood
- Vacuum pump
- Vacuum manifold (or DIY version: vacuum flask with perforated rubber stopper equipped with PCV tube sections. JPG DIY 3x manifold.jpg 93.5KB)
- Swing-bucket centrifuge equipped for 50-mL Falcon tubes.

Other materials:

- For large-scale PCPs: Zymo-Spin V-PS midiprep column assembly (with the DNA-binding matrix removed) with a 15-ml reservoir-X (Zymo Research)
- Pipette filter tips (standard and wide-bore)
- Serological pipettes (standard and wide-bore)
- Micropore Surgical Tape (3M)
- 250-ml glass shake flasks closed with aluminium foil (2 layers)

Safety warnings

- Plant cell suspensions are prone to microbial contamination. Always use a laminar flow hood for handling and propagating suspension cultures. The use of filter pipette tips is recommended. We further recommend maintaining backup cultures on solid medium (propagation once per month), from which fresh liquid cultures can be prepared in case of contamination.
- Different Arabidopsis cell lines might need further optimization for this protocol to work (see guidelines).

Before start

Maintaining the *A. thaliana* suspension cultures in a healthy state is a prerequisite for the success of this protocol. Low and high cell densities can both lead to poor culture viability. Healthy cell suspensions sediment within minutes, resulting in a clear upper phase above the sedimented cells. A green or yellowish colour of the cells (depending on the light conditions) indicate good health, as opposed to white cell colour. It is recommended to weekly passage the cultures as follows:

Col-0 cell line:

Inoculate 7 mL dense culture into 40 mL of Col-0 Growth Medium. Propagate weekly and incubate in an incubator-shaker. 100 rpm, 25°C darkness

MM1 cell line:

Inoculate 2 mL dense culture into 48 mL of MM1 Growth Medium. Propagate weekly and incubate in an incubator-shaker. 100 rpm, 22°C , 16h light/ 8h darkness

Plant cell suspension precultures

1 **Col-0 cell line:**

Inoculate 7 mL dense culture into 40 mL of Col-0 Growth Medium. Grow for 3-7 days before PCP preparation in an incubator-shaker. 100 rpm, 25°C , darkness

MM1 cell line:

1. Inoculate 2 mL dense culture into 48 mL of MM1 Growth Medium. Grow for 1 week in an incubator-shaker. 100 rpm, 25°C darkness
2. Inoculate 2 mL dense culture grown in the dark into 48 mL of MM1 Growth Medium. Grow for 4-7 days before PCP preparation in an incubator-shaker. 100 rpm, 25°C , darkness

Note

- Growth of the MM1 preculture under darkness differs from routine propagation for MM1, which is under a 16-h photoperiod. Growing MM1 precultures under light conditions leads to a much lower transformation efficiency.
- Replacing the spent medium with fresh medium the day before PCP preparation can increase transformation efficiency.

Agrobacterium tumefaciens preparation

3d

2 3d

1. Streak *A. tumefaciens* strain GV3101::pMP90 containing a plant expression vector onto LB agar containing [M] 25 µg/mL rifampicin, [M] 40 µg/mL gentamycin and the appropriate amount of antibiotic to select for your expression vector. Incubate for 2-3 days at 28 °C .
2. From the plate prepared in the previous sub-step, inoculate a 10-mL liquid LB culture containing the same antibiotic concentration as above. Scale up culture if more than 150 small PCPs or more than 3 large PCPs are prepared. Incubate for 24:00:00 in an orbital shaker at 28 °C .
3. Harvest *A. tumefaciens* cells by centrifugation. 5000 x g, Room temperature, 00:10:00
4. Washing: Resuspend the pellet in 20 mL *Agrobacterium* Infiltration Buffer and centrifuge again as above.

5. Resuspend the pellet in 5 mL *Agrobacterium* Infiltration Buffer and determine the OD_{600nm}.
6. Adjust the OD_{600nm} to 0.4 with *Agrobacterium* Infiltration Buffer.

Note

- The last sub-step should be carried out immediately before PCP infiltration. Keeping the diluted *A. tumefaciens* suspension for an extended time can lead to cell aggregation, which results in lower transformation efficiency.
- A final OD_{600nm} of 0.1 has also been successfully tested. However, incubation times for infiltrated PCPs might change.
- Sub-steps 2 and 3 can be omitted, resupending *A. tumefaciens* cells grown on LB agar directly in *Agrobacterium* Infiltration Buffer for washing (alternative sub-step 4). However, lower transformation efficiencies are sometimes observed following this short-cut.

Plant cell pack preparation and infiltration

2h 15m

3 **Washing of plant cells (done on the day of PCP infiltration):** 15m

1. Transfer the plant suspension preculture to a 50-mL Falcon tube.
2. Remove the liquid medium by pressing the tip of a serological pipette against the tube bottom followed by slow aspiration, leaving a cell pellet behind. Alternatively, slow centrifugation (200 g) followed by removing the supernatant with a serological pipette may be used.
3. Slowly add Paul's Medium to the cell pellet until it is resuspended. Only add as much volume as needed to be able to barely aspirate the suspension with a wide-bore 1000-µL pipette tip. This volume depends on the pellet size and the cell line.

4 **Casting and infiltration of PCPs:** 2h

Small PCPs:
Col-0 cell line:

1. Load 500 µL washed Col-0 suspension cells onto the top part of a 1000-uL pipette filter tip. The use of wide bore pipette tips is crucial to transfer the dense cell suspension.
2. Remove excess liquid using a vacuum manifold for approximately 00:00:10 to generate the PCPs.
3. Place the pipette filter tips containing the PCPs in a sterile tip box.
4. Add 300 µL *A. tumefaciens* suspension to each PCP.
5. Close the tip box and incubate for 01:00:00 at 25 °C .

6. Remove excess liquid using a vacuum manifold for approximately 00:00:10 to dry the infiltrated PCPs and place the pipette filter tips back into the tip box.
7. Place wet sterile filter papers into the tip box next to the PCP-containing filter tips to maintain high humidity.
8. Incubate the PCPs inside the tip box for 2-3 days at 25 °C .
9. To harvest the PCPs, place the filter tip upside-down in a 2-mL Eppendorf tube and tap it on the laminar flow hood working surface.

MM1 cell line:

1. Load 150 µL washed suspension cells onto the top part of a 200-uL pipette filter tip. The use of wide bore pipette tips is crucial to transfer the dense cell suspension.
2. Remove excess liquid using a vacuum manifold for approximately 00:00:10 to generate the PCPs
3. Place the pipette filter tips containing the PCPs in a sterile tip box.
4. Add 90 µL *A. tumefaciens* suspension to each PCP.
5. Close the tip box and incubate for 01:00:00 at 25 °C
6. Remove excess liquid using a vacuum manifold for approximately 00:00:10 to dry the infiltrated PCPs and place the pipette filter tips back into the tip box.
7. Place wet sterile filter papers into the tip box next to the PCP-containing filter tips to maintain high humidity.
8. Incubate the PCPs inside the tip box for 2-3 days at 25 °C
9. To harvest the PCPs, place the filter tip upside-down in a 2-mL Eppendorf tube and tap it on the laminar flow hood working surface.

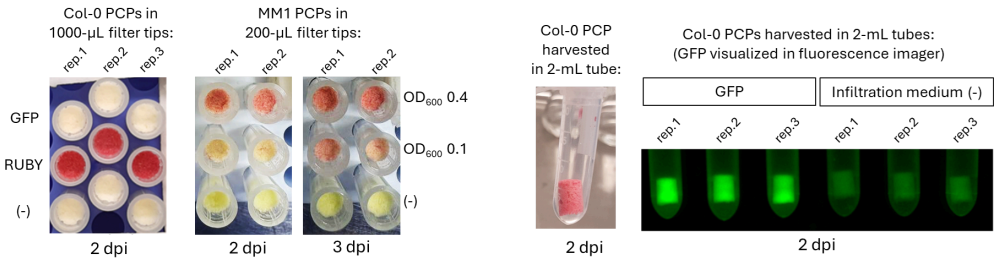
Note

- Transient expression levels can be increased by including a constitutively expressed gene encoding the suppressor of silencing p19 on the expression vector. Alternatively, p19 can be employed by co-infiltration of a second *A. tumefaciens* strain carrying an expression vector with *p19*. In that case, we recommend mixing the two *A. tumefaciens* suspensions (OD_{600nm}=0.4) in a 1:1 ratio before PCP infiltration.
- For protein extraction from harvested PCPs, the following protocol is recommended:

1. Add a 7-mm steel ball and 2 µL of Lysis Buffer per mg of harvested PCP to the 2-mL tube
2. Homogenize for in a Qiagen Retsch MM300 TissueLyser. 00:01:00
3. Incubate on a tube rotator. 00:15:00 4 °C
4. Optional: To obtain the soluble protein fraction, centrifuge and harvest the supernatant. 20000 x g, 4°C, 00:15:00

Expected result

If a control construct harbouring the RUBY reporter is included, PCPs are expected to appear red after 2-3 days. MM1 generally exhibits less RUBY expression than Col-0.



Large PCPs (same protocol for all cell lines):

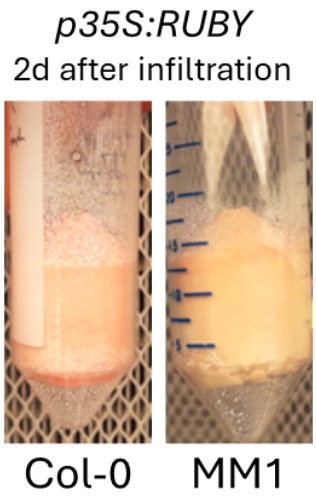
1. Load 20 mL washed suspension cells onto a Zymo-Spin V-PS midiprep column assembly (with the DNA-binding matrix removed) with a 15-ml reservoir. The column+reservoir may be placed in a 50-µL Falcon tube or in a similar sterile container for this step.
2. Remove excess liquid by gravity or by applying a mild vacuum to generate PCPs.
3. Add 20 mL *A. tumefaciens* suspension to each PCP. This is done stepwise; the excess liquid is allowed to drip out of the column.
4. Incubate for 01:00:00, starting from the first addition of the *A. tumefaciens* suspension at Room temperature. Keeping the infiltrated PCPs in the laminar flow hood is recommended.
5. Place the PCP-containing column+reservoir in a 50-mL Falcon tube, if not already done in step 1, and close the tube with a screw lid. It is recommended to seal the lid with Micropore tape to reduce the risk of contamination in the following step.
6. Remove excess liquid using a centrifuge with a swing-bucket rotor 200-300 x g, Room temperature, 00:02:00. The resulting PCP should appear lighter-coloured than before centrifugation, indicating sufficient liquid removal. Repeat this step if the PCP has not been sufficiently dried.
7. Transfer the infiltrated and dried PCP to a sterile 50-mL Falcon tube. The transfer is facilitated by placing the column+reservoir upside down on the Falcon tube and

gently tapping them on the laminar flow hood working surface.

8. Partially close the the Falcon tube with a screw lid and seal it with Micropore tape to allow gas exchange.
9. Place in a humid box at 25 °C in the dark for 2-3 days.

Expected result

If a control construct harbouring the RUBY reporter is included, PCPs are expected to appear red after 2-3 days. MM1 cells sometimes exhibit only a slight colour change; however, the generation of stable transformant from such PCPs is still possible following the steps described in the following section.



Generation of stable transformant cell cultures from infiltrated PCPs

1h

5 **Small PCPs:** 1h

Col-0 cell line:

1. Add 1 ml of Col-0 Selection Medium to the harvested PCPs placed inside a 2-mL Eppendorf tube.
2. Gently disrupt the PCP by pressing it against the tube wall and by up-and-down pipetting with a wide-bore 1000-µL pipette tip.

3. Transfer the disrupted PCP together with all the liquid into a 25-mL shake flask containing 3 mL of Col-0 Selection Medium. Incubate at in an incubator shaker. 100 rpm, 25°C in darkness
4. Once the culture reaches a density comparable to a 7-day-old untransformed culture, propagate it as described under Guidelines, but with Col-0 Selection Medium for several passages.

MM1 cell line:

1. Add 1 ml of MM1 Selection Medium **without hygromycin** to the harvested PCPs placed inside a 2-mL Eppendorf tube.
2. Gently disrupt the PCP by pressing it against the tube wall and by up-and-down pipetting with a wide-bore 1000-µL pipette tip.
3. Transfer the disrupted PCP together with all the liquid into a 25-mL shake flask containing 3 mL of the same medium (described in sub-step 1). Incubate in an incubator-shaker. 100 rpm, 22°C 16h light/8h dark
4. After 3-5 days of culturing, add hygromycin to a final concentration of [M] 20 µg/mL .
5. Once the culture reaches a density comparable to a 7-day-old untransformed culture, propagate it as described under Guidelines, but with in MM1 Selection Medium for several passages.

Large PCPs:

Col-0 cell line:

1. Add 10 mL of Col-0 growth medium to the PCP inside the 50-mL Falcon tube.
2. Gently disrupt the PCP by pressing it against the tube wall and by up-and-down pipetting with a wide-bore serological pipette.
3. Transfer the disrupted PCP together with all the liquid into a 250-mL shake flask containing 40 mL growth medium. Incubate in an incubator-shaker. 100 rpm, 25°C, 00:30:00
4. Replace the growth medium containing washed-off *A. tumefaciens* cells with fresh Col-0 Selection Medium. Incubate for 4 days at 100 rpm, 25°C .
5. Discard the spent medium and replace it with 40 mL fresh Col-0 Selection Medium. Equally inoculate four 250-mL shake flasks containing 50 mL Col-0 Selection Medium with the transgenic cell suspension. Incubate for 7 days at 100 rpm, 25°C .

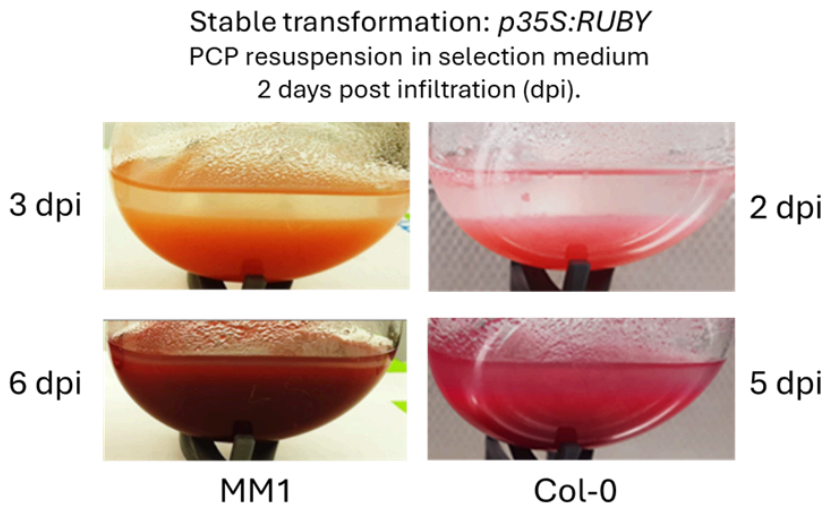
MM1 cell line:

1. Add 10 mL of MM1 growth medium to the PCP inside the 50-mL Falcon tube.

2. Gently disrupt the PCP by pressing it against the tube wall and by up-and-down pipetting with a wide-bore serological pipette.
3. Transfer the disrupted PCP together with all the liquid into a 250-mL shake flask containing 40 mL growth medium. Incubate in an incubator-shaker.
   100 rpm, 25°C, 00:30:00
4. Replace the growth medium containing washed-off *A. tumefaciens* cells with fresh MM1 Selection Medium **without hygromycin**. Incubate for 4 days at
   100 rpm, 22°C 16h light/8h dark .
5. Discard the spent medium and replace it with 40 mL MM1 Selection Medium. Equally inoculate four 250-mL shake flasks containing 50 mL MM1 Selection Medium with the transgenic cell suspension. Incubate for 7 days at
   100 rpm, 25°C .

Expected result

Starting from one large PCP of approximately 8 g (fresh weight), around 100 g of transgenic cells are expected to be produced in total in the four flasks prepared in sub-step 5.



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