

Arabidopsis transformation

I. Protocol

A. Electrotransformation of *Agrobacterium* with a plasmid that has been replicating in *E. coli*.

1. Streak out the host Agro strain from the -80°C onto the appropriate media (e.g., GV3101 onto YEP-Gen₅₀). It will take 2-3 days to establish good size single colonies.

Agro does not seem to last more than a month or two on selectable media plates in the fridge so it is wisest to always use a freshly streaked culture.

Competent Cells: Liquid Culture Method

Additional items needed:

~2500 ml of ice-cold sterile (autoclaved) dH₂O and ice-cold 10% glycerol

2. Pick a single colony of Agro into ~ 5ml YEP. Grow overnight at 28°C.
3. Inoculate 1000 ml of YEP with the 5ml overnight culture.
4. Grow overnight (or until the OD₆₀₀ is ~ 1.5).
5. Chill cells by placing the flask into an ice bath.
6. Spin cells down at 2,500 X g (~4200 RPM in SLA1500 rotor) for 5min at 4°C.
7. Resuspend cells in 1000ml ice cold sterile dH₂O.
8. Spin cells down 10,000 X g (~8500 RPM in SLA1500 rotor) for 15min at 4°C.
9. Repeat with a second 1000ml volume of ice-cold sterile dH₂O.
10. Repeat with 500ml ice-cold sterile dH₂O.
11. Resuspend in 20ml ice-cold sterile 10% Glycerol.
12. Transfers cells to an Oak Ridge tube.
13. Spin cells down at 10,000 X g (~9000 RPM in SS34 rotor) for 15min at 4°C.
14. Resuspend in 2ml ice-cold sterile 10% glycerol.
15. Aliquot into convenient volume (50 - 125μl).
16. * Quick-freeze tubes in liquid N₂.
17. Store cells at -80°C.

Competent Cells: Plate Method

2. Pick a single colony and resuspend it in 100 – 200 μ L of sterile T₁₀E_{0.1}, H₂O or media.
3. Plate this suspension onto a fresh media plate containing the appropriate antibiotic.
4. Grow for ~ 48h.
5. Using sterile technique, scrape off the cells with a loop and resuspend them into 1ml sterile dH₂O in a microfuge tube.
6. Spin the cells down (30 – 60 sec in the microfuge) and decant off s/n, keeping the cells.
7. Repeat the washing of the Agro cells with 1ml volumes of sterile dH₂O for several additional resuspension/decantation cycles.
8. After the final washing and decantation, resuspend the cells in a convenient volume (e.g., 50 μ L volume per electrotransformation is good).

Electroporation

I'm not sure but I think the DNA should be cleaned up some how, e.g., CsCl, Qiagen (or some other Company's cleaning kit) or phenol extracted mini-prep DNA.

1. Electroporate (see Electroporation) ~50 μ l Agro cells with ~1 μ l plasmid DNA from E. coli.
2. Outgrow the cells in antibiotic free medium for 1-3 h.

1.5ml microfuge tubes work fine for out growing the cells. Afterward simply spin the cells down in a microfuge to concentrate them. Since the objective is to just obtain a single colony of the transformed construct it really makes no sense to plate serial dilutions. Therefore after outgrowing and pelleting the cells, simply flame a loop and streak out transformation mix onto selectable medium.

3. Plate transformation mix onto appropriate selectable media plate (usu. YEP-Gen₅₀Strep₃₀₀, YEP-Gen₅₀Spec₁₀₀ or YEP-Gen₅₀Kan₅₀). Grow 2 – 3 days.
4. Make a glycerol stock of this construct so you can go back to it later if needed.

Note: in a pinch and if you only have one or a few constructs to transform, a single colony can be picked off of a plate and prepared for transformation (i.e., skipping steps 2 –5 above).

B. Agro growth and preparation

1. Pick a single colony of the Agro transformed with the plasmid into 5ml YEP containing the appropriate antibiotic. Grow o/n.
2. Inoculate 250ml of YEP containing the appropriate a/b with the 5ml o/n. Grow o/n.
3. Spin cells down 9000 rpm in the Sorval SLA1500 for 5 min.

4. Decant the spent media into bleach or autoclave.
5. Resuspend the Agro cells in 250ml 5% sucrose / 0.05% Silwet-L77.

C. Growth of *Arabidopsis thaliana*

Each lab strain has a different time to bolting (flowering). If plants are being grown for transformation; i.e., floral dipping, each strain will be at the optimum accordingly; i.e., WS2 is at its optimum three weeks after sowing when grown at 22°C constant temp, 18h day and 6h night. Nø2 is 7d to 10d later; RLD takes an additional two weeks, i.e., 3 weeks after WS2, to reach the appropriate stage for dunking.

1. Prepare pots with soil fitted with fiberglass netting secured with a rubber band.

The soil netting prevents soil escaping from the pot when it is inverted for dunking the plants in the Agro solution; this step is not necessary otherwise.

2. Sow seed such that you end up with 10 – 20 plants per pot.

(One way to do this is to make a suspension of *Arabidopsis* seed in 0.1% phytagar and then to pipette the seed onto the netting. Make sure the seed contact the soil, e.g., use a squirt bottle to ensure the seed are through the netting. There are approximately 50 seeds per mg).

3. Place pots in a green tray. Fill tray with about 1inch of tap distilled water.
4. Cover with plastic wrap until the plants are visible, ~3–5d. Place in growth chamber on bottom shelf. Florescent lamps are the best. If one high-pressure sodium lamp (essential to induce flowering) and one metal halide lamp (for vegetative growth) are being used then when the plants are placed on the bottom shelf the light intensity works out to $100 \mu\text{Mm}^{-2}\text{min}^{-1}$. I have not tried inducing with HPS and then switching to all MH. Supposedly *Arabidopsis* don't like HPS (Linda Danhof).

Note: if the seed are fresh and/or have not been completely dried down, they will need to be stratified. To do this either imbibe the seed and then place them in the refrigerator (in a microfuge tube) or, alternatively put the pots and trays into a cold room for 3 or 4d.

5. Thin plants to 10 – 20 per pot before they get too crowded.
6. The day before dunking, make sure the plants are well watered.

D. *Arabidopsis* dunking

1. Bring the plants to be transformed from the growth chamber into the lab. Invert the pot and dunk/dip the inflorescences (flowers) and leaves of the plants into the bacterial suspension (a 1000ml plastic beaker works well for this) and swirl for about 5 sec. Add more 5% sucrose / 0.05% Silwet-L77 if needed (250 ml is a convenient volume to work with because it fits in the 250ml centrifuge bottle).

Note that several pots can be successively dipped in the same Agro suspension.

2. Drain the Agro from the plants.

3. Lay the plants on their side in the tray and cover tray with plastic wrap and place in low light (the lab). Leave o/n.

4. The next day remove the plastic wrap and set the plants upright. Return the plants to the growth chamber. Refrain from watering the plants again until they show signs of needing water ~ 5 d. Otherwise grow and harvest seed as normal.

E. Continued growth and maintenance of plants

1. Generally when transgenic plants are created via the floral dip method the plants are growing in 4-inch pots.

2. Once the plants are unable to support their inflorescence, put a single stake (hyacinth stake) into the pot and use string to tie up all the plants in that pot. This essentially prevents cross contamination between adjacent pots of plants.

3. Once the plants have all set seed and start to show signs of senescence they may be brought into the lab to dry down; i.e., they no longer require light to finish setting seed.

F. Seed Harvesting

1. Plants should essentially be bone dry.

2. Remove the stake and string.

3. Place the plant on top of a white piece of paper and run your fingers up the stem to strip the seeds and siliques off of the plant and onto the piece of paper below. Scrunch the siliques one additional time to remove any remaining seed from the siliques.

4. With another white piece of paper below, pass the seed through a nylon mesh to remove all debris. Repeat this process one or two additional times until a relatively clean preparation of seed is obtained.

5. To prevent cross contamination during seed harvest between each successive transgenic plant seed isolation always thoroughly brush the lab counter top area & paper and shake out the mesh.

6. Store the seed in a 1.5ml microfuge tube. They are quite stable at RT.

G. Plating and screening for transgenic Arabidopsis.

1. Prepare GB5 plates containing the appropriate antibiotic.

2. Seed Sterilization.**

a) Transfer a portion of the seed harvest to a second microfuge tube.
(1mg ~ 50 seed).

b) Add 1ml of freshly prepared H₂O/Bleach/Triton X-100 to the seeds.

- c) Vortex well.
- d) Shake 10 – 20 min.^{##}
- e) Vortex well a second time.
- f) Quick spin.
- g) Aspirate (or decant) off H₂O/bleach/triton X-100 soln.
- h) Add ~ 1ml sterile H₂O.
- i) Vortex, quick spin and aspirate off liquid.
- j) Repeat seed washing with sterile H₂O **TWO** additional times.
- k) Add ~ 1ml sterile 0.1% phytagar to seeds and plate.
- l) Parafilm the plates and place them under lights.^{^^}

H. Transfer of plants from tissue culture

1. As soon as possible remove the transgenic plants from the antibiotic containing media plates and transfer the plants to soil.
2. Gently remove the plants from the agar surface. It might help to first loosen the agar surrounding the plants using forceps and then give the plant a gentle tug and (Note that in lower concentrations of phytagar; e.g., ~0.8%, the roots tend to penetrate the agar whereas in higher concentrations; e.g., ~1.0% the roots tend to grow more on the surface.)
3. If the objective is to grow numerous independent transgenic plants in very close proximity, then transfer single individual plants in an individual cell in one of the cell packs (these are like what you buy bedding plants from a nursery in; they come in all sizes: 6 pack 8 packs etc).
4. Cover the newly transplanted plants with plastic wrap. Leave this on for several days until visible signs of growth from the transplants.
5. To prevent cross contamination of seed between adjacent plants, tie each plant to a hyacinth stake once large enough.

II. Solutions, media and other stuff

A. YEP 1L

1. Yeast Extract 10g
2. Peptone 10g
3. NaCl 5g
4. dH₂O to 1L
5. Agar (for plates only) 15g
6. A/C
7. Cool to ~ 50°C
8. Add appropriate quantity of antibiotic.

B. Antibiotics:

Prepare each needed antibiotic by dissolving it in the appropriate solvent (the following are all water soluble except*):

Antibiotic	Stock conc	For 10ml of stock	Final conc in media
1. Gen	50mg/ml	0.5g	50µg/ml
2. Kanamycin	50mg/ml	0.5g	50µg/ml
3. Hygo	50mg/ml	0.5g	15-50µg/ml
4. Rif*	50mg/ml (in DMSO)	0.5g	50µg/ml
5. Strep	300mg/ml	3g	10µg/ml <i>E. coli</i> 300µg/ml <i>Agro</i>
6. Spectinomycin	100mg/ml	1g	100µg/ml <i>Agro</i>
7. Timenton	300mg/ml	3g	300µg/ml Arabidopsis T ₁

Filter-sterilize a/b using syringe and 0.2µ filter unit
Add a/b to media after it has cooled to ~50°C.
Store a/b at -20°C.

C. 0.1% phytagar

1. Phytagar 0.1g
2. dH₂O 100ml
3. A/C

D. 5% sucrose / 0.05% Silwet-L77

1. dH₂O 250ml
2. Sucrose 12.5g
3. Silwet-L77 125µL Silwet-L77
4. No need to sterilize if used the same day.

E. Gamborg's B5 medium (GB5)

1. GB5* 23.2g
2. MES 0.5g
3. dH₂O to 1L
4. pH to 5.7
5. Phytagar 8-10g[#]
6. A/C
7. Cool to ~ 50°C
8. Add appropriate quantity of antibiotic.

F. H₂O/bleach/triton X-100 soln[^]

1. dH₂O 50%
2. Bleach 50%
3. Triton X-100 0.2%

4. Prepare fresh. Add the Triton to the dH₂O first and then apply heat (or heat the water and then add the Triton), cool, then add the bleach (supposedly heat reduces the effectiveness of the bleach).

NOTES:

*GB5 comes either with- or without- sucrose. The above recipe is based on the assumption that sucrose is present in the GB5 formulation. If it is straight GB5, then only 3.2g of the GB5 is required **but then the media must be augmented with 20g of sucrose.**

[#]0.8% phytagar results in a soft surface which the plant roots will more than likely penetrate whereas 1.0% phytagar results in a less permeable surface which causes the roots to grow along the surface of the phytagar.

[^] The concentrations of the bleach and Triton are not absolutely critical. Some people use 30% bleach and 0.02% Triton X-100. I like 50% bleach and 0.2% Triton X-100.

** Although I perform the addition of the 0.1% phytagar and the plating of the seeds in the laminar flow hood, I perform all other sterilization and washing operations at my bench.

^{##} Don't leave the seeds in contact with the Bleach/Triton X-100 solution too long. This solution will eventually kill the embryo. I think one hour is pushing things but probably still safe.

^{^^} If the seed are fresh, refrigerate (to vernalize) either the seed after the last washing step or the plates for 3-4d.

These are recombinant Agro cells; they must be killed either by autoclaving or by mixing with bleach.

References:

General Information on Arabidopsis and Protocols:

<http://www.arabidopsis.org/>

Follow the appropriate links.

Vacuum infiltration:

Bechtold, N., Ellis, J., and G. Pelletier (1993) *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C.R. Acad Sci. Paris, Life Sciences 316: 1194-1199.

Dipping/Dunking:

Bent, A.F. and S.J. Clough. "Agrobacterium germ-line transformation: Transformation of *Arabidopsis* without tissue culture." In: Plant Molecular Biology Manual (Second Edition), S.B. Gelvin and R.A. Schilperoort, eds. (Kluwer Academic Pub., Dordrecht, The Netherlands).

Desfeux, C., Clough, S. J., and Bent, A. F. Female reproductive tissue are the primary target of Agrobacterium-mediated transformation by the Arabidopsis floral-dip method. Plant Physiol. 123: 895-904.

GV3101:

Koncz, Csaba, and Jeff Schell (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector. Mol. Gen Genet. 204:383-396.