APPENDIX 1: Complete Hairy Root Protocol

Ron et al: Hairy root transformation: a cell type-specific tool in tomato

Section I: Electroporation Protocol
Section II: Hairy Root Transformation
Section III: Media recipes

I: *Agrobacterium rhizogenes* transformation by electroporation

Adapted from: Wen-jun, S. and Forde, B. **Efficient transformation of *Agrobacterium* spp. by high voltage electroporation.** *Nucleic Acids Research* 1989, Vol. 17, Num 20

Preparation of competent cells:

1. Grow cells in 500ml of MG/L broth at 28°C for 24-30 hours. Final OD_{600} 0.5-0.7. (Culture can be diluted to this range as long as undiluted density is less than 3.0)
2. Cool cells on ice for 15 min.
3. Spin at 5000 rpm for 10 min.
4. Wash successfully in 1.0x, 0.5x, 0.02x, and 0.02x the culture volume with 10% sterile glycerol.
5. Resuspend in 0.01x the culture volume with 10% sterile glycerol.
6. Aliquot 50µl to eppendorf tubes and freeze in liquid nitrogen. Store at –80°C.

Transformation:

1. Thaw aliquot(s) on ice.
2. One µl of plasmid DNA is mixed with the cell suspension.
3. Transfer 40µl of cells to a pre-cooled electroporation cuvette.
4. Parameters for the pulse are:
    a. (Field strength of 12.5 kv/cm)
    b. Capacitance of 25 µF
    c. Resistors of 200 ohms in parallel with the sample
    d. (Time constants of 4 msec)
    e. Set voltage to 1.80 kV for 0.1 cm cuvettes
       Set voltage to 2.50 kV for 0.2 cm cuvettes
5. Cells are immediately transferred to 1ml of MG/L broth and shaken at 28°C for 3hrs.
6. Whole aliquot is plated on MG/L with agar containing appropriate antibiotics (cells are spun down 30 s at 8K rpm, decant liquid, and resuspended pellet in remaining liquid ,~ 100 µl, for plating) and incubated for 3-5 days at 28°C.

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II: Hairy Root Transformation for Tomato

Adapted from: Harvey J, Lincoln J, Gilchrist D: **Programmed cell death suppression in transformed plant tissue by tomato cDNAs identified from an *Agrobacterium rhizogenes*-based functional screen.** *Molecular Genetics and Genomics* 2008, **279:**509-521.

NOTE: For any of these steps, you should be working inside a Laminar Flow Hood to keep a sterile environment.

Seed sterilization and germination (7-10 days)

Materials:

- 2.0ml eppendorf tubes
- 70% EtOH
- 50% bleach
- sterile dH_2O
- 1X MS in magenta boxes
- forceps[1]
- laminar flow hood
- growth chamber (22-25^{o}C, 16:8 photoperiod)

Place 30 seeds in an eppendorf tube. Soak seeds in 1ml 70% EtOH for 5 min then soak in 1ml of 50% commercial bleach for 15 min inverting the tubes every 5 min. Rinse 3X with sterile dH_20. Plate seeds ~1cm apart on 1X MS media in magenta box. Incubate under lights (16:8 photoperiods) for 7-10 days (cotyledons are ready for transformation when they have expanded and the first true leaves have just emerged).

Preparation of *A. rhizogenes* cells (3-5 days)

Materials:

- MG/L broth
- Antibiotic(s)
- 50ml Falcon tubes or large 20x150mm culture tubes
- 28^{o}C shaker

1. **If using newly grown cells from electroporation plates…**
   Scape cells from the plate and grow overnight in 10ml MG/L + suitable antibiotic. Shake in 28^{o}C shaker.
2. **If using a freezer stock…**
   Take a 100μl aliquot to start a 10ml overnight culture in MG/L + antibiotic.

[1] We recommend using FST #11080-02, Miltex #6-184 or similar.

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3. If using cells from an old plate…

Restreak cells onto new MG/L + antibiotic plate to get fresh growth. Incubate in 28°C incubator for 3 days. Pick a colony from this fresh plate to start a 10ml overnight culture in MG/L + antibiotic.

Transformation and Co-cultivation (3 days)

Materials:

- 80% glycerol
- cryotubes
- spectrophotometer cuvettes
- MS liquid
- 100 x 15 mm sterile, disposable petri plates
- forceps
- scalpels
- No. 10 scalpel blades
- serological pipettes (5ml, 10ml, 25ml)
- pipette bulb
- sterile Whatman filter paper (90mm)
- MS plates (no antibiotics)
- MS + cefotaxime + antibiotic plates
- Tomato seedlings
- *A. rhizogenes* overnight cultures
- Parafilm or Micropore surgical tape

Prepare *A. rhizogenes* for infection[2]

- Before anything else, **make a freezer stock**. Take a 500 μl aliquot and place it in a 1.5ml screw top tube. Add 500 μl of 80% glycerol and mix thoroughly. Freeze the tubes in liquid nitrogen before storing in –80°C freezer.
- The optical density is checked using a spectrophotometer at 600nm fixed wavelength. The optimal range is an OD_{600} of 0.2 – 0.4.
- Dilute your *A. rhizogenes* with liquid MS so that you have at least 10ml (more if you have a larger number of explants) of the diluted *A. rhizogenes* culture for the infection process. You want to make sure you have enough bacterial solution to thoroughly submerge your explants.

[2] Note: For maximum efficiency of your transformation, your agrobacterium culture should be growing at exponential phase at the time of infection.

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Cut explants[3]

In a 100 x 15 mm sterile, disposable petri plate, add 20ml MS liquid. Hold cotyledon pair with forceps, and cut first the base off, and then cut the top off (two cut edges).
Place cut explants immediately into the MS liquid (the latter cut can be made in MS). Make sure to be gentle with the forceps so that you don't cause excessive wounding to the tissue.

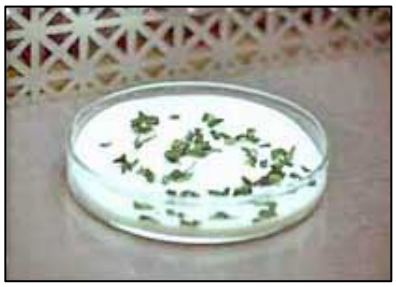
You will need 15-20 plants (30-40 cotyledons) per culture.

Infect explants

Remove the MS liquid from the plate of explants. Add your diluted *A. rhizogenes* and soak for 20 min.

Transfer explants onto co-culture plates

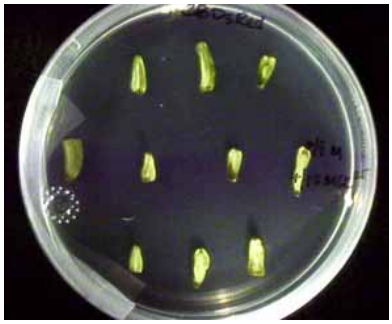
Afterwards, remove the liquid and blot explants dry on sterile Whatman filter papers. Transfer onto solid MS plates (3% sucrose, no antibiotics), twenty explants per plate, bottom side up.



Co-cultivate for 3 days at 22-25^{o}C, covered from lights.

Selection and Root Induction (2-4 weeks)

Ten explants are plated bottom-side up on MS + 3% sucrose + 200mg/L cefotaxime + antibiotic plates. Using the back of the forceps, gently work the explants onto the agar so as to break the surface tension (be careful not to tear your explants in the process). Try to lay the explant as flat on the medium as possible, emphasizing contact of the wounded end onto the medium.



[33] The optimal explant material may be different in different species. For example, *S. pennellii*, Arabidopsis, and *L. hyssopifolium* transformations were successful in young true leaves instead of cotyledons.

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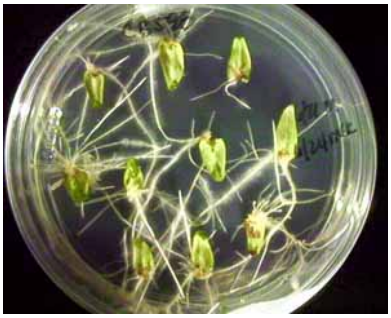
Incubate at 22-25°C until roots grow.

Subclone the hairy roots[4]



Once roots are at least 1.0cm long, roots can be excised from the explant for amplification. Transfer to individual MS + 3% sucrose + 200mg/L cefotaxime + antibiotic plates.

(After 2 rounds of subculture, the cefotaxime and antibiotic may be dropped out of the media and the MS salt concentration dropped to 1/2.)

[4] Note: Theoretically, each root is an independent transformation event. Subclone roots into individual plates and keep lines independent. Routine subcloning can keep lines alive indefinitely, but some markers (kan, gfp) have been shown to get silenced over time.

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III: Media

MG/L [5]

Yield: 1L
40 plates (100 x 15 mm)
10 bottles (100ml bottles)

5g	mannitol
1g	L-glutamic acid
0.25g	K_2HPO_4
0.10g	NaCl
0.10g	$MgSO_4 \cdot 7H2O$
1mg	biotin
5g	tryptone
2.50g	yeast extract
15g	bactoagar

1. Place all ingredients (slowly) in 1L beaker with spin bar.
2. Add ~900ml MQ H_2O to dissolve ingredients completely.
3. Bring volume up to 1L.
4. Adjust pH to 7.0 with 1M NaOH.
5. Dispense 100ml into each bottle

   Or

   Add bactoagar to a 2L flask. Add liquid medium.

6. Autoclave for 30 min @ 121^{o}C
7. If making plates, pour ~25ml into each 100 x 15 mm plate.

[5] NOTE: This media can now be ordered from Caisson (Caisson MQP04-1LT)

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MS

Unknown
Field Code

Yield: 1L (40 plates)

4.43 g/L	MS with vitamins (Murashige & Skoog with micronutrients, macronutrients, and vitamins)
0.5g/L	MES
30g/L	sucrose
10g/L	agar (Difco)

1. Fill a 1L beaker with ~ 900mL MQ H_20
2. Add MS, MES and sucrose in a beaker
3. Adjust pH to 5.8 with 1M KOH
4. Bring volume up to 1L
5. Add agar to 2L flask
6. Add liquid medium to flask
7. Autoclave for 20 min. @ 121^{o}C
8. Allow to cool to 65^{o}C before adding antibiotics if needed. (Plant selection as per your construct, 200mg/L cefotaxime (Caisson C032-10GM) for killing the *A. rhizogenes*)
9. Pour plates.