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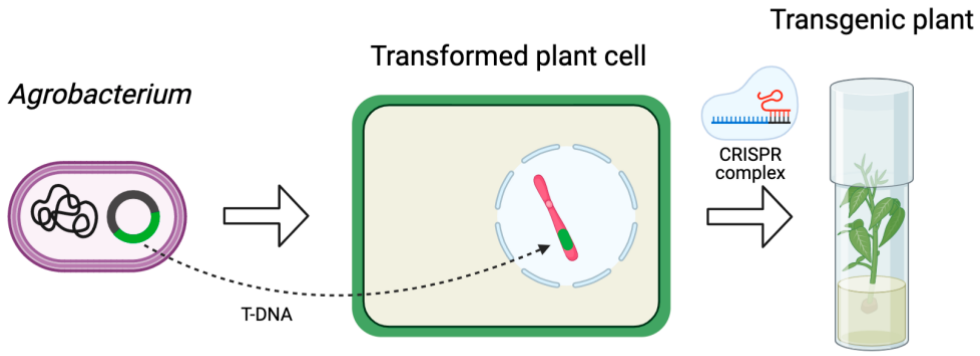
Agrobacterium-mediated *Tragopogon* Transformation



Protocol

Agrobacterium Culture

- Streak the *Agrobacterium tumefaciens* onto a LB + Kan + Rif plate. Grow at 28 °C for 2 days.
    - pCambia 1300 vector has Kan- and Hyg-resistance genes; *Agrobacterium* stain EHA105 has Rif-resistance gene
    - To knock out the *PDS* gene, bacteria stock 093 (1300-AtCas9-GmUbi-GFP-AtU6-1-gTraPDS2-AtU6-29-gTraPDS1) will be used
- *Agrobacterium* colonies are isolated and inoculated into 20 ml liquid Ty medium with AS and appropriate antibiotics (in an autoclaved flask). Shake (dark condition) at 100 rpm, 28 °C for ~24 h.
    - Avoid large lumps of bacteria; brush the bacteria against the inside surface of the flask
    - The streaked *Agrobacterium* plate can be used for 1-2 weeks, but no longer than that (e.g., 1 month)

Infection Medium Preparation

- Resuspend *Agrobacterium* pellet in liquid AAM infection medium (containing AS).
    - Add 1 ml *Agrobacterium* culture to a 1.5 ml microcentrifuge tube
    - Centrifuge at 2,000 rpm for 10 min
    - Remove the Ty medium, and re-suspend the *Agrobacterium* pellet with 1 ml AAM medium

    - Adjust the *Agrobacterium* cell density to OD_{600} = 0.1 (blank: AAM + AS without *Agrobacterium*)

Explant Preparation

- Place wounded leaf segments (i.e., explants) on M5 medium in dark for 1-2 days at room temperature.
    - Wounded-side down; shallow wounds act as sites for *Agrobacterium* infection

Explant Infection

- Immerse explants in a Petri dish containing *Agrobacterium* suspension culture for 30 min at room temperature.
    - Transfer ~20 ml AAM infection medium (containing *Agrobacterium*) into 100 ml X 15 ml Petri dish
    - Swirl the *Agrobacterium* suspension culture (with the explants) every 10 min
    - Blot explants completely on a sterile filter paper; this step usually takes ~20 min
- Transfer infected explants to Cocultivation medium covered with autoclaved filter paper (preventing rampant growth of *Agrobacterium*, and therefore, avoid tissue necrosis/browning) and put the plates in dark for 2-3 days at room temperature.

Callus induction, shooting and rooting are carried out at 25 °C with 14 h photoperiod in an incubator

Callus induction

- Transfer the explants to Selective M5 medium for callus induction; it usually takes 3 weeks.

Shooting

- Transfer the callus to Selective COCO-1 medium for 2 weeks for shoot induction.
- Transfer the callus to Selective WPM medium for shoot formation; shoots will start forming after 2 weeks.

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Rooting

- Excise regenerated shoots from the callus and transfer the shoots to COCO-R medium.
- Transfer the rooted shoots to soil.
    - Transfer rooted shoots to soil inside a tray with a lid on. Put the tray in the growth chamber for 2 weeks. Repot the plants and put the pots inside the greenhouse

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Timeline

Day	*Agrobacterium* preparation	Explant preparation
1	Streak the stock on plate in the morning; take ~2 days to grow	
2		Place leaf segments on M5 medium in dark for 1-2 days at RT
3	Pick colonies and shake in Ty medium for 24 hours	
4	Prepare the AAM with *Agrobacterium* and transform the explants; cocultivation of explants and *Agrobacterium* in dark for 2-3 days at RT	
5		
6	Transfer explants to selective M5 medium	
27	Transfer callus to selective COCO-1 medium	
41	Transfer callus to selective WPM medium for shooting	
62	Transfer shoots to COCO-R medium for rooting	
83	Move regenerated seedlings to soil	

Abbreviations

Abbreviation	Description
AS	Acetosyringone (乙酰丁香酮): a signal (phenolic compound) exuded by wounded eudicot plant cells and involved in plant-pathogen recognition; AS is recognized by a receptor encoded by the *virA* gene, which facilitates the processing and transfer of T-DNA from *Agrobacterium* to the nuclear genome of the plant host; AS, a *vir* gene inducer, allows higher transformation efficiency
Cefo	Cefotaxime (头孢噻肟): often used in transformation of plant species for the elimination of *Agrobacterium tumefaciens*
Hyg	Hygromycin B (潮霉素 B): an antibiotic produced by *Streptomyces hygroscopicus*; active against both prokaryotic and eukaryotic cells by inhibiting polypeptide synthesis
Kan	Kanamycin (卡纳霉素): works by interfering with protein synthesis – it binds to the 30S subunit of the bacterial ribosome
LB	Lysogeny broth
Rif	Rifampicin (利福平): an antibiotic works by decreasing the production of RNA by bacteria
Tim	Timentin (特美汀): used most commonly in the regeneration medium for elimination of the *Agrobacterium* post-transformation

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Medium

Ty medium (liquid)

	For 1 L	For 200 ml
Tryptone	5 g	1 g
Yeast extract	3 g	0.6 g
pH 5.5 (use 1 M HCl) Before use add: • Rif (1,000 X; 50 mg/ml); final [c] = 50 mg/L • Kan (1,000 X; 50 mg/ml); final [c] = 50 mg/L • AS (1,000 X; 100 mM); final [c] = 0.1 mM		

AAM infection medium (liquid)

	For 1 L	For 500 ml
AA macro (10 X)	100 ml	50 ml
AA micro (1,000 X)	1 ml	0.5 ml
AA amino acid (100 X)	10 ml	5 ml
MS vitamins (100 X)	10 ml	5 ml
Fe_2EDTA (100 X)	10 ml	5 ml
Casamino acids	0.5 g	0.25 g
Sucrose	68.5 g	34.25 g
Glucose (0.5 g/ml)	72 ml	36 ml
pH: • 5.33 before autoclaving (using KOH); add filter-sterilized glucose; the final pH 5.15 (should be around 5.2) Before use add: • AS (1,000 X; 100 mM); final [c] = 0.1 mM		

M5 medium

	For 1 L	For 400 ml
MS salt	4.44 g	1.776 g
Sucrose	30 g	12 g
BAP (1 mg/ml)	1 mg	0.4 mg
NAA (1 mg/ml)	0.5 mg	0.2 mg
Gelrite	5 g	2 g
pH: set to 5.8		

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Cocultivation medium

	For 1 L	For 400 ml
MS salt	4.44 g	1.776 g
Sucrose	30 g	12 g
BAP (1 mg/ml)	1 mg	0.4 mg
NAA (1 mg/ml)	0.5 mg	0.2 mg
Gelrite	5 g	2 g
pH: set to 5.35; will reach 5.2 (the desired pH) after autoclaving Add AS (1,000 X; 100 mM) after autoclaving; final [c] = 0.1 mM		

Selective M5 medium

	For 1 L	For 400 ml
MS salt	4.44 g	1.776 g
Sucrose	30 g	12 g
BAP (1 mg/ml)	1 mg	0.4 mg
NAA (1 mg/ml)	0.5 mg	0.2 mg
Gelrite	5 g	2 g

pH: set to 5.8

After autoclaving, add:

- Tim (500 X; 200 mg/ml); final [c] = 300 mg/L
- Hyg (50 mg/ml stock)
    - 15 mg/L Hyg for *T. porrifolius*; add 300 µl to 1 L medium

Selective COCO-1 medium

	For 1 L	For 400 ml
Woody Plant Medium	2.41 g	0.964 g
Sucrose	30 g	12 g
BAP (1 mg/ml)	2.5 mg	1 mg
Agargellan	8.5 g	3.4 g
Volume	950 ml (+ 50 ml coco)	380 ml (+ 20 ml coco)

pH: set to 5.7

After autoclaving, add:

- filter-sterilized coconut water (final 5%)
- Tim (500 X; 200 mg/ml); final [c] = 300 mg/L
- Hyg (50 mg/ml stock)
    - 15 mg/L Hyg for *T. porrifolius*; add 300 µl to 1 L medium

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Selective WPM medium

	For 1 L	For 400 ml
Woody Plant Medium	2.41 g	0.964 g
Sucrose	30 g	12 g
Agargellan	5 g	2 g
pH: set to 5.7 After autoclaving, add: • Tim (500 X; 200 mg/ml); final [c] = 300 mg/L • Hyg (50 mg/ml stock) ○ 15 mg/L Hyg for *T. porrifolius*; add 300 µl to 1 L medium		

COCO-R medium

	For 1 L	For 400 ml
Woody Plant Medium	2.41 g	0.964 g
Sucrose	30 g	12 g
IBA (1 mg/ml)	1.5 mg	0.6 mg
Agargellan	8.5 g	3.4 g
pH: set to 5.7		

AA Macro 10X solution

	For 1 L	For 500 ml
KCl	29.5 g	14.75 g
$MgSO_4 \cdot 7H_2O$	2.5 g	1.25 g
$CaCl_2 \cdot 2H_2O$ (or $CaCl_2$)	1.5 g (or 1.13 g)	0.75 g (or 0.565)
$NaH_2PO_4 \cdot H_2O$	1.5 g	0.75 g
Store at 4 °C		

AA Micro 1000X solution

	For 1 L	For 500 ml
$MnSO_4 \cdot 4H_2O$	10 g	5 g
H_3BO_3	3 g	1.5 g
$ZnSO_4 \cdot 7H_2O$	2 g	1 g
KI	750 mg	375 mg
$Na_2MoO_4 \cdot 2H_2O$	250 mg	125 mg
$CuSO_4 \cdot 5H_2O$ (or $CuSO_4$)	25 mg (or 15.98 mg)	12.5 mg (or 7.99 mg)
$CoCl_2 \cdot 6H_2O$	25 mg	12.5 mg
Store at 4 °C		

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AA Amino Acids 100X solution

	For 1 L	For 500 ml
L-glutamine	87.6 g	43.8 g
L-aspartic acid	26.6 g	13.3 g
L-arginine	17.4 g	8.7 g
Glycine	750 mg	375 mg
Store at 4 °C; heat to dissolve		

MS Vitamins 100X solution

	For 1 L	For 500 ml
Myo-inositol	10 g	5 g
Nicotinic acid	50 mg	25 mg
Pyridoxine-HCl	50 mg	25 mg
Thiamine-HCl (10 mg/ml)	1000 μL	500 μL
Glycine (100 mg/ml)	20 μL	10 μL
Store at 4 °C		

Fe_2EDTA (Iron Chelate) 100 X Solution

- Dissolve 2.78 g of $FeSO_4 \cdot 7H_2O$ in 350 ml H_2O
- Dissolve 3.726 g of disodium EDTA (MW: 372.24) in 350 ml H_2O. Heating is required
- Combine the solutions and bring up to the final volume of 1 L (clear yellow solution)
- Storage: amber bottle (or covered by foil) to protect it from exposure to light; store at 4 °C

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Additional Links

Acetosyringone's function in plant transformation
https://bmcbiotechnol.biomedcentral.com/articles/10.1186/s12896-018-0459-5

pCAMBIA 1300 Vector
https://www.snapgene.com/resources/plasmid-files/?set=plant_vectors&plasmid=pCAMBIA1300

T-DNA Binary Vectors
http://www.plantphysiol.org/content/146/2/325