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A protocol for *Agrobacterium* mediated transformation of *Mimulus guttatus* from leaf petiole explants

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1 *Works for me* dx.doi.org/10.17504/protocols.io.8vghw3w

Mimulus


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ABSTRACT

This is a protocol for Agrobacterium mediated transformation of Mimulus guttatus from leaf petiole explants including the following procedures:

1. Surface sterilization of seeds
2. Agrobacterium culture preparation
3. Agrobacterium infection and co-cultivation
4. Callus induction and shoot induction
5. Rooting of shoots

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GUIDELINES

1. Check the contents on herbicide formula to verify the active ingredient (Basta or Phosphinothricin) before use so that you can modify the selection protocol as appropriate.
2. A pilot kill-curve test may be necessary to choose the optimum concentrations of herbicide for each *M. guttatus* population.
3. Antibiotics and hormones need to be filter sterilized and added to medium after autoclaving. For best results, add antibiotics and hormones when the contents cool to 45 °C – 50 °C or lower after autoclaving.
4. Fresh medium should be used for best results. Leave the solid medium overnight at room temperature after pouring into petri-dish to cool and solidify. This will minimize condensation on the lids.
5. This protocol was standardized for herbicide resistance selection.

MATERIALS

NAME	CATALOG #	VENDOR
Timentin	T-104-2	Gold Biotechnology
Cefotaxime	C-104-25	Gold Biotechnology
Phosphinothricin	P-165-250	Gold Biotechnology
4-CPPU	C279	Phytotech Labs
Meta-toplin	T841	Phytotech Labs
Murashige & Skoog basal salts with Gamborg Vitamins	M404	Phytotech Labs
Acetosyringone	2478-38-8	Sigma Aldrich

MATERIALS TEXT

Growth medium composition

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Murashige & Skoog basal salt medium (MS salts)	4 g/L
Sucrose	20 g/L
Calcium gluconate	1.3 g/L
MES 2-(NMorpholino) ethanesulfonic acid hydrate)	0.25 g/L
Adjust pH to 5.6 using KOH before adding gelrite	
Gelrite	0.25 %

Agrobacterium virulence induction medium

Murashige & Skoog basal salt medium (MS salts)	2 g/L
Sucrose	10 g/L
MES 2-(NMorpholino) ethanesulfonic acid hydrate)	0.5 g/L
Adjust pH to 5.5 using KOH and autoclave	
Acetosyringone (Dissolved in DMSO and add before use)	200 µM

Co-cultivation medium

Murashige & Skoog basal salt medium (MS salts)	4 g/L
Sucrose	20 g/L
Calcium gluconate	1.3 g/L
MES 2-(NMorpholino) ethanesulfonic acid hydrate)	0.25 g/L
Adjust pH to 5.6 using KOH before adding gelrite	
Gelrite	0.25 %
CPPU*	1 mg/L
Acetosyringone*	100 µM
* Filter sterilize before adding to the medium	

Callus induction medium

Murashige & Skoog basal salt medium (MS salts)	4 g/L
Sucrose	20 g/L
Calcium gluconate	1.3 g/L
MES 2-(N-Morpholino)ethanesulfonic acid hydrate	0.25 g/L
Adjust pH to 5.6 using KOH before adding gelrite	
Gelrite	0.25 %
CPPU*	1 mg/L
Timentin (Ticarcillin-cluvanate)*	200 mg/L
Ceftaxime*	50 mg/L
Phosphinothrocin*	6 mg/L
* Filter sterilize before adding to the medium	

Shoot induction medium

Murashige & Skoog basal salt medium (MS salts)	4 g/L
Sucrose	20 g/L
Calcium gluconate	1.3 g/L
MES 2-(NMorpholino) ethanesulfonic acid hydrate)	0.25 g/L
Adjust pH to 5.6 using KOH before adding gelrite	

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Gelrite	0.25 %
Meta-toplin*	0.1 mg/L
Timentin (Ticarcillin-cluvanate)	200 mg/L
Ceftaxime	50 mg/L
Phosphinothrocin	6 mg/L
* Filter sterilize before adding to the medium	

Root induction medium

Murashige & Skoog basal salt medium	2 g/L
Sucrose	10 g/L
Calcium gluconate	1.3 g/L
MES 2-(N-Morpholino)ethanesulfonic acid hydrate	0.25 g/L
Adjust pH to 5.6 using KOH before adding gelrite	
Gelrite	0.25 %
Napthelene AceticAcid (NAA)*	0.1 mg/L
Timentin (Ticarcillin-cluvanate)*	200 mg/L
Ceftaxime*	50 mg/L
Phosphinothrocin*	6 mg/L
* Filter sterilize before adding to the medium	

SAFETY WARNINGS

For safety information and warnings, please refer to the SDS (Safety Data Sheet).

Establishing invitro cultures for explant source (2.5-3 months): **Surface sterilization of seeds**

1 Collect mature seeds from plants grown in greenhouse or growth chamber to reduce contamination in tissue culture. Seeds collected from natural sites/fields may increase the risks of endogenous contaminations in cultures.

1.1 Place seeds in a **1.5 ml** sterilized eppendorf tube(s) then fill the tube(s) with surface sterilization solution (**5 % laundry bleach** and a **drop** of hand-soap). Shake the tube(s) vigorously for **00:08:00** - **00:10:00** .

1.2 Move the tube(s) to laminar flow hood. Discard the sterilizing solution and add sterile water to rinse the seeds by shaking for at least **00:00:30** . Repeat rinse at least 5 — 6 times to completely wash-off bleach and soap from seeds. After the last rinse add at least **1 ml sterilized water** to later plate the seeds.

1.3 Store the seeds at **4 °C** for at least **2 — 3 weeks** to cold stratify seeds.

1.4 Bring back tubes to laminar flow hood. Transfer the sterilized seeds onto growth medium. Spread the seeds uniformly. Grow around 5 — 6 seeds per jar (**200 ml**) or polypropylene Deli jars (**quart-size**).

1.5 Incubate jars cultures at **20 °C** — **21 °C** under cool fluorescent lamps.

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1.6 When the plants produce proliferating axillary shoots, harvest shoots with roots and subculture onto new jar. Adjust frequency of sub-culturing to avoid senescence.

Agrobacterium mediated transformation of explants: **Agrobacterium culture preparation (4 days)**

2 Streak EHA105 *Agrobacterium* strain harboring binary plasmid on LB or YEP agar plate with rifampicin ([M] **40 µg/mL**) and appropriate antibiotic resistance cassette on the binary plasmid. Incubate plates at **28 °C** for **two days**.

3 Inoculate a single colony of *Agrobacterium* into **10 ml** YEP liquid medium with rifampicin ([M] **40 µg/mL**) and an appropriate antibiotic (Kanamycin at [M] **50 µg/mL** or Spectinomycin at [M] **100 µg/mL** or Amplicillin at [M] **100 µg/mL**). Shake the culture at **28 °C** for **36:00:00** — **48:00:00** at **200 rpm** .

4 Centrifuge the cultures to pellet *Agrobacterium* at **4000 rpm** . Remove the medium and resuspend in **5 ml** virulence induction medium for at least **03:30:00** — **04:00:00** with gentle shaking (**50 rpm** — **80 rpm**) in dark at **Room temperature** . Adjusting to pH **5.5** is critical. Glucose can be substituted for sucrose, because glucose enhances *virulence* induction. However, glucose may enhance senescence of older leaves; if leaves are old, then use sucrose.

5 Adjust agrobacterium OD to **0.2** using liquid half-strength MS medium to achieve a very light color suspension in half-strength MS medium. Higher OD is **not desirable**.

Agrobacterium mediated transformation of explants: **Agrobacterium infection and co-cultivation (3-4 days)**

6 Bring plant culture jar to laminar flow hood. Pull shoots onto a sterile petri dish. Using a sterile scalpel cut a leaf explant off the plant and dip the cut petiole in agrobacterium and plate on co-cultivation medium. Perform infections in small batches to avoid desiccation of petiole explants.

7 Incubate plates in dark or low intensity light at **Room temperature** for **2 — 3 days**. Avoid overgrowth of agro on plates. Overgrowth of agrobacterium may kill explants, thus the use dilute agro culture to dip petioles.

8 On **day 3**, wash the explants in sterile timentin ([M] **100 mg/L**) + cefatoxime ([M] **50 mg/L**) in hood and transfer the explants to callus induction medium with phosphinothrocin. This is appropriate selection medium for plasmids with *Blp* resistance cassette. Incubate plates at **21 °C** under cool fluorescent lamps.

Agrobacterium mediated transformation of explants: **Callus induction and shoot induction (4-5 months)**

9 Subculture explants after **21 — 24 days**. Retain only small part of callus and culture on callus induction medium to allow callus growth. It is critical to grow long enough on CPPU with routine subculture (**21 — 24-days** interval) for sufficient growth and maturation of callus. Premature transfer to Meta-toplin may kill explants. Increasing sucrose to [M] **2.5 %** at later stages may help in getting larger callus. At least 3 — 4 subcultures are necessary.

10 When the compact callus turns friable with signatures of differentiation, pick the healthy callus and plate on shoot induction medium to regenerate shoots from callus.

Agrobacterium mediated transformation of explants: **Rooting of shoots (3 weeks)**

11 When the shoots emerge and reach **0.5cm — 1cm** tall, separate individual shoots and plate them on rooting medium.

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12 For good rooting and hardening, subculture on half-strength rooting medium.

13 When roots proliferate, move the shoots to potting medium in greenhouse and leave the pots on a mist bench to harden for at least **two weeks**.

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