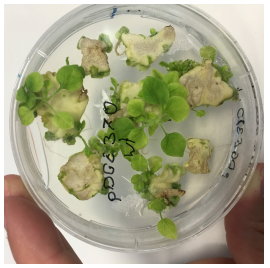
Jul 30, 2019

Stable transformation of Nicotiana benthamiana



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Protocol status: Working
We use this protocol and it's working

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Abstract

Detailed protocol with pictures for the transformation of *Nicotiana benthamiana*. The same protocol will also work for transformation of *Nicotiana tabaccum*, but regeneration of transformed plants will take more time.

Attachments

Nicotiana benthamian...

2.1MB

Guidelines

Plant tissue culture has to be carried out under sterile conditions.

Materials

Required Material

YEB medium:
5 g/l beef extract, 1 g/l yeast extract, 5 g/l peptone, 5 g/l sucrose, 0.5 g/l $MgCl_2$ [add 1.5 % agar for plate preparation)

Antibiotics:
Rifampicin: stock 100 mg/l in DMSO or DMF; 1000 x for bacterial selection
Gentamycin: stock 15 mg/l in H_2O; 1000 x for bacterial selection
Kanamycin: stock 50 mg/l in H_2O; 1000 x for bacterial, 500 x for plant selection
Spectinomycin: stock 50 mg/ml in H_2O; 1000 x for bacterial selection
Carbenicillin: stock 100 mg/ml in H_2O; 1000 x for bacterial selection

Induction medium:
YEB medium, 10 mM MES pH 5.7 – 5.8 [KOH], 20 µM Acetosyringone, antibiotics

MMA medium:
4.3 g/l MS salts including vitamins (Duchefa M0222), 20 g/l sucrose, 10 mM MES pH 5.7 - 5.8 [KOH]

MS medium:
4.3 g/l MS salts including vitamins (Duchefa M0222), 20 g/l sucrose, 10 mM MES pH 5.7 - 5.8 [KOH],
8 g/l Gelrite (Duchefa G1101). Verify pH, and adjust with KOH if required.

MSII medium:
MS medium, 1 mg/l 6-Benzylaminopurine (BAP), 0,1 mg/l Naphthalene acetic acid (NAA), 100 mg/l Kanamycin, 200 mg/l Cefotaxime (Claforan, Duchefa C0111)

MSIII medium:
MS medium, 100 mg/l Kanamycin, 200 mg/l Cefotaxime (Claforan, Duchefa C0111)

Tobacco plants:
We use 3-5 week-old, greenhouse-grown *Nicotiana benthamiana*plants (growth conditions: 16h light period, 60 % relative humidity at 24/20 °C (day/night), 130-150 µE m^{-2} s^{-1} light intensity).

Preparation of Agrobacterium suspension

1

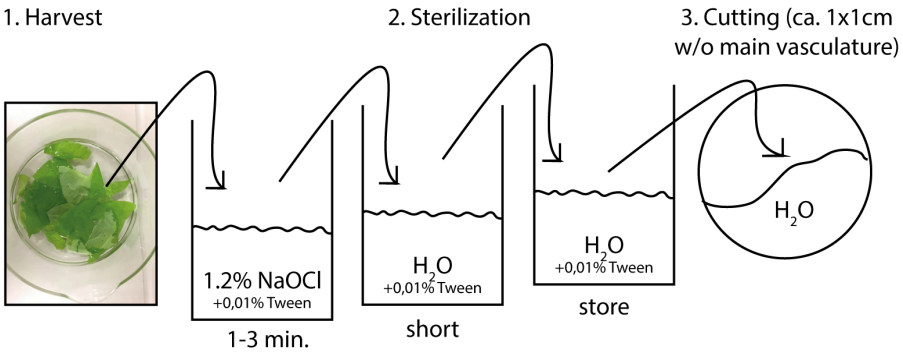
- Streak the *Agrobacterium tumefaciens* (*A. tumefaciens;* GV3101 pMP90) strain carrying the transformation construct on YEB plates containing required antibiotics (Rifampicin, Gentamycin + construct-specific antibiotic).
- Two days prior to transformation: Inoculate 5 ml YEB liquid culture (with antibiotics); grow for 24 h with shaking at 30 °C.
- One day prior to transformation: Inoculate 100 ml culture with 1 ml of the pre-culture in induction medium (containing antibiotics), shake over night at 30 °C.
- Day of transformation: Pellet bacteria by centrifugation (40 ml in Falcon tubes, 20 min, 4000 x g. Resuspend bacteria in MMA medium (without antibiotics) to an OD_{600}=0.8 (50 ml).

Preparation of *N. benthamiana* leaf tissue for transformation

2

Try to injure plant tissue as little as possible! All steps following the surface-sterilization have to be carried out under sterile conditions.

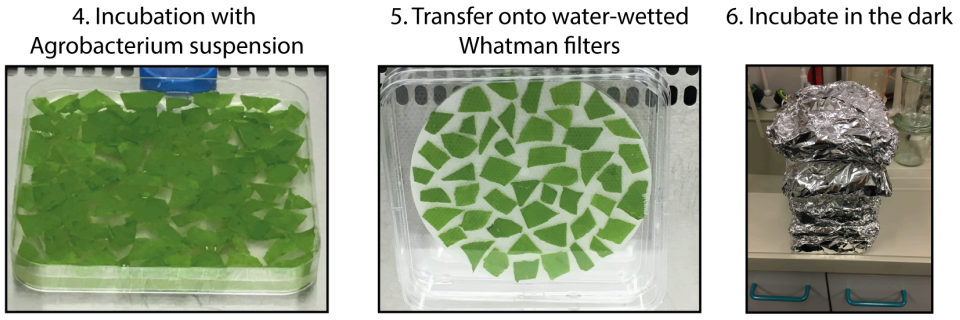
- Harvest the 2-3 youngest, but fully expanded leaves of 3-5 weeks-old *Nicotiana benthamiana* plants (3-5 leaves are required for each transformation).
- Cut leaves into pieces, omitting main veins.
- Incubate leaf cuts for 30 sec in 1.2 % NaOCl (+0.01 % Tween).
- Wash twice in H_2O (+0.01 % Tween) to get rid of the bleach.
- Store leaf cuts in H_2O.



Transformation

3

- Further cut surface-sterilized leaves into smaller pieces (ca. 1×1 cm).
- Incubate leaf cuts in *A. tumefaciens* suspension:
  - Pour *A. tumefaciens* suspension in a square petri dish.
  - Place leaf cuts on the surface of the suspension. When the surface is covered, you will have more than enough leaf cuts for each transformation.
  - Incubate at least 30 min.
- Prepare a fresh square petri dish with a sterile piece of water-wetted Whatman paper.
- Transfer leaf cuts onto Whatman paper.
- Seal dishes with Leukopor tape and incubate for 2 days in the dark (24 °C, wrap in aluminium foil to protect from light).



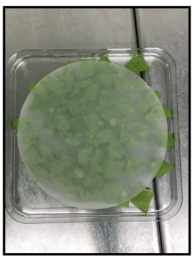
Selection and shoot induction

4

- Prepare square dish with 50 ml H_20 (containing Kanamycin (100 mg/l) and Cefotaxime (250 mg/l)) for washing of the leaf cuts.
- Pick up the Whatman paper with the leaf cuts using sterile forceps and place it upside-down onto the surface of the wash solution to release leaf cuts from the filter into the wash solution. Gently shake the petri dish, and incubate for > 3 min.
- Dry leaf cuts by gently swiping on a fresh, sterile Whatman filter.

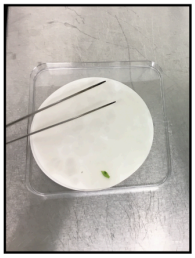
- Place dried leaf cuts on shoot induction medium (MS-II). Use standard round petri dishes to minimize the risk of contamination. Place 8-10 leaf cuts on each plate.
- Seal plates with Leukopor tape. Incubate in a light cabinet until shoots occur (5-6 weeks under our conditions: 23°C, 24h light, intensity 105 – 125 µE $m^{-2}s^{-1}$).
- It may be necessary to transfer leaf cuts/ developing calli to new MS-II plates during the incubation if media start drying out.

7. Wash & sterilize leaf cuts



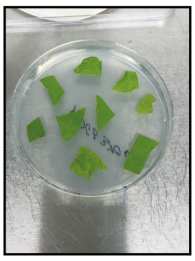
- turn Whatman filter upside down
- in square plastic dish with H_2O + antibiotics + Claforan
- incubate pieces for at least 3 min.

8. Dry on Whatman filter



gently swipe to dry leaf cuts

9. Place pieces on MSII medium



- ca. 8-10 pieces per plate
- MSII medium + antibiotics + Claforan
- close plates with Leukopor
- incubate for 5-8 weeks

Root induction and transfer to soil

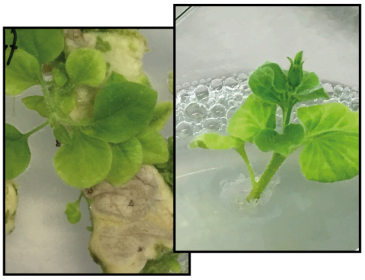
5

Shoots start developing from calli on MS-II plates and need to be transferred to MS-III plates to induce rooting. Since calli do not develop homogeneously, shoots may be cut and transferred at different time points.

- Cut well-developed shoots with a sterile blade. Try to cut as low as possible, but avoid transferring any callus tissue, as this will prevent further development of the shoot.
- Stick shoots with the cut surface into MS-III medium, and incubate under the same conditions as before for further development of the shoot and rooting.
  Note: Red light will prevent rooting!
- We use 0.5 l Weck glasses for rooting and place max. 3 shoots in each jar.
- Shoots originating from a single callus are, in general, originating from the same transgenic event. Thus, to only sample independent transformants, we only transfer one single shoot per callus to rooting medium.
- Calli can be kept as a back-up on MS-II medium, as shoots will continue to develop.

- Shoots will further develop in MS-III media, and will eventually form roots (2-3 weeks).
- Transfer well-developed shoots to soil. Remove MS medium from shoots/ roots to avoid contamination. We transfer shoots directly into potting soil that had been passed through a sieve. Again, shoot development is not homogeneous, and some shoots will not start rooting in a timely manner. Indeed, even rather small shoots without any roots will, in many cases, develop well once transferred to soil.
- It is important to keep plants under high humidity after transfer to soil. Place respective plants in a tray covered with a lid for ~ 2 weeks. Once plantlets appear sufficiently vigorous, reduce humidity in the course of 2-3 days by first displacing the lid before completely removing it.
- Grow plants as usually to obtain seeds. In our hands (GV3101 pMP90, pVS1 origin for transformation constructs), most plants will contain multiple insertions of the T-DNA.

10. Move shoots w/o callus on MSIII medium



11. Place plants into soil when roots are formed