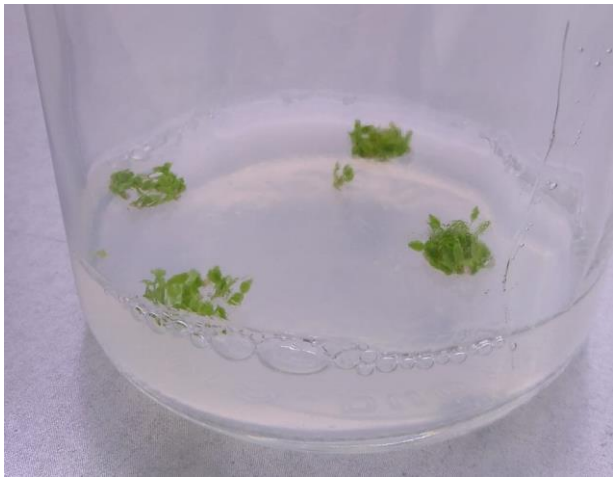
Protocol for tobacco and *N. Benthamiana* stable transformation

1. **Seed sterilisation** (work in sterile bench)**:**
wash the seeds in:
70% ethanol for 3 min (shaking)
1.25% hypochlorite containing some drops of 0.001% Triton W-100 for 8-10 min (shaking)
three times in sterile distilled water (3x 200 ml)
resuspend in 0.1% agarose and distribute on agar plates
keep the plates for 2 days in the fridge (4°C dark)

2. **Preparing the plants**
Nicotiana tabacum L. var. Samsun NN used for transformation should be **4-6 weeks old** and you need 2 plants for a transformation
or *N. Benthamiana* should be **7-8 weeks old** and you need 4 plants for a transformation.
Temperature: 23°C, humidity: 60%, light: 13 h

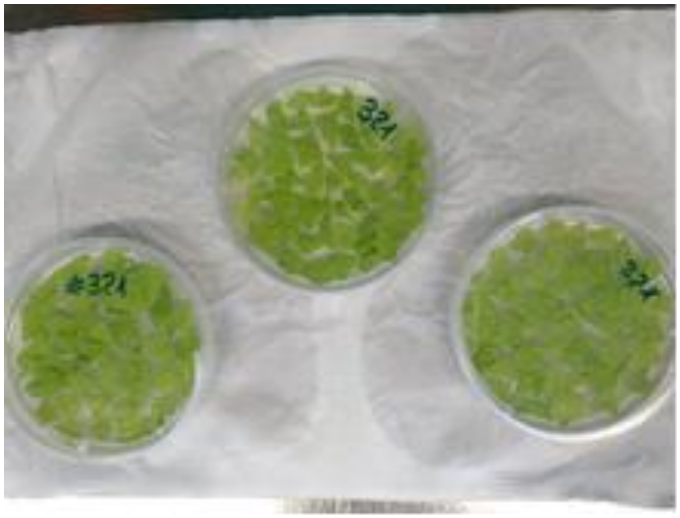


N. Benthamiana 1 week old

N. Benthamiana 7 weeks old

3. **Preparing the *Agrobacterium* suspension**
Few days before the transformation: streak out the recombinant *Agrobacterium* (GV3101, pMP90) on plates with LB-media (with Rif/Gen/$), incubate them for 2 days at 28°C.
Monday: two days before transformation, inoculate 3 ml low-salt **LB medium** (Rif/Gen/$) with one single colony of *Agrobacterium*. Shaking for 24 h at 28°C.
Tuesday: the day before transformation, start a 250 ml LB culture ($) with 2 ml subculture. Shake overnight at 28°C.
($: antibiotic corresponding to your binary plasmid).
Wednesday: centrifuge bacteria culture (5500 rpm 10 min), adjust $OD_{600} \approx 1.0$ and re-suspend the pellet in $MgCl_2$ 10 mM without antibiotics

4. **Infection and co-cultivation of plant leaves with Agrobacterium**:
Put the suspension into sterile Petri dishes (9 cm ∅).
Cut the leaves in pieces in a sterile Petri dish (*N. tabacum* 0.5 x 0.5 cm – *N. Benthamiana* 1 x 1 cm), without the middle vein.
Incubate the explants with the bacteria suspension for 3 min
Put the explants on plates with **MS medium with 2% sucrose**, leaf surface up. Their whole lower surface should be in contact with the medium.
About 100 pieces are enough for 1 transformation (about 3 Petri dishes).
The leaves are sensitive to any kind of injury, burned or too harsh handled tissue will die. Therefore, never squeeze the tissue and let the forceps, after sterilisation, cool down. Try to use also sharp razor blades.
Use green and healthy leaves.
Incubate them for 2 days in the dark at RT
If the *Agrobacteria* are overgrowing the leaves, the plates should be exchanged

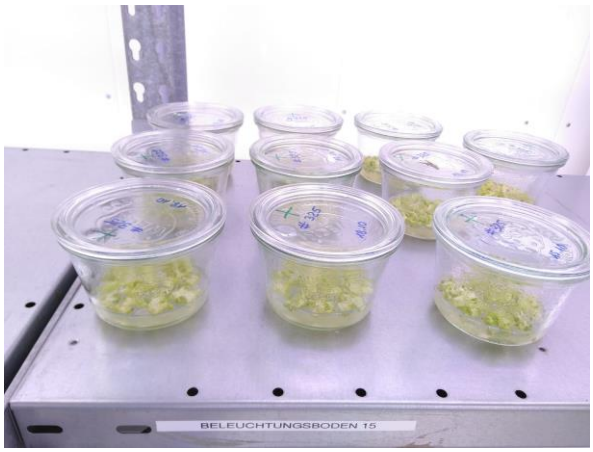


Co-cultivation of pieces of N. Benthamiana on Petri dishes

5. **Selection of transformed tissue** (in 0.25 l glassware[1])
Friday: Transfer leaves to *Selection Medium* (about 6 plates).

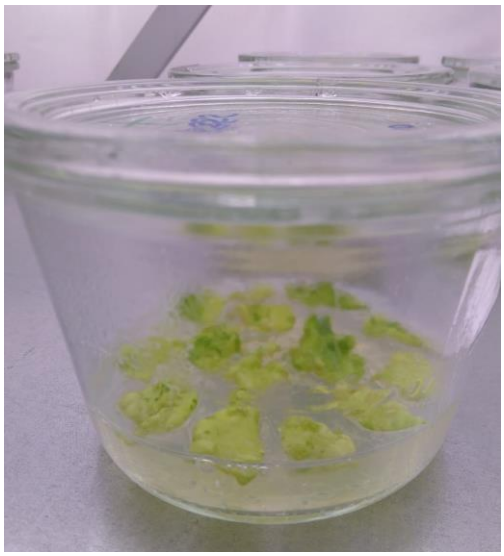
At the beginning, leaves with strong *Agrobacterium* infection should be discarded or washed in 10 mM $MgSO_4$ containing Cefotaxime and incubated separately on plates. When callus is forming, it is worthwhile to cut off the healthy tissue and transfer it to separate glasses.

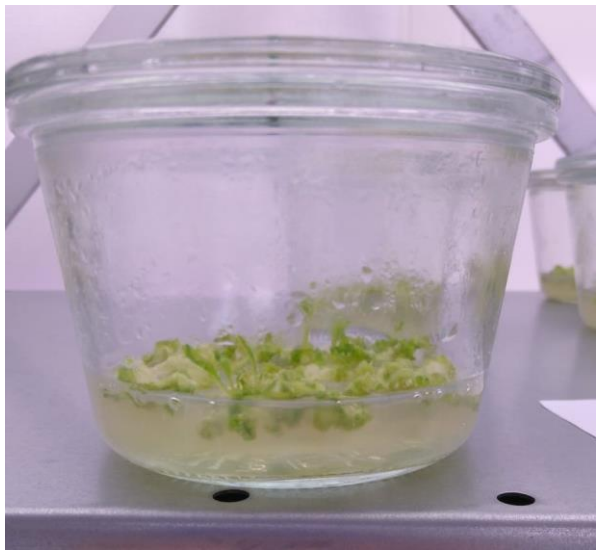
The leaves must be transferred to fresh medium every 10 days.



[1] WECK-Sturzglas ½ oder ¼ Liter (Rundrand 100) - http://www.shop-weck.de/shopindex.htm

6. **Regeneration of transgenic plants**
After a couple of weeks the first shoots are forming.





After approx. 2 months the shoots are cut and transferred to *rooting medium* (max. 3 plants / 0.5 l glass). The callus should be kept because more shoots will form by time. It is not advisable to let them touch the top of the glassware. Thus, they must be transferred from 0.25 l glassware to 0.5 or 1 l glassware[1]

To maintain axenic culture the top of the plants is cut off and transferred to fresh medium. The old bottom part can be thrown away as soon as the upper part has roots. If plants are transferred to the greenhouse, the bottom part (with roots) can be transplanted into soil.

7. **Assignment**

Shoot cuttings from the same callus obtain the same number (e.g., 19.1, 19.2, and 19.3). It is advisable to number the callus from which these shoots were cut off as well (here: callus 19) in case more shoots form.

8. **Media for growth of *Agrobacterium***

YEB-Medium:

5 g/l Beef-Extract
1 g/l Yeast-Extract
5 g/l Peptone
5 g/l Sucrose
0.49 g/l $MgSO_4 \bullet 7H_2O$
For plates add Agar direct into the bottle
Autoclave

LB-Medium:

10 g/l Bacto-Tryptone
5 g/l Bacto-Yest Extract
10 g/l NaCl (5 g/l for low salts medium)
For plates add Agar direct into the bottle
Autoclave

Add antibiotics to 60°C warm medium, stir well and put immediately in plates.
If plates contain antibiotics, they should not be kept longer than a month.

Ampicillin 100 mg/l (stock 100 mg ddH_2O, filter sterile)
Rifampicin 100 mg/l (stock 50 mg DMSO)
Kanamycin 25 mg/l (Stock 50mg/ml ddH_2O, filter sterile)
Gentamycin 40 mg/l (stock 10 mg/ml ddH_2O, filter sterile)

9. **Media for plants**

9.1. ***Medium for the plates for the first 2 days (MS2%)***

dissolve in 900 ml ddH_2O:
add 4.31 g/l MS-salt (Duchefa, Haarlem, The Netherlands, or GIBCO BRL, Parsley, Scotland)
add 20 g/l sucrose
add 5 ml/l Vitamin mix
adjust pH to 5.7-5.8, with about 8-10 droplets of a KOH stock1M and fill up to 1 l
add agar direct into the bottle
autoclave
cool to 60°C (hand warm)
stir well and pour immediately in Petri dishes (9 cm ∅)

9.2. ***Selection medium (MG + hormones + antibiotics****) in 0.25 l glassware*[2]

dissolve in 900 ml ddH_2O:
add 4.31 g/l MS-salt (Duchefa, Haarlem, The Netherlands, or GIBCO BRL, Parsley, Scotland)
add 16 g/l glucose (0.089M – 17,6 g/l glucose monohydrate)
add 5 ml/l Vitamin mix
adjust pH to 5.7-5.8, with about 8-10 droplets of a KOH stock1M
fill up to 1 l
add agar direct into the bottle
autoclave
cool to 60°C (hand warm)
add hormones:
1 mg/l BAP,
0.2 mg/l NAA
add antibiotics:
500 mg/l Cefotaxime
50 mg/l Kanamycin or 15 mg/l Hygromycin or 4 mg/l PPT
stir well and pour immediately in Petri dishes (9 cm ∅) or glassware (0.25 l).

9.3. ***Rooting medium (MS2% + antibiotics****) in 0.5 l glassware*[2]

dissolve in 900 ml ddH_2O:
add 4.31 g/l MS-salt (Duchefa, Haarlem, The Netherlands, or GIBCO BRL, Parsley, Scotland)
add 20 g/l sucrose
add 5 ml/l Vitamin mix
adjust pH to 5.7-5.8, with about 8-10 droplets of a KOH stock1M and fill up to 1 l
add agar direct into the bottle
autoclave
cool to 60°C (hand warm)
add antibiotics:
500 mg/l Cefotaxime
50 mg/l Kanamycin or 15 mg/l Hygromycin or 4 mg/l PPT
stir well and pour immediately in glassware (0.5 l).

In general: Always store medium always at 4° C. Let it warm to RT before use.
Do not use medium, which is older than a month due to decrease in the activity of hormones and antibiotics
If fungal infection occurs, add Amphotericin (5 mg/l) to the medium

[2] WECK-Sturzglas ½ oder ¼ Liter (Rundrand 100) - http://www.shop-weck.de/shopindex.htm

10. Stocks

10.1. **α - Naphtalenacetic acid (NAA)** (MW 186.2; # N-0640 Sigma,)
stock concentration: 1 mg/ml
first add 1/10 vol. 0,1M NaOH and solve it, then add 9 /10 vol. ddH_2O
filter sterile (0,2 µm)
store 1 ml aliquots at 4°C or for longer at -20°C

10.2. **Amphotericin B** Fungicide (A-2411 Sigma; durable 3 days at 37°C, powder, stored at 4°C)
Stock concentration: 5 mg/ml
Dissolve in DMSO or in water (Sigma's Catalogue)
Store 1ml aliquots at -20°C

10.3. **Basta (PPT)** Herbicide (AgrEvo, 183 g/l Glufosinate, stored at RT)
stock concentration: 10 g/l
dilute in ddH_2O
filtre sterile (0.2 µm)
store in 100 ml Erlenmeyer in RT

10.4. **6-BenzylAminoPurin (BAP)** (MW 225,3; # B-3408 Sigma, powder)
Stock concentration: 1 mg/ml
add 1/10 vol. 0,1M NaOH, then 9/10 vol. sterile ddH_2O
filter sterile (0.2 µm)
store 1 ml aliquots at 4°C or for longer at -20°C

10.5. **Cefotaxime sodium (C)** Anti-bacterial (Duchefa C0111, stored at 4°C)
stock concentration: 250 mg/ml
dilute in ddH_2O
filter sterile (0.2 µm)
store 1 ml aliquots at -20°C

10.6. **Hygromycin B (H)** (Duchefa Bioch. 2 ml solution: 502 mg/ml, H0192, 1 g = 107 €, durable 2 years at 4°C; Roth CP 12.1 - solution 50 mg/ml, 10 ml, € 99.90; from Sigma the powder is stable at least 5 years if stored at 2-8°C)
stock concentration: 15 mg/ml
dilute in ddH_2O
filter sterile (0.2 µm)
store 1 ml aliquots at 4°C - freezing should be avoided[3]

10.7. **Kanamycin (KAN)** (K-1377 Sigma; 30S; salt store at RT)
stock concentration: 35 and 50 mg/ml
dilute in ddH_2O (60 mg Kan-salt contains approx. 50 mg Kan !!!)
filter sterile (0,2 µm)
store 1ml aliquots at -20°C

10.8. **Vitamin mix** (stock stored in 25 ml aliquots at -20°C)
Nicotine acid 0.1 g/l (final conc. 0.5 mg/l)
Pyridoxine-HCl 0.1 g/l (final conc. 0.5 mg/l)
Thiamine-HCl 0.02 g/l (final conc. 0.1 mg/l)
myo-Inositol 20 g/l (final conc. 100 mg/l)
Glycine 0.4 g/l (final conc. 2.0 mg/l)

[3] http://www.sigmaaldrich.com/catalog/product/sigma/H9773?lang=de&region=DE