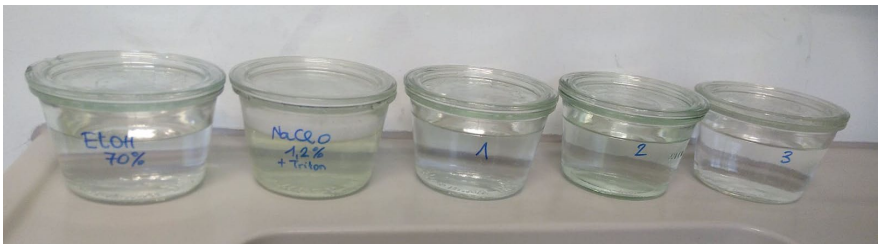
Protocol for tomato stabile transformation[1]

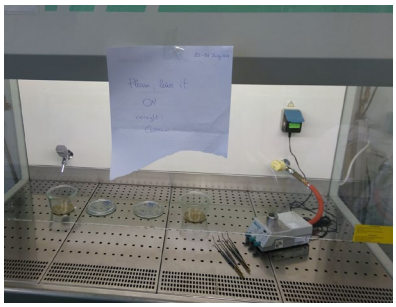
1. Seed sterilisation

At least 2 weeks before the transformation, wash about 300 seeds (approx. g 0.5 *Lycopersicon esculentum* L. var. Moneymaker) in autoclaved metal-double-teasieves.
Work in sterile bench with sterile jars:

- 3 min shaking in 200 ml 70% ethanol,
- 10 min shaking in 200 ml 1.5% hypochlorite containing some drops of 0.001% Triton W-100,
- wash in 3 x 200 ml sterile distilled water.



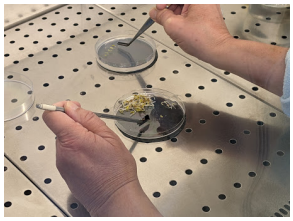
Let the seeds drying in the sterile bench over night.
Keep the dried seeds 2 days in the fridge (4°C dark) or longer.

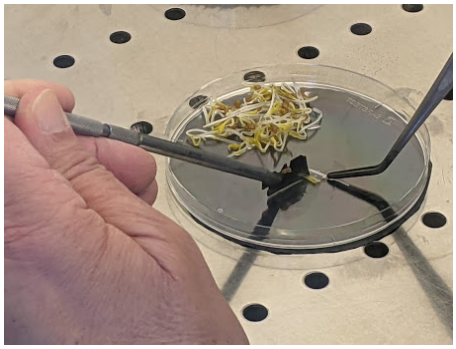


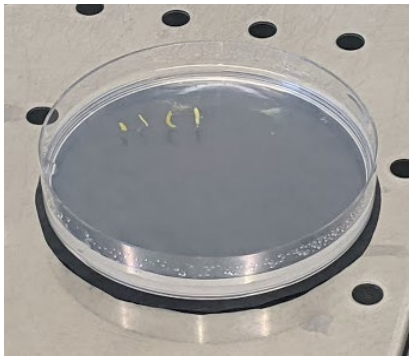
2. Preparing the cotyledons

1st day (**Friday**, 12 days before the transformation):
sow up to 30 single seeds per jar on **Tomato medium (ToMe)**, 10 jars are enough to get about 400 suitable cotyledons
let germinating the seeds for **12 days** in the dark and at 22°C (the cotyledons to cut should be 1 cm long and the first real leaves not yet developed).

12th day (**Wednsday**, it needs about 2 hours work for one construct):
Give some drops of **Liquid Germination medium** (**LGM**) on a sterile Petri-dish and cut the seedlings under the cotyledons. Remove the top and the basis of the leaf and make a little cut in the middle along the vein on the lower surface. Do not squeeze the cotiledonds.
If possible, use only cotyledons that are not rolled, because it will be possible to lay them on plates (10 Petris for one construct should be enough) containing **Conditioning medium** (**CM**) with full contact of the upper surface with the hormones.
About 20 leaves per Petri-dish (9 ∅) are incubated for 2 days in darkness at 22°C. The upper part of the leaves should lay flat on the medium.







[1] Published at: Development of a tomato plant resistant to *Clavibacter michiganensis* using the endolysin gene of bacteriophage CMP1 as a transgene. Wittmann J et al. *Plant Pathology* (2015).

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/$), incubate them for 2 days at 28°C.
12th day (Wednsday): Two days before transformation, inoculate 3 ml low-salt **LB medium** (Rif/Gen/$) with one single colony of *Agrobacterium*. Shaking for 24 h at 28°C.
13th day: The day before transformation, start a 100 ml culture (with the antibiotic corresponding to your binary plasmid) with 1 ml subculture in a **Bacteria-growth medium** (**BGM**). Shake overnight at 28°C.

4. **Transformation: infection and co-cultivation of plant leaves with Agrobacterium**

14th day (Friday): Before using it bring the OD_{590} to 1 with 10 mM $MgSO_4$ + Acetosyringon 0.1-0.2 mM (centrifugation 4000 rpm, 10 min, RT).
Drop the Agro-suspension with a pipette on the cotyledons (about 2 drops each cotyledon ≈ 2 ml for each Petri-dish) incubate the cotyledons with the bacteria suspension for 2 days in the dark at 22°C

5. **Selection of transformed tissue** (in 0,25 l jars)

17th day (Monday): put the cotyledons on plates with **Selection medium (**with 35 mg/l Kanamycin or 2 mg/l Basta[2] or 6 mg/l Hygromycin[3]), leaf surface up, in the light (14 h light and 10 h dark at 23°C, 50% humidity) and leave them for 3 days on it.
After 3 days (**Wednsday**) transfer again the cotyledons on fresh Selection medium with 35 mg/l Kanamycin or 2 mg/l Basta or 6 mg/l Hygromycin.
Then transfer the cotyledons every week:

- 2 x 7 days on fresh Selection medium with 50 mg/l Kanamycin or 2 mg/l Basta or 6 mg/l Hygromycin
- from now transfer the cotyledons every week on fresh Selection medium with 100 mg/l Kanamycin or 2 mg/l Basta or 6 mg/l Hygromycin

After a couple of weeks the first shoots are forming (first shoots and green calli are to be cut and put directly on the medium)

6. **Regeneration of transgenic plants**

After approx. 2 months the shoots are cut and transferred to **Rooting medium (**with 20 mg/l Kanamycin or 2 mg/l Basta or 6 mg/l Hygromycin). Max. 3 plants in a 0.5 l jar.
If there are problems by rooting, let the Kanamycin or Basta or Hygromycin away.
The callus should be kept, because more shoots will form by time.
It is not advisable to let the shoots touch the top of the jar, after they have rooted let them grow on soil.
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.

Assignment

Shoot cuttings from the same callus obtain the same number (e.g. 1.1, 1.2, 1.3). It is advisable to number the callus from which these shoots were cut off as well (here: callus 1) in case more shoots form.


[2] Kind information of Dr. Markus Albert, ZMBP, Uni Tuebingen.
[3] Kind information of Viktoria Troester, ZMBP, Uni Tuebingen: Methods in Molecular Biology, Vol. 343 p. 468. Joyce van Eck, Boyce Thompson Institut, Cornell University.

Media for growing *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 15 g/l Bacto Agar Difco direct into the bottle
Autoclave

LB-Medium:
10 g/l Bacto-Tryptone
5 g/l Bacto-Yeast Extract
10 g/l NaCl (5 g/l for low salts medium)
For plates add 15 g/l Bacto Agar Difco direct into the bottle
Autoclave

Bacteria-growth medium (BGM)
10 g/l Yeast-Extract
10 g/l Bacto-Peptone
5 g/l NaCl
Autoclave
0.2 mM Acetosyringone (from 400 mM stock-solution in DMSO)
Antibiotics

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, filtre sterile)
rifamycin 100 mg/l (stock 50 mg DMSO)
kanamycin 25 mg/l (Stock 50mg/ml ddH_2O, filtre sterile)
gentamycin 40 mg/l (stock 10 mg/ml ddH_2O, filtre sterile)

Media for plants

Tomato Medium *(ToMe - 2 Litres for about 30 big jars[4])*
dissolve in 900 ml ddH_2O:
- 4.3 g/l Murashige & Skoog Salt
- 30 g/l sucrose
- 100 mg/l myo-Inositol
- 1 ml/l NPT Vitamins stock-solution

adjust pH to 5.8, with about 6 droplets of a KOH stock1M and fill up to 1 l
add agar direct into the bottle
autoclave
stir well and pour immediately in big jars.

Liquid Germination Medium *(LGM – 0.5 litre in two bottles)*
dissolve in 450 ml ddH_2O:
- 2.15 g Murashige & Skoog Salt
- 15 g sucrose
- 50 mg myo-Inositol
- 0.5 ml NPT Vitamins stock-solution

adjust pH to 5.8, with 1-2 droplets of a KOH stock1M and fill up to 0,5 l
autoclave

Conditioning medium *(CM – 1 litre for about 30 Petri-dishes)*
Tomato Medium
cool to 60°C (hand warm)
add hormones: 0,1 mg/l BAP and 1 mg/l NAA
stir well and pour immediately in Petri dishes (9 cm ∅)

Selection medium *(about 10 litres)*
Tomato Medium
cool to 60°C (hand warm)
add 1 mg/l trans-Zeatin
add antibiotic against Agrobacterium: 250 mg/l Ticarcillin-clavulanate;
add antibiotics to select: Kanamycin mg/l 35, 50 or 100, or Basta 2 mg/l, or Hygromycin 6 mg/l
stir well and pour immediately in 0,25 l little jars[5]

Rooting medium *(about 5 litres)*
Tomato Medium
cool to 60°C (hand warm)
add 0.1 mg/l IAA
add antibiotics to select: 20 mg/l Kanamycin, or 2 mg/l Basta or 6 mg/l Hygromycin
add antibiotic against Agrobacterium: 500 mg/l Vancomycin
stir well and pour immediatey in big jars (0,5 l or 0,75 l).
If there are problems by rooting, leave Kanamycin or Basta away

In general:
Always store medium 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, you could add Amphotericin (5 mg/l) to the medium but it is better not to open the contaminated jars in order to avoid the diffusion of fungial spora! Contaminated jars must be autoclaved!

[4] WECK-Sturzglas 1/2 Liter (Rundrand 100) - http://www.shop-weck.de/shopindex.htm
[5] WECK-Sturzglas 1/4 Liter (Rundrand 100) - http://www.shop-weck.de/shopindex.htm

Stocks

α - Naphtalenacetic acid (NAA) (MW 186.2; # N-0640 Sigma,)
stock concentration: 1 mg/ml
add 1/10 vol. 0,1M NaOH, then 9 /10 vol. ddH_2O
filter sterile (0.2 µm)
store 1 ml aliquots at -20°C

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
Store 1ml aliquots at -20°C

Basta (PPT) Herbicide (AgrEvo, 183 g/l Glufosinate; liquid, stored at RT)
stock concentration: 20 g/l
dilute in ddH_2O
filter sterile (0.2 µm)
store 100 ml bottle at Room Temperature

6-BenzylAminoPurin (BAP) (MW 225,3; # B-3408 Sigma, powder)
Stock concentration: 1 mg/ml
dissolve 40 mg in 1ml NaOH 1M and add sterile ddH_2O up to 40 ml for 40 Eppis
filter sterile (0.2 µm)
store 1 ml aliquots at -20°C

Indole-3-Acetic Acid (IAA)
MW 175.2; # N-I 2886 Sigma, Dessiccate stored at less than 0°C
Merck: store at +5°C to +30°C
stock concentration: 1 mg/ml
add 1/10 vol. 100% EtOH, then 9/10 vol. sterile ddH_2O
filter sterile (0.2 µm)
store 1 ml aliquots at -20°C

Hygromycin B (H)
Sigma the powder is stable at least 5 years if stored at 2-8°C;
Roth 10 ml solution 50mg/ml – CP12.1 – steril
Duchefa Bioch. 2 ml solution: 502 mg/ml, H0192, 1 g = 107 €, durable 2 years at 4°C;
stock concentration: 12 mg/ml
dilute in ddH_2O
filter sterile (0.2 µm)
store 1 ml aliquots at 4°C - freezing should be avoided[6]

Kanamycin Monosulfat (KAN) (K-1377 Sigma, oder Duchefa K0126, salt stored at RT)
stock concentration: 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

Ticarcillindisodium/potassium-clavulanate (TiCla)(T0190 Duchefa Bioch., stored at 4°C, 10 g = € 150)
stock concentration: 250 mg/ml
dilute in ddH_2O
filter sterile (0.2 µm)
store 1 ml aliquots at -20°C

trans-Zeatin (t-Z) (MW 219,25; # Z 0876 Sigma, 5 mg = € 30 or Duchefa Z0917, 50 mg = 87 €, stored at –20°C)
stock concentration: 1 mg/ml
add 1/10 vol. 0.1M HCl for Sigma or 1N NaOH for Duchefa, then 9 /10 vol. ddH_2O
filter sterile (0.2 µm)
store 1 ml aliquots at -20°C

Vancomycin hydrochloride (VAN) (# V0155, Duchefa, 5 g = € 200, stored at 4°C)
stock concentration: 500 mg/4 ml
dilute 5 g in 40 ml sterile ddH_2O
filter sterile (0.2 µm)
store in 4 ml aliquots at -20°C

NPT Vitamins mix (NPT) (stock stored in 1 ml aliquots at -20°C)
Thiamine-HCl stock concentration: 10 mg/ml, final conc. 10 mg/l
Nicotine acid stock concentration: 1 mg/ml, final conc. 1.0 mg/l
Pyridoxine-HCl stock concentration: 1 mg/ml, final conc. 1.0 mg/l
Filter sterile (0.2 µm) and store 1 ml aliquots at -20°C

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

Timetable for tomato transformation:

			Media
1st Week	1st day Friday	Seeds sterilization Seeds sowing	Germination medium in jars (0,5 l).
2nd Week		Seeds germination Streak out Agrobacterium on plates	
3rd Week	12th day Wednsday	Prepare the cotyledons (about 400)	Liquid Germination medium: 0,5 l bottle Conditioning medium: about 10 Petri dishes (9 ∅) in the dark
	12th day Wednsday	Inoculate the Agro-pre-culture	YEB with Antibiotics (5 ml)
	13th day Thursday	Inoculate Agros	Medium 3 with 50% Antibiotics and Acetosyringon
	14th day Friday	Drop the Agros	
	17h day Monday	Transfer the cotyledons	Selection medium with 35 mg/l Kanamycin or 2 mg/l Basta (in the light) or 6 mg/l Hygromycin: 0,25 l jars (about 10)
4th Week	20th day Wednsday	Transfer the cotyledons	Selection medium with 35 mg/l Kanamycin or 2 mg/l Basta (in the light) or 6 mg/l Hygromycin: 0,25 l jars (about 10)
5th Week		Transfer the cotyledons and cut away the shoots if there are some	Selection medium with 50 mg/l Kanamycin or 2 mg/l Basta or 6 mg/l Hygromycin: 0,25 l jars (about 10)
6th Week		Transfer the cotyledons and cut away the shoots if there are some	Selection medium with 50 mg/l Kanamycin or 2 mg/l Basta or 6 mg/l Hygromycin: 0,25 l jars (about 10)
7th Week		Transfer the cotyledons and cut away the shoots if there are some	Selection medium with 100 mg/l Kanamycin or 2 mg/l Basta or 6 mg/l Hygromycin: 0,25 l jars (about 10)
8th week		Transfer the cotyledons and cut away the shoots if there are some On Rooting Medium	Selection medium with 100 mg/l Kanamycin or 2 mg/l Basta or 6 mg/l Hygromycin: 0,25 l jars (about 10) Big jars with Rooting Medium
9th Week		Transfer the cotyledons and cut away the shoots if there are some On Rooting Medium	Selection medium with 100 mg/l Kanamycin or 2 mg/l Basta or 6 mg/l Hygromycin: 0,25 l jars (about 10) Big jars with Rooting Medium

Approx. costs for consumable materials: 350 €