Transformation Protocol for Tobacco – Abbreviated

Streak out bacteria onto YM

↓ 2-3 days 28°C

Co cultivation on RMOP

↓ 3-11 days in dark

Selection on RMOP-TCH

↓ Subculture every 2 weeks

Plantlets onto MST-TCH

↓

Transfer plants to the glasshouse

Transformation Protocol for Tobacco

Plant material: *In vitro* plants of *Nicotiana tabacum* L. cv Wisconsin 38

Day 1 Prepare bacteria**:**

- Streak **YM plus antibiotic** plates with bacteria.
- Incubate plates for 2-3 days at 28°C.

Day 3 Transformation**:**

- Measure about 20mL Minimal A medium for each bacterial strain.
- Scrape or wash bacteria from plate with sterile loop and suspend in 20mL of Minimal A.
- Adjust density to an OD_{600} 0.9-1.0.
- Take first healthy fully expanded leaves from 4-5 week old tissue culture grown tobacco plant, cut into 0.5cm squares (or can use a cork borer, which is about 1.0cm diameter) in deep petri dish, under sterile **RMOP** liquid medium. Store tissue pieces in **RMOP** in deep petri dish.
- Transfer leaf pieces (about 20 per transformation) to deep petri dish containing bacterial suspension.
- Swirl to ensure bacteria has contacted cut edge of leaf and let stand for 5 minutes.
- Remove leaf pieces from suspension and blot dry on filter paper or on the edge of the container.
- Place leaf pieces with adaxial side (upper leaf surface) on solid **RMOP**, about 10 pieces per plate.
- Incubate plates in dark at 28°C for:
  2-3 days *A. tumefaciens*
  5 days *S. meliloti*
  5 days *M. loti*
  5-11 days *Rhizobium sp*. NGR234

Day 6-14 Selection**:**

- Transfer leaf pieces onto solid **RMOP-TCH**, with abaxial surface (lower surface of leaf) in contact with media.
- Incubate plates for 2-3 weeks in the light at 28°C, with 16 hours daylight per day.
- Subculture every 2 weeks.

Plantlet formation**:**

- When shoots appear transfer to **MST-TCH** pots.
- Incubate plantlets with 16 hours daylight for 1-2 weeks.
- When roots form the plants can be transferred to soil in the glasshouse.
- Plants can be maintained in tissue culture but will need to be subcultured every two weeks. This is done by cutting the growing tip of plant, removing excess leaves and transferring this to fresh media.

Media and Solutions for Tobacco Transformation

YM Media

	1L
Mannitol	10g
Yeast extract	0.4g
K_2HPO_4 (10% w/v stock)	1 ml
KH_2PO_4 (10% w/v stock)	4 ml
NaCl (10% w/v stock)	1 ml
$MgSO_4.7H_2O$ (10% w/v stock)	2 ml

- pH 6.8
- Agar 15g/L
- Autoclave
- When ready to pour add antibiotic selection if required

Keep poured plates for 2 days at room temperature to visualise any contamination, then store at 4°C.

RMOP + RMOP-TCH media

(Svab, *et al*, 1975)

	1L	Final conc.
Sucrose	30g	3%
Myo-inositol	100mg	0.1%
MS Macro 10x	100mL	1x
MS Micro 1000x	1mL	1x
Fe_2EDTA Iron 100x	10mL	1x
Thiamine-HCl (10mg/mL stock)	100µL	1mg
NAA (1mg/mL stock)	100µL	0.1mg
BAP (1mg/mL stock)	1mL	1mg

- pH 5.8
- Phytagel 2.5g/L for solid
- autoclave
- for RMOP-TCH, when ready to pour add:
  - Timentin (200mg/mL stock) 1mL
  - Claforan (250mg/mL stock) 1mL
  - Hygromycin (50mg/mL stock) 1mL

BAP (1mg/ml) (6-Benzylaminopurine)

Add 1N KOH drop wise to 100mg BAP until dissolved. Make up to 100mL with Milli-Q H_2O

- Store 4°C

Media and Solutions for Tobacco Transformation

NAA (1mg/ml) (Naphthalene acetic acid)

Dissolve 100mg NAA in 1mL absolute ethanol. Add 3mL 1N KOH. Make up to 80mL with Milli-Q H_2O. Adjust pH to 6.0 with 1N HCl, make up to 100mL with Milli-Q H_2O.

- Store 4°C

Cefotaxamine® (250mg/ml)

Add 8ml sterile Milli-Q H_2O to 2g Claforan

- Store 4°C in dark

Timentin® (200mg/ml)

Add 15ml sterile Milli-Q H_2O to 3g Timentin

- Store 4°C

MST + MST-TCH media

(Svab, et al, 1975)

	1L	Final concentration
Sucrose	30g	3%
MS Macro 10x	100mL	1x
MS Micro 1000x	1mL	1x
Fe_2EDTA Iron 100x	10mL	1x

- pH 5.8
- Phytagel 2.5g/L
- autoclave
- for MST-TCH, when ready to pour add:
    - Timentin® (200mg/mL stock) 1mL
    - Cefotaxamine® (250mg/mL stock) 1mL
    - Hygromycin (50mg/mL stock) 1mL

MS Macro 10x

(Murashige and Skoog, 1962)

	10x (g/L)	Final concentration 1 x mM
KNO_3	19.0	18.8
$NH_4 N0_3$	16.5	20.6
$CaCl_2.2H_2O$	4.4	3.0
$MgS0_4.7H_2O$	3.7	1.5
KH_2PO_4	1.7	1.25

- Store 4°C
- Substituting chemicals:
    - $CaCl_2$ 3.3g/L
    - $MgS0_4$ 1.8g/L

Media and Solutions for Tobacco Transformation

MS Micro 1000x (Murashige and Skoog, 1962)

	1000x (g/L)	Final concentration 1 x µM
$MnS0_4.4H_20$	22.3	100
$ZnS0_4.7H_20$	8.6	30
H_3BO_3	6.2	100
KI	0.83	5.0
$Na_2MoO_4.2H_2O$	0.25	1.0
$CuSO_4.5H_2O$	25mg	0.1
$CoCl_2.6H_2O$	25mg	0.1

- Store 4°C
- Substituting chemicals:
  - $MnS0_4.H_20$ 16.9/L

$FeSO_4$EDTA Iron 100x

	g/1L	1 x mM
$FeS0_4.7H_20$	2.78	0.1
Na_2EDTA	3.72	0.1

- Store 4°C in dark bottle

References:

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. **Phys. Plant. 15**: 473-497.

Svab, Z., P. Hajdukiewicz and P. Maliga. 1975. Transgenic tobacco plants by co-cultivation of leaf disks with pPZP *Agrobacterium* binary vectors. In "Methods in Plant Molecular Biology-A Laboratory Manual", P. Maliga, D. Klessig, A.. Cashmore, W. Gruissem and J. Varner, eds. Cold Spring Harbor Press: 55-77.