WEB313 - SOMT Golden Gate Cloning

cloning to pICH41308 and subcloning, for SOMT2 expression in *Nicothiana benthamiana* (with Sylvestre Marillonet)

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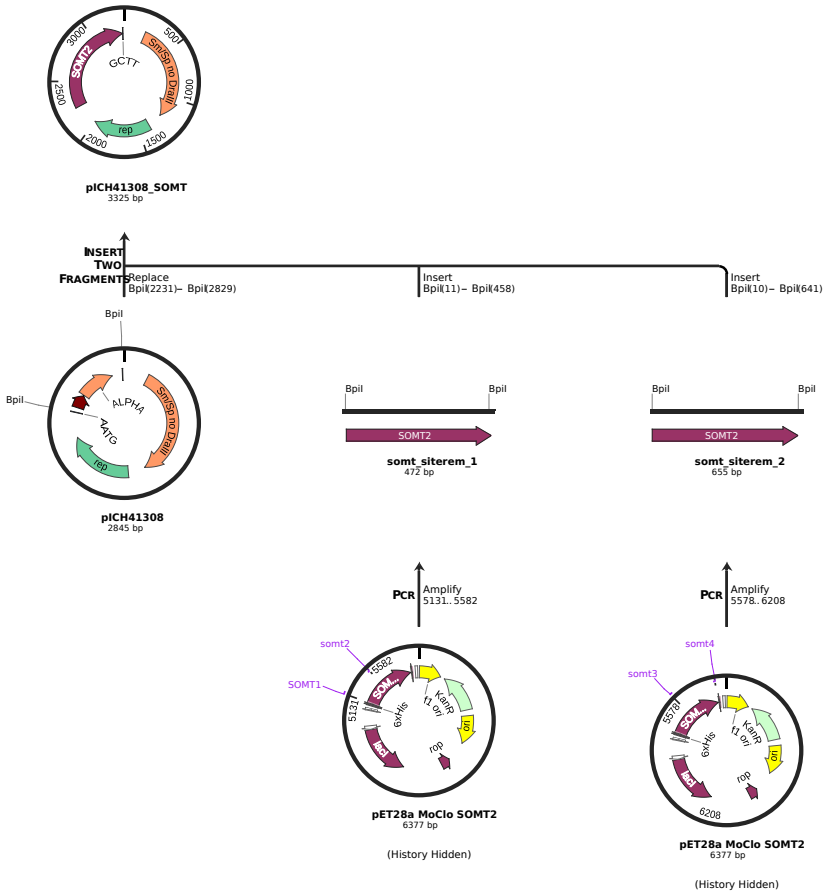

Figure 1: Schematic drawing of the **Level 0** cloning of SOMT2 into pICH41308

1 Introduction

- according to literature SOMT can methylate 4'-hydroxy group of isoflavones and flavones, namely naringenin
- no activity could be shown *in vitro*, neither from refolded, nor from solubly expressed (*E. coli*, periplasmatic expression) protein
- to verify the activity described in literature → eucaryotic expression *in planta (N. benthamiana)*
- cooperation with Sylvestre Marillonet (SZB)
- SZB already has the pathway cloned up to naringenin (Figure 2)
- **Aim**:

  A. clone SOMT in vector for expression in *N. benthamiana*
  B. infiltrate plant using *Agrobacterium tumefaciens*
  C. (hopefully) detect product, 4'-O-methyl naringenin

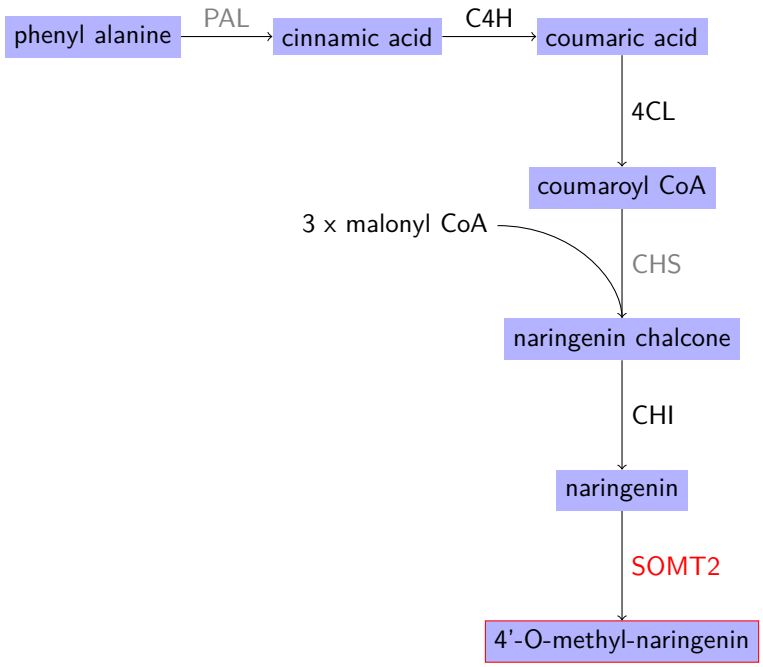

Figure 2: Semi-synthetic pathway to naringenin and 4'-O-methyl naringenin in *N. benthamiana*. Enzymes not endogenous to *N. benthamiana* are in gray. PAL - phenylalanine ammonia lyase, C4H - cinnamic acid 4-hydroxylase, 4CL - 4-coumaric acid:CoA ligase, CHS - chalcone synthase, CHI - chalcone isomerase, SOMT2 - soy O-methyl transferase 2

2 Cloning to pICH41308

ATTENTION: SOMT gene has an endogenous BsaI cleavage site → site needs to be removed

- four primers used for cloning (due to endogenous BsaI site) (see Table 1 for sequences)

  a) *somt1* - forward primer for first fragment

b) *somt2* - reverse primer for first fragment (also primer with **C▷G** mutation to remove endogenous BsaI site)
c) *somt3* - forward primer for second fragment
d) *somt4* - reverse primer for second fragment

Table 1: The primers used for cloning.

name	sequence [5'→3']
somt1	tt gaagac aa aatggcttcttcattaaacaatggccg
somt2	tt gaagac aa ggacaccccaaatactgtgagatcttcc
somt3	tt gaagac aa gtccttaggaacacctttctgggac
somt4	tt gaagac aa aagctcaaggatagatctcaataagagac

2.1 PCR SOMT2

- cloning from pET28a MoClo SOMT2 (2) [51 $\frac{ng}{\mu l}$]

Reaction mix:

substance	volume	per 50 μl
10x KOD Buffer	10 μl	1x
25 mM $MgSO_4$	6 μl	
2 mM dNTPs	10 μl	0.2 mM
51 ng/μl template	0.5 μl	12.5 ng
KOD HS Polymerase	2 μl	1 μl
water	65.5 μl	

Reactions:

Reaction 1	Reaction 2
47 μl Reaction mix	47 μl Reaction mix
1.5 μl somt1	1.5 μl somt3
1.5 μl somt2	1.5 μl somt4
Theoretical size of fragment	
472 bp	655 bp

PCR Program:

Step	Description	Temperature	Time	
1	Denaturation	95°C	2 min	
2	Denaturation	95°C	20 sec	
3	Annealing	55°C	10 sec	
4	Extension	70°C	12 sec	repeat **2-3** 24x
5	Final Extention	70°C	2 min	
	Pause	4°C	∞	

3 Level 0: Digestion-Ligation

Reaction:

- **1:10 diluted DNA is used**

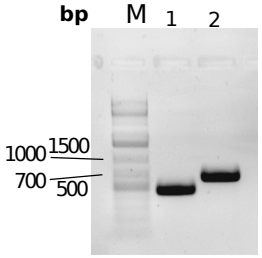

Figure 3: Agaroase gel electrophoresis results of PCR. Bands correspond to the theoretical size of the fragments (472 and 655 bp).

DNA	concentration $\frac{ng}{\mu L}$		$(\frac{ng}{\mu L})$
pCH41308	420	dilute (1:10) $\longrightarrow$	42
SOMT.f1	305		30.5
SOMT.f2	275		27.5

component	amount	volume
pICH41308	20 fmol	0.89 μl
SOMT.f1	20 fmol	0.20 μl
SOMT.f2	20 fmol	0.31 μl
T4 Ligase (Fermentas)	5 U	1 μl
BpiI (Fermentas)	5 U	0.5 μl
Ligase buffer (Fermentas)	1x	1.5 μl
ad to 15 μl MQ-H_2O (10.6 μl)		

Cycler Program:

step	temperature	time
	37°C	4 h
	50°C	5 min
	80°C	5 min
Pause	4°C	∞

4 Level 1: Digestion-Ligation

DNA	concentration $\frac{ng}{\mu L}$		$(\frac{ng}{\mu L})$
pCH51266	428	dilute (1:10) $\longrightarrow$	42
pCH41421	155		15
pICH75044	241		24
pICH41308_SOMT	285		28

Reaction:

- **1:10 diluted DNA is used**

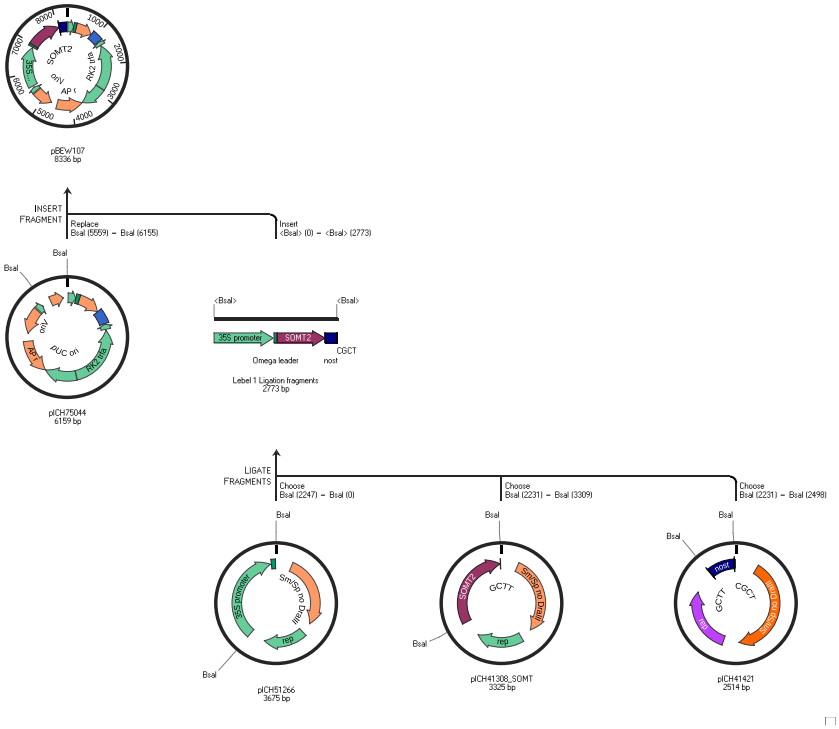

Figure 4: Level 1 Cloning into expression vector for *Agrobacterium tumefaciens* transformation.

component	amount	volume
pICH75044	20 fmol	3.4 μl
pICH51266	20 fmol	1.2 μl
pICH41421	20 fmol	2.9 μl
pICH41308_SOMT	20 fmol	1.2 μl
T4 Ligase (Fermentas)	5 U	1 μl
BsaI (Fermentas)	5 U	0.5 μl
Ligase buffer (Fermentas)	1x	1.5 μl
ad to 15 μl MQ-H_2O (3.3 μl)		

Cycler Program:

step	temperature	time	
	37°C	2 min	⎫ 50 x
	16°C	5 min	⎭
	50°C	5 min	
	80°C	10 min	
Pause	4°C	∞	

- transform 5 μl ligation reaction into 50 μl DH5α cells

- add 500 μl SOC
- plate 100 μl onto LB + S-Gal + Ampicillin Agar
- incubate at 37°C $\rightarrow$ $\sim$ 50 colonies, no blacks (blue)
- 3 mL cultures of two colonies $\rightarrow$ plasmid prep and digestion with BpiI

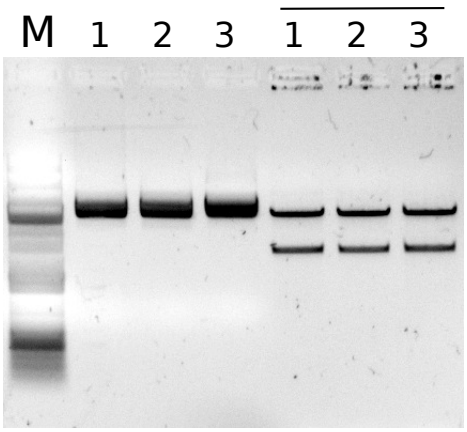

Figure 5: BpiI digestion of pBEW107 (overlined, right) and undigested vector controls (left).

5 Infiltration of *Nicotiana benthamiana*

5.1 Transformation into *Agrobacterium tumefaciens*

! cool cuvettes and DNA on ice, work quickly
- thaw electrocompetent agrobacteria on ice
- add 0.5 μl plasmid to 50 μl electrocompetent agrobacterium suspension and mix by tapping
- place into electroporation cuvette and pulse
- add 1 mL LB and transfer culture to 1.5 mL tube
- shake for 3-4 hours at 28°C
- centrifuge and remove 900 μl of supernatant
- plate remaining suspension onto LB + 40 ug/mL rifampicin + 50 ug/mL carbencillin plates
- grow at 28°C for 2-3 days
- store plate in fridge

5.2 General infiltration of *N. benthamiana*

- inoculate 5 mL LB + 40 $\frac{\mu g}{mL}$ rifampicin and 50 $\frac{\mu g}{mL}$ carbencillin with *agrobacterium*
- grow over night at 28°C and 220 rpm
- measure OD^{600} in 1:10 dilution in water (use LB + rif in water as blank)
- for infiltration make dilutions of cell culture with $OD^{600} = 0.2$ (10^{-1} dilution) in **infiltration buffer)**
- infiltrate reduced (cut back leaves, except for the ones that will be infiltrated) *N. benthamiana* plants using 2 mL syringe

5.3 Special infiltration for this experiment

- everything as in general protocol (5.2)
- ! two infiltration solutions were prepared, both containing 10406 and 10733 (PAL and CHS genes) and either pBEW107, or the control plasmid

Table 2: OD^{600} values measured for the *agrobacterium* cultures.

Sample	OD^{600}	comment
pBEW107	1.72	measured against water
control (75044)	1.32	"
pBEW107	1.15	measured against LB + rif in water (Ramona)
control (75044)	0.88	"
PAL? (10406)	1.86	"
CHS? (10733)	2.08	"

Table 3: Recipes for infiltration solution. Two infiltrations solutions were prepared. One with the SOMT2 gene (pBEW107) and one with the vector control (75044). Both infiltration solutions also contained the PAL and CHS genes (10406, 10733).

Sample	component	volume (mL)
pBEW107	pBEW107	3.478
	10406	2.15
	10733	1.923
	inf. buffer	12.449
control	75044	4.545
	10406	2.15
	10733	1.923
	inf. buffer	111.382

Infiltration pattern (from leaf top)

- left side of leaf → SOMT2
- right side of leaf → control

6 Preparation and extraction of plant material

6.1 Preparation of leaves

The infiltrated areas of the plants were cut out. Separate samples for each plant, top and bottom leaves and infiltration sides (left/right) were prepared. Twelve (12) samples were prepared in total (Table 4). The cut out areas were weighed (wet weight), frozen in liquid nitrogen, ground into powder and stored at -80°C .

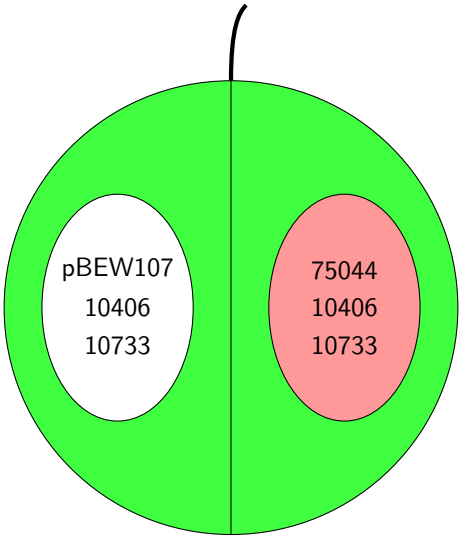

Figure 6: Schematic drawing if the infiltrated leaves (view from top). On the right side the SOMT2 gene is infiltrated, left is the control.

Table 4: Leaf samples from agrobacterium infiltration. The plants were grown for 7 days at (**???**) after infiltration.

sample	plant	leaf position	leaf side	wet weight	dry weight	tube tara
1tl	1	top	left	0.4204		3.1688
1tr	1	top	right	0.2986		3.1456
1bl	1	bottom	left	1.6333		3.1480
1br	1	bottom	right	1.2322		3.1567
2tl	2	top	left	0.3349		3.1499
2tr	2	top	right	0.4363		3.1523
2bl	2	bottom	left	1.1756		3.1621
2br	2	bottom	right	1.3726		3.1412
3tl	3	top	left	0.7079		3.1464
3tr	3	top	right	0.6861		3.1539
3bl	3	bottom	left	1.0370		3.1380
3br	3	bottom	right	1.1720		3.1539

6.2 Extraction of flavonoids

2-mL tubes were weighed. Two tips of a small spatula of plant material (approx. 6 mg) was added and the tube weighed again. The plant material was extracted with 500 μl of 1 mM ascorbic acid, 0.2% formic acid, 0.1 mM flavone in 75% MeOH. For extraction the suspension was vortexed for 30 s, rotated in an orbital shaker for 10 min and vortexed again for 30 s. The suspension was centrifuged at 10.000 x g and 4°C for 10 min. The supernatant was centrifuged again, and the resulting supernatant solution was applied to HPLC.

6.3 Hydrolysis of glycosylated compounds

To analyse the aglycone flavonoid, the glycosylated flavonoids were hydrolyzed. Therefore 500 μl of 2 N 75% methanolic HCl were added to the solid residual plant material obtained from the extraction. The suspension was heated to 90°C for 1 hour.

The result was centrifuged for 10 minutes at 10.000 x g and 4°C , and the supernatant was evaporated in a *SpeedVac* set to 60°C . The residue was resuspended in methanol containing 1 mM ascorbic acid and 0.2% formic acid. The resulting solution was centrifuged at 10.000 x g and 4°C for 10 minutes. Consequently, the supernatant was analyzed by HPLC.

Attention: Some of the tubes sprung open during heating.

7 Buffers and Recipes

7.1 Antibiotics

Rifampicin (40 mg/mL): dissolve 200 mg rifampicin in 5 mL MeOH
Carbencillin (50 mg/mL): dissolve 250 mg carbencillin in 5 mL H_2O

7.2 Buffers

Infiltration buffer: 10 mM Mes/NaOH, 10 mM $MgSO_4$ pH 5.5