

**For one construct:****1<sup>st</sup> day:**

1. Prepare **3 square Petris** 12 cm with one bottle 1% Phytoagar (Duchefa) (8 g Agar in 800 ml water)
2. Scarify 100 (about 0.1 g) Lotus “Gifo” seeds by rubbing with sand paper
3. Surface-sterilize:
  - a. 1.5% sodium hypochlorite solution (make it fresh) with autoclaved water in one Epis
  - b. Shake 8 minutes in one Epis
  - c. Wash seeds in 6 shifts of autoclaved water
  - d. Shake the seeds circa 2 hours in *Conserve* solution (Spinosad, insecticide: 30 ml distilled water + 22.5 microliter sterile filtered *Conserve*) in one Epis
  - e. Throw away the *Conserve* solution
4. Distribute the seeds on three 1%-Phytoagar-Petris (12 cm square), seal with Parafilm and keep vertical at 26°C, 3 days dark (aluminium) and 3 days light: **sewing on Thursday, to light on Monday and transform on Wednesday.**

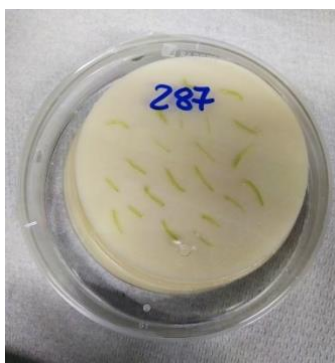
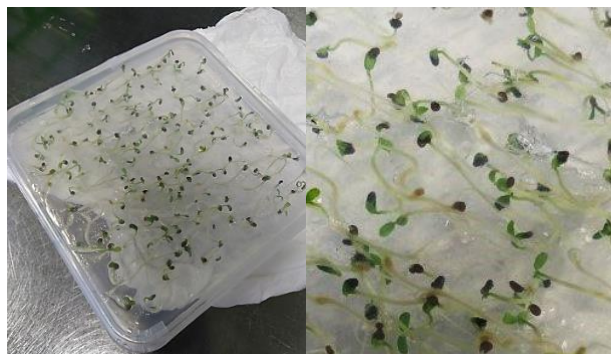
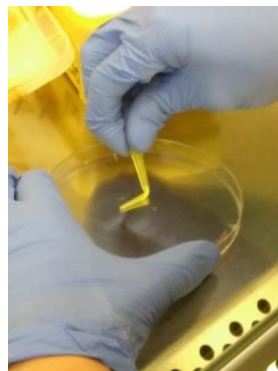
**5<sup>th</sup> day:**

5. On day 5 after start of seeds, scrape *Agrobacterium* colonies off a selective plate with a blue tip with bent top and resuspend them in one Epis with 1 ml sterile water, streak the diluted *Agrobacterium* strain on 2 fresh selective plates with a yellow tip with bent top.

<sup>1</sup> Modified from: Transgenic Plants: *Agrobacterium*-Mediated Transformation of the Diploid Legume *Lotus japonicus* - Kurt Handberg, Jiri Stiller, Thomas Thykjaer and Jens Stougaard - Cell Biology: A Laboratory Handbook 1994 (pages 119-127)

Stable Agrobacterium Transformation of *Lotus japonicus* Protocol**7<sup>th</sup> day:**

6. On day 7 after germination scrape 2 days old Agrobacterium colonies off a selective plate with a tip with bent top and resuspend them in YMB medium by whirling (for 1 construct you need about **20 ml**).
7. Leave bacteria as milky cultures at room temperature while preparing the cocultivation plates.
8. For **1 construct**, prepare cocultivation plates by placing 0.5-cm-high platforms of sterile filter paper in **8 Petri dishes** (Rundfilter MN615 - 7 cm diam. Macherey-Nagel); add Cocultivation Medium (CoM) until free liquid appears (12-15 ml for each Petri = about **200 ml for 1 construct**).
9. Prepare a new set of **4 Petri dishes** with 2 layers of sterile filter paper for cutting the hypocotyls; soak filter papers with **4 ml** of Agrobacterium YMB suspension (=> 4 ml x 4 Petris = **16 ml** or 4 ml of YMB for control plates).
10. Transfer 1 seedling from the germination plate to one of 4 Petris with *Agrobacterium*-soaked filter paper (or with YMB-soaked filter paper for control). Using scalpel and forceps, cut seedlings below the shoot primordia and at the stem base; hold hypocotyl explant between forceps and cut in-between. Transfer hypocotyls to the cocultivation plates. Give **20 hypocotyls on each plate and 8 plates** for each construct. Cutting for one transformation needs **about 2 hours**.
11. Incubate cocultivation plates for **7 days 21°C in the dark** (with aluminium).



Stable Agrobacterium Transformation of *Lotus japonicus* Protocol**2<sup>nd</sup> week:**

12. To eliminate *Agrobacterium* transfer explants *resting on the top filter paper* from the cocultivation plate to Callus Medium (CM) with 300 mg/l of Cefotaxime (8 Petris). Incubate for **7 days 26°C under continuous light**.

**3<sup>rd</sup> week:**

13. Start selection of transgenic calli by transferring explants, without filter paper, to Selective Callus Medium (SCM) containing 300 mg/l of Cefotaxime (8 Petris). For selection use the following concentrations: kanamycin 100 mg/l, Hygromycin 15 mg/l, G418 25 mg/l.

Conditions for subsequent selection and regeneration steps are **continuous light, 26°C and 7-day incubation intervals**.

Continue selection and propagation of transgenic calli on Selective Callus Medium (SCM) with cefotaxime 300 mg/l for **5 weeks** (about 5 weeks x 8 Petris = 40 Petris). Separate transgenic green calli from explant as soon as possible and propagate in one piece unless clonal material is needed.

**8<sup>th</sup> week:**

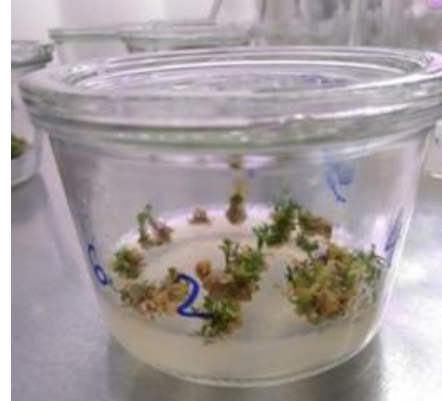
14. Start shoot induction by transferring green transgenic calli to Shoot Induction Medium (SIM) with cefotaxime 300 mg/ml. Shoot structure will develop in the dark green zone appearing at the perimeter of the calli. Keep calli undivided; remove brown and light-yellow tissue (10-15 jars x about 10 weeks), **continuous light, 26°C**. *The calli without emerging shoots are kept on SIM and are transferred every week on fresh jars (about 15-20 jars each week for several weeks).*



Stable Agrobacterium Transformation of *Lotus japonicus* Protocol**About 14<sup>th</sup> week:**

15. Transfer calli with emerging shoot structures to Shoot Growth Medium (SGM) for **2 weeks**.

*The calli without emerging shoots are kept on SIM and are transferred every week on fresh jars (about 15-20 jars each week for several weeks), continuous light, 26°C.*

**+ 2 weeks:**

16. For shoot elongation, transfer calli with short shoots onto Shoot Elongation Medium (SEM) for **2 weeks or longer at 21 °C, continuous light.**

**+ 2 weeks:**

17. Individual shoots, 2-4 cm long, are cut off and moved to Root Induction Medium (RIM) (about 10 shoots can be set in the same big jar) for **1 week, 21°C, continuous light.**

18. After **1 week** individual shoots are moved to Root Elongation Medium (REM) at **21°C, continuous light.**

19. Transfer shoots with short roots to greenhouse.



Stable Agrobacterium Transformation of *Lotus japonicus* Protocol

## Media

**Cocultivation Medium (CoM):** mix 387 mg readymade B5 salts including vitamins in 1 liter of water and autoclave. Before use add 5 mM MES – pH 5.2, 3 mg/l kinetin and 3 mg/l 2,4-D, 11.2 mg/l B5-vitamins.

**LO<sup>+</sup> Medium:** for 1 liter add 3.87 g of readymade B5 salts including vitamins and 20 g of sucrose, adjust pH to 5.5, put 10 g of Agar Noble (Fisher Scientific J10907 - # 15875078) into the bottle and autoclave.

- **Callus Medium (CM):** prepare 1 liter of LO<sup>+</sup> Medium, cool it and add 3 mg/l kinetin and 3 mg/l 2,4-D, 112 mg/l of B5 vitamins, 300 mg/l fresh made Cefotaxime solution. Pour it into 9-cm Petri dishes.
- **Selective Callus Medium (SCM):** prepare 1 liter of LO<sup>+</sup> Medium, cool it and add 3 mg/l kinetin and 3 mg/l 2,4-D, 112 mg/l of B5 vitamins, 300 mg/l fresh made Cefotaxime solution, 15 mg/l Hygromycin or 25 mg/l G418. Pour it into 9-cm Petri dishes.
- **Shoot Induction Medium (SIM):** prepare 1 liter of LO<sup>+</sup> Medium, add 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to **hot** medium, then cool it and add 0.2 mg/l of BAP, 112 mg/l of B5 vitamins, 300 mg/l fresh made Cefotaxime solution, 15 mg/l Hygromycin or 25 mg/l G418.
- **Shoot Growth Medium (SGM):** prepare 1 liter of LO<sup>+</sup> Medium, cool it and add 0.2 mg/l of BAP, 112 mg/l of B5 vitamins.
- **Shoot Elongation Medium (SEM):** prepare 1 liter of LO<sup>+</sup> Medium, cool it and add 112 mg/l of B5 vitamins.

**L<sup>+</sup> Medium:** for 1 liter of half-strength B5 Medium add 1.94 g of readymade B5 salts including vitamins and 10 g of sucrose, adjust pH to 5.5, put 15 g of Agar Noble (Fisher Scientific J10907 - # 15875078) into the bottle and autoclave.

- **Root Induction Medium (RIM):** prepare 1 liter L<sup>+</sup> Medium, cool it and add 56 mg/l of B5 vitamins and 0.5 mg/l of NAA stock solution.
- **Root Elongation Medium (REM):** prepare 1 liter L<sup>+</sup> Medium, cool it and add 56 mg/l of B5 vitamins.

### Stock solutions

- **2,4-D at 3 mg/ml:** dissolve 75 mg 2,4-D in 12 ml 96% ethanol, and add water to 25 ml. Filter-sterilized into 1 ml aliquots and stored at -20°C.
- **B5 Vitamins 112 mg/ml:** dissolve mg112 x 40 = g 4.48 in 40 ml H<sub>2</sub>O. Filter-sterilized into 1 ml aliquots and stored at -20°C.
- **BAP at 1 mg/ml:** dissolve 25 mg BAP in 0.5 ml 1 M NaOH, and add water to 25 ml. Filter-sterilized into 1 ml aliquots and stored at -20°C.
- **Cefotaxime at 300 mg/10 ml** for 1 litre medium: dissolve 300 mg in 10 ml H<sub>2</sub>O, filter-sterilize, and use the same day.
- **Conserve** (Spinosad, insecticide): 30 ml distilled water + 22.5 microliter sterile filtered *Conserve* in one Epi
- **Geneticindisulfat (G418)-Lösung 50 mg/ml, sterile:** Roth Nr. CP11.2, 20 ml, € 82.90.
- **Hygromycin B-Lösung 50 mg/ml, steril:** Roth Nr. CP12.1, 10 ml, € 106.50.
- **Kinetin at 3 mg/ml:** add 75 mg kinetin to 0.5 ml 6 M HCl \*  
(do not add HCl to kinetin: it doesn't dissolve!) and dissolve (difficult), add 20 ml water, titrate solution to pH 2.0 with 1 M KOH, and add water to 25 ml. Filter-sterilized into 1 ml aliquots and stored at -20°C.
- **MES 1 M:** dissolve g 9.76 in 50 ml H<sub>2</sub>O, pH 5.2, filter-sterilize.
- **NAA at 0.5 mg/ml:** dissolve 12.5 mg in 12 ml 96% ethanol and add water to 25 ml. Filter-sterilized into 1 ml aliquots and stored at -20°C.
- **(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 4 M:** dissolve 52.85 g in 100 ml H<sub>2</sub>O and filter-sterilize (difficult to filter, put only 20 ml into a syringe).

\* HCl 37% ~ 12 M -> 0.5 ml 6M HCl ~ (0.300 HCl 37% + 0.200 ml H<sub>2</sub>O)

weight Kinetin on weigh paper;

give ml 0.300 HCl 37% into one Epi, add ml 0.200 ml H<sub>2</sub>O and mix it;

give a little kinetin into the Epi very slowly and mix,

again, give a little kinetin into the Epi very slowly and mix,

repeat it, etc., do not allow the formation of clumps!

The solution becomes yellow. Give it into a 50 ml Falcon, add water until 20 ml and mix, the little clumps will disappear!

Put it on magnetic mixer and check pH, it would be about 1.2

Give KOH 1M until pH will be 2

Add water until 25 ml

Filtrate Aliquot 1 ml

-20°C

Stable Agrobacterium Transformation of *Lotus japonicus* Protocol

	Medium to autoclave				
	CoM	LO <sup>+</sup>	LO <sup>+</sup>	L <sup>+</sup>	L <sup>+</sup>
	1 Liter	1 Liter	5 Liters	1 Liter	5 Liters
<b>B5 Salts including Vitamins</b>	mg 387	g 3,87	g 19,35	g 1,94	g 9,70
<b>Sucrose</b>		g 20	g 100	g 10	g 50
<b>pH</b>		5,5	5,5	5,5	5,5
<b>Agar Noble in the bottle</b> (Fisher Scientific J10907 - # 15875078)		g 10	g 10	g 15	g 15

Weeks	Medium	Stocks/liter					
<b>CoM</b> dark 21°C 1 week	CoM	MES 5 ml	B5 vit. 0,1 ml	Kin 1 ml	2,4-D 1 ml		
<b>CM</b> light 26°C 1 week	LO <sup>+</sup>		B5 vit. 1 ml	Kin 1 ml	2,4-D 1 ml	Cefotaxime 300 mg in 10 ml	
<b>SCM</b> light 26°C 5 weeks	LO <sup>+</sup>		B5 vit. 1 ml	Kin 1 ml	2,4-D 1 ml	Cefotaxime 300 mg in 10 ml	300 µl Hygromycin or 500 µl G418
<b>SIM</b> light 26°C 3 weeks or longer	LO <sup>+</sup>	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ml 1.25	B5 vit. 1 ml		BAP ml 0.2	Cefotaxime 300 mg in 10 ml	300 µl Hygromycin or 500 µl G418
<b>SGM</b> light 26°C 2 weeks	LO <sup>+</sup>		B5 vit. 1 ml		BAP ml 0.2		
<b>SEM</b> light 21°C 2 weeks or longer	LO <sup>+</sup>		B5 vit. 1 ml				
<b>RIM</b> light 21°C 1 week	L <sup>+</sup>		B5 vit. ml 0.5		NAA 1 ml		
<b>REM</b> light 21°C 2 weeks or longer	L <sup>+</sup>		B5 vit. ml 0.5				
<b>Greenhouse</b>							

**Stable Agrobacterium Transformation of *Lotus japonicus* Protocol**

Transformation #.....Plant.....

Plant selection.....

Construct made by ..... Chair group.....

Note (see back)	Date	Week	Petris			Little Jars (Liter 0.25)			Big Jars (Liter 0.50)	
			Agros	CM	SCM	SIM	SGM	SEM	RIM	REM
		1								
		2								
		3								
		4								
		5								
		6								
		7								
		8								
		9								
		10								
		11								
		12								
		13								
		14								
		15								
		16								
		17								
		18								
		19								
		20								
		21								
		22								
		23								
		24								
		25								
		26								
		27								
		28								
		29								
		30								
		31								
		32								
		33								
		34								
		35								
		36								
		37								
		<b>Sum</b>								



Stable Agrobacterium Transformation of *Lotus japonicus* Protocol

Date	Week	Petris		Little Jars (Liter 0.25)		Big Jars (Liter 0.50)																																																												
		Agros	CM	SCM	SJM	SGM	SFM	RJM	REM																																																									
24/06/2020	1	8																																																																
01/07/2021	2		8																																																															
08/07/2021	3			8																																																														
15/07/2021	4				7																																																													
22/07/2021	5			6																																																														
29/07/2021	6			6																																																														
10/08/2021	7			6																																																														
20/08/2021	8				7																																																													
01/09/2021	9			10																																																														
09/09/2021	10				12																																																													
16/09/2021	11				14																																																													
23/09/2021	12				16																																																													
30/09/2021	13				17	1																																																												
07/10/2021	14				17	1																																																												
14/10/2021	15				16	1	1																																																											
22/10/2021	16				15	2	1																																																											
28/10/2021	17				13	1	1																																																											
04/11/2021	18				7	1	2	1																																																										
11/11/2021	19				6	1	2	0	1																																																									
18/11/2021	20				3	0	3	1	0																																																									
26/11/2021	21				1	1	1	0	1																																																									
02/12/2021	22				0	0	2	3	0																																																									
09/12/2021	23				0	0	1	0	2																																																									
16/12/2021	24						3	3	0																																																									
23/12/2021	25							0	0	4																																																								
30/12/2021	26																																																																	
06/01/2021	27																																																																	
11/01/2021	28								3																																																									
19/01/2021	29								0	3																																																								
26/01/2021	30								3	0																																																								
02/02/2021	31								0	3																																																								
	32																																																																	
	33																																																																	
	34																																																																	
	35																																																																	
	36																																																																	
	37																																																																	
	38																																																																	
	39																																																																	
	40																																																																	
	41																																																																	
	42																																																																	
	Jars Sum	8	8	33	154	9	17	14	14	14																																																								
<p><b>Transformation # 001</b></p> <table border="1"> <thead> <tr> <th>Media</th> <th>Agros</th> <th>CM</th> <th>SCM</th> <th>SJM</th> <th>SGM</th> <th>SFM</th> <th>RJM</th> <th>REM</th> <th>Cost/liter</th> <th>Transformation Cost</th> </tr> </thead> <tbody> <tr> <td>Liter Selective Callus Medium :</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td>14.56 €</td> <td></td> </tr> <tr> <td>Liter Shoot ind.+ Growth + Elong. Medium :</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td>14.56 €</td> <td></td> </tr> <tr> <td>Liter Root Induction + Elongation Medium :</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td>13.08 €</td> <td></td> </tr> <tr> <td><b>Total</b></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td><b>42.20 €</b></td> <td><b>231.22 €</b></td> </tr> </tbody> </table>												Media	Agros	CM	SCM	SJM	SGM	SFM	RJM	REM	Cost/liter	Transformation Cost	Liter Selective Callus Medium :									14.56 €		Liter Shoot ind.+ Growth + Elong. Medium :									14.56 €		Liter Root Induction + Elongation Medium :									13.08 €		<b>Total</b>									<b>42.20 €</b>	<b>231.22 €</b>
Media	Agros	CM	SCM	SJM	SGM	SFM	RJM	REM	Cost/liter	Transformation Cost																																																								
Liter Selective Callus Medium :									14.56 €																																																									
Liter Shoot ind.+ Growth + Elong. Medium :									14.56 €																																																									
Liter Root Induction + Elongation Medium :									13.08 €																																																									
<b>Total</b>									<b>42.20 €</b>	<b>231.22 €</b>																																																								