PLANT TRANSFORMATION BY AGRO INFECTION

- Cut tobacco leaves into 0.5 - 1 cm^2 squares. Avoid the mid-rib and the primary nerves, and don't damage the surface of the squares. Of course, all work is to be done in a laminar flow hood.

- Put 10-20 leaf squares to float on 10 ml of A10 in a Petri dish, upside up (and don't squash the leaves with the forceps in the process).

- Infect the 10 ml with 50-100 µl of Agrobacteria overnight culture (in MGL medium) grown at 28°C. A control without Agrobacterium will suffice.

- Keep the plates for 2-3 days under low light density (avoid direct light).

- Transfer the leaves to a fresh dish with 10 ml of A10. Incubate for 15 minutes, and swirl gently 2-3 times to allow bacteria to come off the plant cells.

- Suck away the liquid with an automatic pipette (disposable plastic pipette) and replace by 10 ml of A10. Incubate a further 5-10 minutes and swirl gently 2-3 times for the same reason as before.

- Repeat wash 3 further times the final wash is carried out using A10 supplemented with 500 µg/ml cefotaxime (a bacteriostaticum).

- Transfer the leaves to solid A11 medium (5-10 leaves /plate) and press them very gently to the surface to allow good contact (but you don't want to have the edges under the surface).

- Transfer to a fresh plate after approx 7 days.

- Transfer to a fresh plate after approx 10 more days.

- Transfer calli of 1-2 mm on A12 medium, 50 calli/plate. This allows good contact with the medium to ensure proper selection as well as nutrition. This incubation can be up to 2 weeks but no longer (cefotaxime is unstable). From this moment on, the orientation of the callus should not be changed.

- Place calli of 5 mm in small jars containing A13 (A12 but no NAA) and incubate for a further 2 weeks. If no shoots of good quality appear after 2 weeks, this step can be repeated (weird looking regions of the calli can be cut off).

- Cut shoots with a sharp scalpel and place on MS2 medium. Do not push the stem too deep in the Agar. You can place 5 shoots in one medium jar, because not every shoot will form roots. No selective pressure is required at this stage (it would just slow down root formation).

- Well developed shoots with roots can be directly analyzed for the gene product (but make sure you label the plant if more than one are present in one jar). Plants that should be kept for further analysis are cut at an internode close to the apex and place on standard MS2 medium.

Usually, 50 -100 independent transgenic plants should be obtained this way. It is no luxury to do this if you want to find good overexpressing plants (make optimal use of multiple insertions, position effect etc...)

	A10	**A11**	**A12**	**A13**	**800ml**
B5	+	+	+	+	2.56 g
NH_4NO_3(250 mg/l)	+	+	+	+	200 mg
MES (500 mg/l)	+	+	+	+	400 mg
Glucose (2%)	+	+	+	+	16 g
Agar (0.75%)	-	+	+	+	6 g
Adenine (40 mg/l)	-	+	+	+	32 mg
pH 5.7 (KOH)					

Added prior to use of medium:-

	A10	**A11**	**A12**	**A13**	**Stock Conc**
BAP	1 μg/ml	1 μg/ml	1 μg/ml	1μg/ml	1mg/ml
NAA	0.1 μg/ml	0.1 μg/ml	0.1 μg/ml	-	1mg/ml
Cefotaxime	-	500 μg/ml	200 μg/ml	200 μg/ml	200mg/ml
Selective pressure					
Kanamycin	-	100 μg/ml	100 μg/ml	100 μg/ml	100mg/ml
or					
Phosphinothricine	-	5 μg/ml	5 μg/m1	2 μg/ml	

BAP = 6-benzylamino purine
NAA = α-naphthalene acetic acid