# The basic protocol used for any *Agrobacteruim* mediated transformation experiments

## Step 1: Identification a suitable explant with regeneration protocol

It includes selection of the explant from which a complete plant can be regenerated through a well-defined regeneration protocol. The protocol may be taken from literature as it is or modified to get better results. In case of absence of previous literature about this plant, researcher must design its own through trying and error.

**Practical:** Indirect regeneration protocol for hypocotyl explants of Canola (*Brassica napus*)….induction by MS + 1 mg/l 2,4-D, Shoot formation on MS + mg/l 4 mg/l BA, Root formation on MS + 0.5 mg/l IBA

## Step 2: Cultivation of *Agrobacterium* in liquid medium

**Note:** *Agrobacterium tumefaciens,* LBA 4404 strain harboring the binary vector pCAMBIA 1301, was used for transformation. The plasmid contains hygromycin phosphotransferase (*hpt*) and ß-glucuronidase (*gus*) genes as plant selectable marker and reporter genes, respectively. The plasmid backbone contains aminoglycoside phosphotransferase gene (for kanamycin resistance) as bacterial selectable marker gene.

So, *Agrobacterim* is inoculated into liquid medium (LB or YEB medium) containing the antibiotic (the resistance for which is encoded by the bacterial selectable marker gene on T-plasmid backbone) to get rid of bacterial cells that lost the vector. Cultures are maintained on shaker till its $O.D_{600}$ reach 1-1.5.

**Practical:** Preparation of YEB medium (1000 ml) supplemented with Kanamycin (50 mg/l). YEB medium containing 5g Peptone, 5g beef extract, 5g sucrose, 1g yeast extract and 0.24g magnesium sulfate in one liter. Divide the media into 10 parts (100 ml per flask) and sterilize the medium in autoclave for 20 minutes at 1.5 atm. pressure and 121 ºC. In laminar flow cabinet, add 0.1 ml filter-sterilized Kanamycin stock solution to each flask using sterile tip. In laminar flow cabinet, inoculate each flask with a single *Agrobacterium* colony. Cultures were incubated for 18 hrs at 25 °C on shaker (150 rpm) to reach $O.D_{600}$ reach 1-1.5.

**Step 3: Co-cultivation of explants with the *Agrobacterium***

The explants were cut and soaked (for certain period) into the previously prepared liquid culture of *Agrobacterium* (diluted with liquid MS medium). This medium was supplemented with acetosyringone (phenolic compound), which increases virulence of *Agrobacterium* by triggering vir genes. Very diluted cultures (as measured with $OD_{600}$) or very short time of incubation highly reduce the transformation efficiency while sever treatments cause necrosis and death of explants.

The explants are subsequently removed from the bacterial solution and placed onto acetosyringone-supplemented MS solid medium. The incubation time of explants with *Agrobacterium* also needed to be optimized to allow maximum transformation efficiency and avoid necrosis of explants.

**Practical:** Preparation of 100 µM / L acetosyringone. In laminar flow cabinet: Add 0.1 ml filter-sterilized acetosyringone stock solution (100 µM /ml) to bacterial solution using sterile tip. In laminar flow cabinet, dilute bacterial culture with liquid MS medium to obtain 100 ml culture having the desired $OD_{600}$. Put hypocotyl explants in bacterial solution for the determined time. Pick explants and place them on sterile filter paper. Culture the explants on solid MS medium containing 100 µM/l acetosyringone. Cultures were incubated for 48 hrs at 25 °C in dark.

**Step 4: Killing the *Agrobacterium* with a suitable antibiotic**

The explants are removed from the medium and washed in antibiotic (cefotaxime) solution that kills *Agrobacterium* cells. In laminar flow cabinet, prepare liquid MS medium containing 500 mg/l cefotaxime. Add 1 ml filter-sterilized cefotaxime stock solution (50 mg /ml) to 100 ml liquid MS medium using sterile tip. Wash explants with liquid MS medium containing 500 µM/l cefotaxime. Collect explants and put them on sterile filter paper.

**Step 5: Selection of transformed explants and regeneration of transformed plants**

The explants are transferred to fresh solid medium (the medium used for regeneration, either directly or indirectly) supplemented with a selective agent (the antibiotic whose resistance is encoded by the plant selectable marker gene on T-DNA). The medium should be also supplemented with cefotaxime to ensure killing any remaining

*Agrobacterial* cells. Only transformed explants will survive and regenerate plants while explants that did not received T-DNA will die.

**Practical:** Preparation of solid callus induction medium containing 500 mg/l cefotaxime and 30 mg/l hygromycin. In laminar flow cabinet, add 1 ml filter-sterilized cefotaxime stock solution (50 mg /ml) and 0.06 ml filter-sterilized hygromycin stock solution (50 mg /ml) using sterile tips. Culture the explants on the medium and incubate them for 2 weeks (with weekly subculture) at 25 °C under cool-white fluorescent light (1000 Lux irradiance) with 16-hour photoperiod.

Preparation of solid regeneration medium containing 500 mg/l cefotaxime and 30 mg/L hygromycin. In laminar flow cabinet, add 1 ml filter-sterilized cefotaxime stock solution (50 mg /ml) and 0.06 ml filter-sterilized hygromycin stock solution (50 mg /ml) using sterile tips. Put explants carrying calli on the medium. Cultures were incubated for 4 weeks (with weekly subculture) at 25 °C under cool-white fluorescent light (1000 Lux irradiance) with 16-hour photoperiod.

Preparation of solid rooting medium containing 500 mg/l cefotaxime and 30 mg/L hygromycin. In laminar flow cabinet, add 1 ml filter-sterilized cefotaxime stock solution (50 mg /ml) and 0.06 ml filter-sterilized hygromycin stock solution (50 mg /ml) using sterile tips. Put explants carrying shoots on the medium. Cultures were incubated for 2 weeks at 25 °C under cool-white fluorescent light (1000 Lux irradiance) with 16-hour photoperiod. Regenerated shoots are micropropagated on MS medium containing 30 mg/L hygromycin.

**TEST:**

Regeneration on media containing the plant selectable marker is not an evidence for transformation. Plant cells may acquire resistance for such marker due to genetic variations occurring in vitro (somaclonal variation). Consequently, the regenerated plants must be subjected to several tests to confirm their transformation. These tests include test for presence of the reporter gene (Gus test), PCR-based test for the gene(s) of interest and southern blot. Western blot can be also used to confirm the presence of protein encoded by transgene(s).

In each test, the percentage of plants giving +ve results must be calculated to determine the transformation efficiency.

Transgenes must be followed in next generations to confirm their presence. The corresponding control for each test is derived from regenerated plant not subjected to transformation step.

**GUS Test:**

Leaves were incubated overnight, at 37 °C, in a staining solution containing:

1.0 $mgl^{-1}$ of 5-bromo-4-chloro-3-indolyl-*β*-glucuronide,

0.5% Triton X-100,

20% methanol

50 mM sodium phosphate buffer (pH 7).

The chlorophyll of leaves was distained by rinsing in 95% ethanol. Transformed leaves were turned to blue colour, while non-transformed leaves remain colorless.