

## **Transformation of *Arabidopsis thaliana* using *Agrobacterium tumefaciens***

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week # 1 (12 February)

week # 8 (08 April)

*Agrobacterium tumefaciens* is a soil bacterium that causes a serious plant disease known as crown gall. *Agrobacterium* causes this disease by inserting a piece of DNA into the plant's nuclear genome. The inserted DNA contains genes coding for enzymes required for the synthesis of unusual amino acids called opines as well as genes that result in aberrant production of plant hormones. Insertion of the DNA into the plant genome thus causes the disruption of normal cell growth resulting in the formation of large unorganized tumors which produce the opines. The bacteria can then grow on the tumors, using the opines as a food source.

The bacterial DNA that is transferred to the plant cell (the T-DNA) is present in the bacterium on a large plasmid called a Ti (tumor-inducing) plasmid. The T-DNA is bordered on each side by special DNA sequences called the T-DNA borders. Any DNA that is present between these borders will be transferred to the plant and inserted into the nuclear genome. This feature allows plant scientists to utilize *Agrobacterium tumefaciens* as a valuable research tool. If the tumor-inducing genes are removed from the Ti plasmid and replaced with a gene(s) that we wish to put into a plant, the altered *Agrobacterium* can then be used as a vehicle for plant transformation.

We will be using a strain of *Agrobacterium* called GV3101 (see Figure 1). This is a "disarmed" strain, which means that the DNA containing the tumor inducing genes has been removed from the Ti plasmid. In order to use this bacterial strain for plant transformation, an additional plasmid, called pBI101, has been added. pBI101 contains a *gusA* gene (which encodes  $\beta$ -glucuronidase) and a *nptII* gene (which provides resistance against the antibiotic kanamycin), both enclosed within T-DNA border sequences. This means that the bacteria will be able to transfer the *gusA* gene and *nptII* gene (but not any tumor-inducing genes) into plant cells. The *gusA* gene represents a useful gene that we would like to insert into our experimental plants. The *nptII* gene serves as a selectable marker, since any plant cells that have incorporated the "T-DNA" will become resistant to kanamycin.

We will inoculate immature flowers of *Arabidopsis thaliana* plants with the *Agrobacterium* strain GV3101 containing plasmid pBI101. Insertion of T-DNA into gametophytic tissues of the flowers can result in the production of transgenic seeds that will grow into transgenic plants (containing both the *gusA* gene and the *nptII* gene).

### **EXPERIMENTAL PROCEDURE**

#### **I. Inoculation**

Obtain a saturated 200 mL culture of *Agrobacterium tumefaciens* (GV3101 containing pBI101) grown overnight at 30° in YEP containing 25  $\mu$ g/ml kanamycin. Take a 1:10 dilution of the bacterial culture and measure its OD<sub>600</sub>. Harvest the cells by centrifugation at 5,500 x g at room temperature for 20 minutes. Resuspend the cells in Inoculation Medium to an OD<sub>600</sub> of approximately 0.8. Place 400 mL of the Inoculation Medium into a 600 mL beaker.

Obtain one pot of young *Arabidopsis* plants containing many flower buds. Invert the pot and place the plants into the Inoculation Medium such that all above ground parts are submerged.

Gently agitate the plants for 3 to 5 seconds and then remove them from the beaker. Place the pot onto a plastic tray and cover the plant to maintain high humidity.

Keep the plants covered up in dim light for 24 hours to allow them to recover from the inoculation procedure.

### **Maintenance and seed collection**

Place the plants in the growth chamber and let them grow for 3 to 4 weeks to produce seeds. As the plants mature, tape the inflorescences together to keep them from falling over.

When the siliques (fruits) are brown and dry, collect the seeds by gently pulling the groups of inflorescences through your fingers over a large white coffee filter. Remove contaminating stem and pod material by gently blowing. Store your seeds in a microcentrifuge tube.

### **II. Selection of transgenic plants**

Measure out 3 aliquots of about 500 seeds (about 30 mg) each and place each aliquot into a 15 mL centrifuge tube. Sterilize each seed aliquot by adding 3 mL of 95% ethanol to each tube and gently agitating for 1 minute. Carefully pour out the ethanol and add 3 mL of 50% bleach containing 0.05% Tween 20 to each tube. Agitate gently for 5 minutes. Working inside a sterile laminar flow hood, pour out the bleach and rinse the seeds 3 times with 10 mL sterile water. Suspend each aliquot of seeds in sterile water.

Continuing to work under sterile conditions, use a pipet to squirt one of your seed suspensions onto a 100 mm petri dish containing Selection Medium. Spread the seeds out as well as you can over the surface of the agar. Place the lid on the petri dish and place the seeds in the dark at 4° for 2 days. Then move the petri dishes to a lighted incubator with a temperature of 24°. All of the seeds should now germinate, but only plants containing the *nptII* gene will be able to produce dark green leaves and well-established roots in the Selection Medium. Once the transgenic (kanamycin-resistant) seedlings can be clearly distinguished from the nontransformed seedlings, the transgenic seedlings should be transferred to soil.

### **RECIPES FOR MEDIA**

#### YEP

10 g Bactotryptone

5 g yeast extract

5 g NaCl

water to 1 liter

autoclave 20 min. at 121°, then add filter sterilized antibiotics

#### INOCULATION MEDIUM

50 g sucrose

500 µL Silwet L-77 surfactant

water to 1 liter

autoclave 20 min. at 121°,

### SELECTION MEDIUM

2.2 g MS salts  
10 g sucrose  
water to 1 liter

adjust pH to 5.7 with KOH  
add 10 g agar  
autoclave 20 min. at 121°, then add filter sterilized antibiotics  
(selection medium should contain 50 mg/l kanamycin)

### **USEFUL REFERENCES**

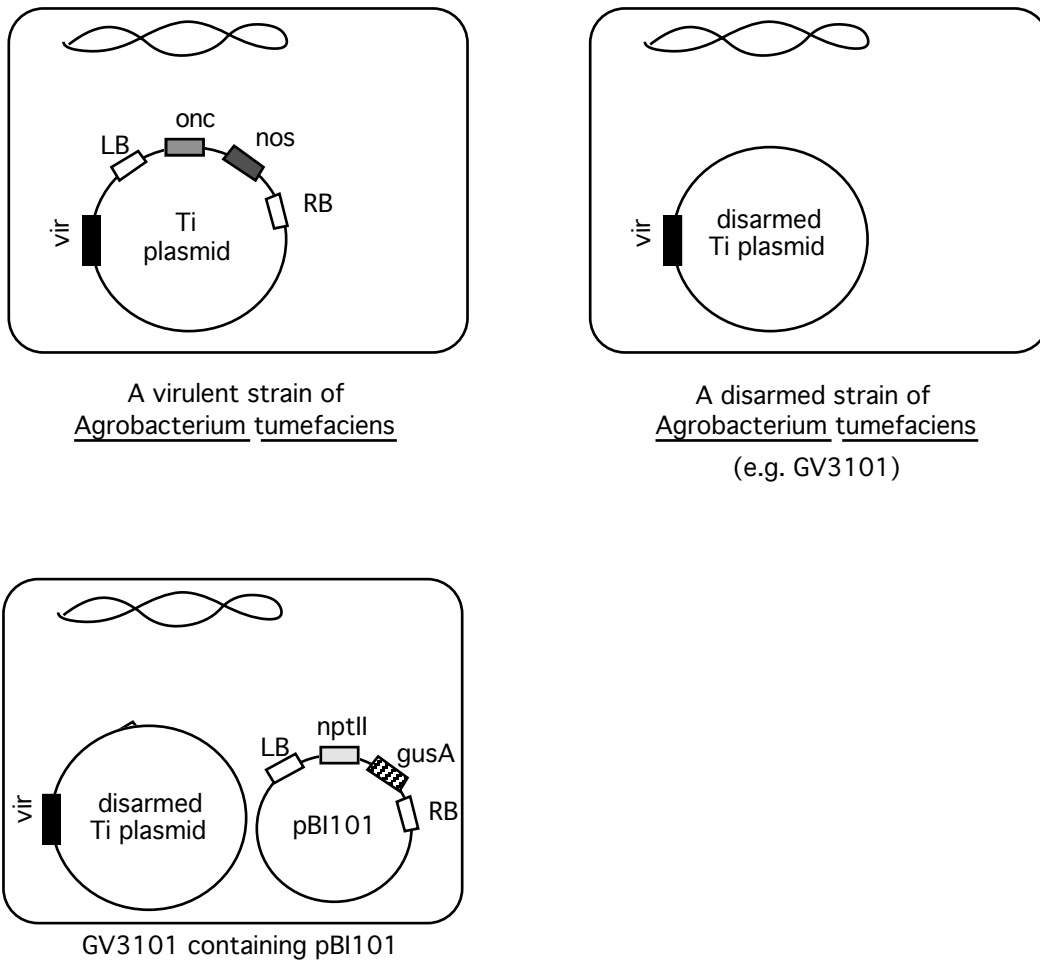
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Hooykaas and Shilperroort (1992) *Agrobacterium* and plant genetic engineering. *Plant Mol. Biol.*  
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Clough, S.J. and Bent A. F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16:735-743

### **PREPARATION OF ARABIDOPSIS PLANTS FOR FLORAL DIP TRANSFORMATION**

Allow *Arabidopsis* plants to grow (about 1 month) until the primary flowering stalk appears. Cut off the primary flowering stalk to allow the growth of more secondary flowering stalks. Do the transformation when the secondary stalks have just a few open flowers.



**Figure 1.** Plasmids present in different strains of *Agrobacterium tumefaciens*.

*vir* genes - genes required for T-DNA transfer

LB - the left T-DNA border

RB - the right T-DNA border

*onc* genes - genes which cause aberrant hormone production in plant cells resulting in the growth in tumors

*nos* gene - encodes an enzyme required for opine synthesis

*nptII* - a gene encoding the enzyme neomycin phosphotransferase. The enzyme inactivates the antibiotics neomycin and kanamycin.

*gusA* - a gene encoding the enzyme  $\beta$ -glucuronidase