PLNT2530 Plant Biotechnology
*Lab1: AGROBACTERIUM TUMEFACIEN*S MEDIATED PLANT CELL TRANSFORMATION
(Part I)

One of several ways of introducing foreign DNA into plant systems (i.e. generation of transgenic plants) is using the Agrobacterium infections system. Agrobacterium strains such as *A. tumefaciens* and *A. rhizogenes* are natural soil bacteria which parasitize susceptible plants by transferring a specific segment of DNA, the T-DNA region, of a bacterial plasmid (Ti plasmid) into plant cells. This DNA is inserted into the nuclear DNA of the plant. Expression of the wild type genes encoded on this T-DNA segment can alter the normal development of the transgenic plant cells in several ways including uncontrolled cell division (tumor production) and synthesis of unique metabolites, opines, which the plant cannot use but which the invading Agrobacterium can.

Agrobacterium does not efficiently infect and transform all plant species. In fact different strains of Agrobacterium exhibit different host ranges. The specific requirements for successful infection are currently being elucidated. Agrobacterium invades a plant only at a site of injury. In this lab you will create the injury by producing a cut surface. This wounding causes the release of specific phenolic compounds at the wound sites. These compounds activate virulence genes in the bacteria which in turn catalyse the binding of the bacterium to a plant cell wall and subsequently the replication of the T-DNA region and its transfer of the T-DNA into the plant cell.

In this experiment the T-DNA region of the Ti plasmid of an *A. tumefaciens* strain which you will be using, has been engineered to remove most of the wild type genes (including those coding for enzymes synthesizing an auxin and a cytokinin) and two new genes incorporated into this region. The single wild type gene retained encodes octapine synthase, an enzyme responsible for the synthesis of octapine, one of the unique metabolites mentioned above. The new genes encode the enzymes β-glucuronidase (GUS) and neomycin phosphotransferase II (NPTII).

While the antibiotic kanamycin is toxic to most plant cells, those cells having and expressing the NPTII gene are tolerant to this antibiotic by virtue of gene product's ability to phosphorylate kanamycin and thereby detoxify it. Thus the presence of this gene allows one to differentiate between transformed cells and non-transformed cells on the basis of their ability to grow on a kanamycin-containing media. The β-glucuronidase is utilized as a readily detectable marker as it can catalyse the release of a fluorescent product from a non-fluorescent substrate. Because of the linearity of the enzyme reaction and the low level of endogenous β-glucuronidase activity in non-transformed plant cells, the level of expression of the T-DNA encoded genes can also be examined using this enzyme.

Procedure (**Bring permanent ink marking pen to labs for labelling plates.)**

Sterilization of Tissue and Preparation of Leaf Disks**:**

Tissue obtained from fully expanded green leaves of 1-2 month old *N. tabaccum* will be used as explants with this transformation system. Leaves which have been removed from healthy plants should be briefly washed with distilled water, followed by immersion in 70% ethanol (2 min). Rinse the material with sterile distilled water and immerse in a 1% NaOCl solution (1/5 dilution of commercial chlorox) for 10-20 min. If younger leaves are used the time of exposure to hypochlorite may need to be reduced to avoid excessive bleaching of the tissue. In a laminar flow hood rinse leaf pieces several times with sterile distilled water and transfer to a sterile petri plate.

Using a sterile hole punch cut approximately 25 discs into a sterile petri plate containing a small volume of sterile water to prevent desiccation (avoid the midrib).

Infection with Agrobacterium**:**

A. tumefaciens strain MP90 which carries the binary T-DNA vector pBI121 will be grown overnight at 28°C in LB media containing 50 µg kanamycin/ml. The cells will be measured at an optical density of 620 nm to determine the concentration (using 5 x10^8 cells/ml for 1 OD_{620}) and then gently centrifuged (5500xg, 10 min) to pellet the bacteria. Cells will be resuspended in MS (Murashige & Skoog) complete media without hormones at a cell density of 10^9-10^{10} cells/ml. This exchange of media removes the kanamycin containing bacterial growth media which would inhibit callus development.

Transfer your leaf disks to a new petri plate containing the bacterial-MS media suspension. Float the disks to incubate for approx. 2 min. Disks should then be individually removed, blotted carefully on a sterile paper towel to remove excess liquid, and placed adaxial side (upper leaf surface) up in petri plates (approx. 12 disks/plate; 2 plates/student) containing the following medium:

Co-cultivation media

MS mineral salts
0.6 g/L MES
B5 vitamins [Plates of all required media will be
3% sucrose prepared in advance by the demonstrator.]
0.8% agar
1.0 mg/l BAP (6-benzylaminopurine) (cytokinin)
0.1 mg/l NAA (α-naphthaleneacetic acid) (auxin)
100 µM acetosyringone

The first students completed should cut additional disks for 3 control plates. These disks should be immersed in MS media (NO Agrobacterium) and blotted and plated as was done for the other plates. These will be used for the regeneration of non-transformed tissue and plants. These plates should be clearly marked as **Controls**.

Label all plates lids with initials, seal with a double layer of parafilm and incubate in the dark (a drawer).

After 48 h. The explants treated with Agrobacterium will show bacterial growth around the edges by this stage. Both control and treated explants must be transferred to plates containing the same medium supplemented with carbenicillin (500 mg/L). This antibiotic kills *A. tumefaciens* as well as other frequent contaminants. (If the initial bacterial population is very high, several subculturings may be required; check the cultures every 24 h and at the first signs of bacterial growth, subculture onto fresh carbenicillin-containing media.) Explants will be maintained on this media to induce the development of callus along the cut surfaces.

Selection and Shoot Development Media
MS mineral salts
MES
B5 vitamins
3% sucrose
0.8% agar
1.0 mg/l BAP
0.1 mg/l NAA
500 mg/l carbenicillin
50mg/ml kanamycin sulfate

The plate conditions are designed to i) select for transformed callus and ii) induce this callus to undergo differentiation and shoot regeneration.

Note: Control disks should be transferred fresh plates of the same media except these should contain no kanamycin

N. tabaccum cells are sensitive to kanamycin levels above 20-30 mg/L. (This can be demonstrated by plating a small number of control explants on a kanamycin containing plate.) Thus, this culture step allows the detection of non-transformed explants, which are visible by the lack of growth or cell proliferation

The calli that show shoot formation on their surface (these are easily recognized by the development of green spots on their surfaces) should be excised and transfered onto fresh media. (Ensure treated and control calli are place on the correct media! ie. +kan/-kan) Under continuous light conditions, the development of green leaflets should be evident after one week.

Once shoot formation is well developed in transformed tissues carefully remove each developing shoots from treated explant derived material and place them onto root induction medium. Note that this media contains no exogenous hormones. If shoot development has occurred auxin will be produced that should provide the necessary hormone for root formation.

Root Induction Media
M.S. salts
MES
B5 vitamins
0.8% agar
3.0% sucrose
50 mg/ml kanamycin

This medium supports the development of roots and plantlet growth, which is evident after 1-2 weeks. Plantlets showing root development are likely to be transformed and will be used for the screening of the β-glucuronidase activity. A small number of control plants might be placed on this media to see the effect of kanamycin on normal plants. Other control plants should be placed on this same media without kanamycin to allow normal rooting of the control plants.

Note: You are required to make weekly observations and comparisons of tissue development as outlined under Lab Report at the end of Part II

Note: Students should be familiar with the types of changes to be anticipated at the various stages of plant regeneration. A weekly log summarizing the **OBSERVED** changes should be maintained, recording development as well as specific changes which occur following any change in media. These changes will need to be summarized and discussed on a scale of days post-infection. You will also be asked to discuss why the hormone composition of the media was modified.

Reference

Chetty VJ, Ceballos N, Garcia D, Narváez-Vásquez J, Lopez W, Orozco-Cárdenas ML. Evaluation of four *Agrobacterium tumefaciens* strains for the genetic transformation of tomato (Solanum lycopersicum L.) cultivar Micro-Tom. Plant Cell Rep. 2013 Feb;32(2):239-47. doi: 10.1007/s00299-012-1358-1. Epub 2012 Oct 26. PMID: 23099543.

This paper identifies strain MP90 as one of Jefferson's *A. tumefaciens* strains carrying pBI121. The cite the 1987 paper as carrying pBI121, although that paper doesn't specifically mentions MP90 by name.