PLNT2530 Plant Biotechnology
*Lab1: Agrobacterium tumefacien*s mediated plant cell transformation

(Part II, Confirmation of Transformation)

Part I of this lab began with the transformation of tobacco explants with two genes (see P 11 of lab manual). Over the course of the term the transformed cells have been regenerated first into callus then through changes in media induced to undergo organogenesis to regenerate whole plants. The first gene (NPTII) provided the means to select for transformed cells by making them immune to the effects of kanamycin, however survival and regeneration of non-transformed "escapes" can happen. Today's lab will provide a more direct method of testing if the recovered plants are transformed or not. The confirmation of successful transformation is based on the demonstration of the presence of the gene product from the second gene. The GUS gene product is an enzyme (β-glucuronidase), which is absent from untransformed tobacco. You will assess the presence of the enzyme by demonstrating if a specific reaction is catalysed or not. In the case of β-glucuronidase the reaction being assessed is the cleavage of methylumbelliferyl β-glucuronide to methyl umbelliferone.

GUS Enzyme Reaction:

β-glucuronidase (GUS)

methylumbelliferyl β-glucuronide (MUG) ----------> methyl umbelliferone (MU)
(non-fluorescent substrate) (fluorescent product)

Extraction:

(NOTE at least one control, non-transformed plant, should be assayed as well as putative transformants)

1. Cut leaf samples (0.5 g) into small pieces and placed into a prechilled mortar (on ice).

2. In a fume hood add 2.0 ml of chilled GUS extraction buffer (50 mM $NaPO_4$, pH 7.0, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% Triton X-100) and homogenize with a pestle. It is very important to homogenize the leaf tissue well, as this results in maximium cell breakage and release of the cytoplasmic contents (including the GUS enzyme). If a sufficient fraction of the leaf cells are not broken the activity, which may be present, will not be detected.

3. Centrifuge (10,000g, 5 min, 4˚C) the homogenate to remove cell debris. Recover the supernatant and store on ice.

GUS Assay:

4. Assay the supernatant for GUS activity by preparing the following digest and blank reactions. The methylumbelliferyl β-glucuronide (MUG) 0.5 mM 4-methylumbelliferyl β-D-glucuronide (MUG) is prepared in GUS extraction buffer. Prepare digest reactions in duplicate with extracts from both non-transformed and transformed plants. Also prepare blanks in duplicate.

	MUG substrate	extract supernatant	GUS extraction buffer
Digest	1.0 ml	0.1 ml	0 ml
Substrate Blank	1.0 ml	0 ml	0.1 ml
Extract Blank	0 ml	0.1 ml	1.0 ml

All digest and blank reactions should be incubated at 37 °C for 30 min.

Several blank reactions must be run to account for fluorescence which may arise from other sources other than the enzymic conversion. The extract blank assesses if there is any contribution to overall flourescence from molecules in the extract while the substrate blank takes into consideration the fact that there may be some non-enzymic breakdown of the substrate prior to or during the incubation period. These contributions need to be measured and subtracted from the total fluorescence so that only fluorescence due to enzyme cleavage is measured.

5. After the 30 min incubation, a 0.2 ml aliquot from each reaction and blank is removed and added to separate 1.8 ml aliquots of Stop Buffer.

6. Mix and measure fluorescence of blanks and diluted digests with the fluorometer. Read the display immediately.

To quantitatively measure the amount of product produced by GUS activity, the level of fluorescence must be measured using a fluorometer (λ_{ex}=365nm, λ_{em}=460nm) and compared to the amount of fluorescence emitted from a known molar amount of the fluorescent product, 4-methyl umbelliferone (MU). The fluorometer will be standardized with 0 and 50 nM solutions of MU.

7. Record your results on the lab whiteboard. You will need to record the results of everyone in the lab for your report.

Lab Report

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 should be summarized and discussed on a scale of days post-infection.

References:

R.W. Old & S.B. Primrose, Principles of Gene Manipulation 3rd Ed. Blackwell Scientific Publications, 1985 p 215-230 (there are later editions as well)

T.M. Murphy & W.F. Thompson, Molecular Plant Development, Prentice Hall, 1988, p 184

R.A. Jefferson et al., J. Molecular Biology 193: 41-46, 1987