NOTES FOR DEMONSTRATOR

*Agrobacterium tumefaciens* Mediated Plant Cell Transformation

PLANT MATERIAL:

Tobacco plants should be started for this 2nd term laboratory 6-8 weeks before required ie. November 10-15.

AGROBACTERIA:

Two weeks before the laboratory, the *Agrobacteria tumefaciens* (strain MP90 obtained from B. Crosby at PBI) carrying the GUS gene and NPTII gene should be taken from long-term culture (-70C) and streaked on an LB agar plate containing 50 ug kanamycin/ml. The plate normally requires 2 days at 20-25C for adequate growth. Growth on kanamycin containing media ensures selection of bacteria carrying the Ti plasmid. This plate is used to inoculate 5 ml liquid cultures which in turn is used (0.5 ml) to inoculate 30 ml overnight liquid cultures.

For preparation of the Agrobacteria for this laboratory we require:

4 -LB agar plates with Kan (50 ug/ml)

3 -5 ml LB-Kan liquid media in screw capped pyrex tubes

6 -30 ml LB-Kan liquid media in 250 ml flasks

1 200 ml MS complete liquid media

Prepare necessary volume of LB media, aliquot required volumes into containers (add agar if required) and autoclave. For plate media allow solution to cool (55C) before aseptically adding kanamycin from filter sterilized stock solution (10 mg kanamycin/ml water, in freezer). Liquid LB media can be stored until required at which time an appropriate volume of stock kanamycin solution is aseptically added.

LB media per liter

yeast extract 5 g

NaCl 10 g

tryptone 10 g

Dissolve in 950 ml of deionized water, adjust pH to 7.0, (add 15 g agar/litre for plate media), dilute to 1 litre and autoclave.

The sterile MS liquid media is used to replace the Agrobacteria culture media prior to treatment of leaf discs. Culture metabolites and kanamycin have a negative influence on callus development.

CULTURE MEDIA REQUIREMENTS:

The basic MS media (0.5L) including sucrose, B5 vitamins and hormones (but not antibiotics) can be prepared in bottles, autoclaved and stored at room temperature until required. As required the media can be melted, allowed to cool to 55-65C before the appropriate amounts of filter sterilized antibiotics are added and the media poured into plates.

Cocultivation Plating Media

MS mineral salts (from 10X stock) 2 plates/student plus a few extra

0.6g/L MES

1.0ml B5 vitamins (from 1000X stock)

3% sucrose

0.8% agar

1.0 mg/L BAP

0.1mg/L NAA

100µM Acetosyringone \* Add when media is cooled to 55oC

pH to 5.7 before autoclaving

Selection/Shoot Regeneration Media

(Bactericide, Transformant Selection)

MS mineral salts

0.6g/L MES 2 plates/student

1.0 ml B5 vitamins

3% sucrose

0.8% agar

1.0 mg/l BAP

0.1mg/L NAA

200 mg/l carbenicillin \*

50 mg/l kanamycin \*

pH to 5.7 before autoclaving

1 plate/student of the same media except lacking kanamycin - for control leaf disks.

Root Induction Media (Bactericide - Transformant Selection)

MS salts

MES 5 plates and 20 vials of this media

B5 vitamins in total.

0.8% agar

3.0 sucrose

50 mg/l kanamycin \*

2 plates and 5 vials of this media but lacking kanamycin.

pH to 5.7 before autoclaving

FOR THE INITIAL LAB

NaOCl (Javex), 2 L Sterile water Parafilm strips

70% EtOH, Sterile forceps Sterile scalpels

 Sterile paper towels Sterile paper punch

**Plasmid DNA Lab**

pBI121 is a binary vector, low copy number plasmid. For better DNA yield grow the pBI121 agro culture for 3 to 4 days. Need to have a large cell culture.

**GUS EXTRACTION AND ASSAY REAGENTS**

Stocks:

a) Na2HPO4 fwt -141.96 For 0.10 M 0.705 g/50 ml

b) NaH2PO4.H2O fwt -137.99 For 0.10 M 0.690 g/50 ml

GUS Extraction Buffer (min 50 ml)

50 mM NaPO4, pH 7.0

 10 mM ß-mercaptoethanol (14.4 M neat)

10 mM EDTA

0.1% Triton X-100

For 50 mM pH 7.0 buffer:

mix 30.5 ml of a) + 19.5 ml of b)

69 ul β-mercaptoethanol

2 ml of 0.5 M EDTA stock

100 ul triton X-100

dilute to 100 ml

GUS Assay Buffer

 0.5 mM 4-methylumbelliferyl β-D-glucuronide (MUG) in GUS extraction buffer.

(Dissolve 4.4 mg of MUG in 20 ml extraction buffer and store in a disposable 50 ml polypropylene tube at 4C)

Methylumbelliferone Stock Solution

 0.5 mM methylumbelliferone (MU) in Stop buffer

(Dissolve 9.9 mg sodium MU in 50 ml in d-H2O, protect from light and store at 4C.)

MU Standards

100 uM 200 ul of MU stock + 800 ul Stop buffer

 10 uM 300 ul of 100 uM dilution + 2700 ul Stop Buffer

1.0 uM 300 ul of 10 uM " + 2700 ul " "

0.1 uM 300 ul of 1.0 uM " + 2700 uL " "

0.05uM 1.0 ml of 0.1 uM “ + 1.0 ml Stop Buffer

Stop Buffer

 0.2 M Na2CO3

(Dissolve 2.12 g Na2CO3 in 100 ml d-H2O)

(1990 - the standard range of the MU recommended 0.1-100uM exceeds the linear range of the instrument and was greater than the range required. The standardization can be done for a range up to 5 uM.) 1993 - the curve is linear up to 1.0 uM, put in an additional standard at 0.5 uM.

(revised May/97)

**MS Stock Media (10X)**

NH4NO3 16.5 g

KNO3 19.0

MgSO4.7H2O 3.7

KH2PO4 1.7

CaCl2.2H2O 4.4

H3BO4 62.0 mg

MnSO4.4H2O 233.0

ZnSO4.7H2O 86.0

KI 8.3

Na2MoO4.2H2O 2.5 ------

CuSO4.5H20 0.25 ------ Prepare 1.0 mg/ml stocks of these

CoCl2.6H2O 0.25 ------ to ensure accurate additions.

EDTA,Na2 372 mg

FeSO4.7H2O 278 mg

An alternative to these last two is to use

sequestrene 400 mg

Inositol 1.0 g

Sucrose 300 g

Dissolve 1.0 liter of water, aliquot into 100 ml lots and freeze in whirlpak bags until required. Each bag will make 1 liter of MS media.

**B5 Vitamins (1000x stock) 100 ml**

Pyrodoxine-HCl 100 mg

Nicotinic acid 100 mg

Thiamine-HCl 500 mg

Dissolve in 100 ml and store at 4C or frozen. Require 1 ml of stock for each liter of media.

**Notes: 1993** By two weeks post infection the control and treated leaf discs have grown to almost double their original size and changed from dark green to a yellow green. Some of the treated discs have not changed. These appear to be water soaked and have neither grown nor changed color. Many have bacteria on the upper surface of the leaf although not at the perifery where there is immediate contact with the media and carbenicillin. Transfer to Kanamycin containing plates was carried out at 2 weeks (Feb 2) as visible callus was apparent at this time. Those discs which had not responded were discarded. The tissues are continuing to expand with some browning at Feb 8. Some callus is showing signs of shoot differentiation

Feb 16 - Transfer to induction plates and under light

Mar 5 - Many of the shoots stopped growing and have not greened. Very few green shoots are showing this year. The control plates are overgrown to the point of totally filling the plate.

Mar 16 - Students transferred control plant into rooting media.

Mar 20 - Transferred potential transformants to a Kan+ rooting media. (late but plants still small).

Mar 23 - Two of ten control plants show root development. Some tissues seem to have 'stalled' on transfer.

**Notes 1995**: The presence of 2,4 D in the callus media should be tested relative to a comparable media without 2,4 D. Shoot initiation in the absence of it was much, much faster in Don's experiment on effects of different hormone conditions.

**Notes 1997:** Tobacco plants -start seedling in a warm growth chamber for faster growth (`25 C, no temp drop at night). MP90 glycerol stock from 1993grew very well with a heavy growth in liquid media after 24 h. Used cells at a density of 1x109 cells/ml - ended up with 150 ml. Calli developed well. Transferred to selection plates within 2 weeks. Calli formation stalled after 1 month. Don’t allow tissue to stay on same plate for more than 2 weeks.

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