Protocol I

Culturing of Plant Cell Suspension Cultures

Purpose: Plant cells can be grown under sterile conditions as suspension or callus cultures and given the appropriate hormonal supplements to the growth media can be induced to regenerate into an entire plant. This phenomenon is unique to plants and is termed totipotency. Cell cultures (plant, animal, microbial, etc.) also offer advantages for certain types of experimental manipulations because the culture conditions are easily manipulated. This protocol details procedures necessary for the maintenance of plant cell suspension cultures.

1. Preparation of Murashige and Skoog nutrient media - 1 liter

    4.3 gm MS salts (contains essential salts, nitrogen, potassium, phosphorus, etc.)
    2 ml Gamborg's vitamins
    5 ml 0.2 mg/ml 2,4-dichlorophenoxyacetic acid (hormone)
    30 gm sucrose
    dissolve mixture in 800 ml distilled or purified water
    adjust pH to 5.4-5.6
    bring volume to 1 liter
    dispense into Erlenmeyer flasks (50 ml/250 ml flask, 100 ml/500, 200 ml / 1 l)

    autoclave 20 min.

    For plates, add 8 gm agar before autoclaving

2. Wipe down laminar flow hood with 70% ethanol

3. Flame tops of flasks

4. Dilute cell cultures with fresh media if necessary

5. Dispense cultures into flasks containing fresh media (25 ml culture into 50 ml fresh media

6. Shake cells at approximately 100 rpm (provides aeration)

7. Subculture the cultures whenever the cultures appear to deplete media source.

Protocol II

Induction of Plant Defense Responses

Purpose: Attempts to investigate plant defense responses are complicated by two factors. One, the interaction is generally limited to a very few cells and thus, biological material to examine is limiting. Two, it is often difficult to discern if a response is derived from the plant or the pathogen when investigating an intact system. Fortunately, plant defense responses to microbial pathogens can be stimulated by challenging plant cell cultures with cell surface components or extracellular proteins of fungal pathogens. The pathogen derived factors that can induce plant defense responses are referred to as elicitors (they elicit a response). The following protocol describes an induction treatment of tobacco cell suspension cultures to cellulase, a fungal extra-cellular enzyme isolated from *Trichoderma viride*.

1. Preparation of Cellulase. Dissolve 1 mg of T.v. cellulase in 1 ml H_2O and then filter sterilize

2. Using standard sterile technique and working in the laminar flow hood, add 5 µl of 1µg/µl cellulase to a set number of microtiter wells., followed by 5 ml of approximately 4 day old tobacco cell suspension culture. (Calculate the final cellulase concentration)

3. At designated times from 1 to 24 h after cellulase addition, add 5 ml of H_2O, then separate and collect the extracellular media using a Büchner funnel lined with miracloth. Samples should be frozen for later analysis.

Protocol III

Extraction of Media for Phytoalexins (Antibiotics)

Purpose: One of the defense responses plants mount to pathogen challenge is the production of antibiotic compounds referred to as phytoalexins. These antibiotics accumulate in intra- and extra-cellular spaces. The purpose of this protocol is to extract and concentrate the phytoalexins produced by elicitor-induced tobacco cell cultures, and to carryout a preliminary examination of the phytoalexins by thin-layer chromatography (TLC).

Extraction Procedure

Equipment:	pear shaped collection flasks separatory funnel glass pipettes rotoevaporator autosampler vials positive displacement pipetters (if available)
Solvents:	chloroform hexane

Protocol

1. Rinse collection flasks and separatory funnels with chloroform

2. Pure entire media sample into separatory funnel with closed stopcock.

3. Add 20 ml chloroform, secure glass stopper, invert funnel, open stopcock, and gently shake for 15 sec.

4. Close stopcock, invert funnel, remove glass stopper and let stand for 1 to 2 min.

5. Drain lower phase into collection flask

6. Repeat extraction

7. Secure collection flask with chloroform extract onto rotoevaporator. Evaporate to dryness

8. Resuspend dried extract with 200 µl hexane and transfer to autosampler vials.

Thin-Layer Chromatography (TLC) Procedure

Equipment:	silica thin-layer sheets chromatography tank glass atomizer spray box positive displacement pipetters (if available)
Developing solvent:	cyclohexane:acetone (1:1)
Indicator reagent:	1.4 g vanillin 40 ml methanol 250 µl H_2SO_4

Protocol

1. Mark TLC plate with pencil (1.5 cm up from bottom, 1.5 cm spacing)

2. Spot 20 µl aliquots of samples and allow samples to dry

3. Develop TLC plate until solvent has moved 7 cm past application zone. Remove plate and dry.

4. Spray plate with indicator reagent and heat with hair dryer for color development.

5. Describe the results.

Protocol IV

Bioassay for Phytoalexins (Antibiotics)

Purpose: The observation of extracellular compounds produced by plants in response to pathogen challenge is not sufficient to concluded that these compounds are defense compounds. Bioassays are a means of assessing the relative toxicity of the putative phytoalexins.

Cladosporium cucumerium bioassay

Equipment:
- TLC plates
- incubation chamber
- plates of *Cladosporium cucumerium*
- cheesecloth
- glass atomizer
- spore harvesting media

Protocol

1. Run samples on TLC plates as in previous laboratory.

2. Allow the TLC plate to dry. Should not be able to smell any solvent.

3. Harvest *Cladosporium* spores. Using 3-5 plates of fungi, flood first plate with 5 ml of harvesting buffer, scrape mycelium mat into solution, swirl, pour onto next plate, and repeat above procedure. Filter mycelium extract through 2 layers of miracloth.

4. Pour spore suspension into atomizer and spray TLC plate until it is almost saturated.

5. Incubate the TLC plate in a moist, dark environment for 5 to 10 days at room temperature.

Protocol V

Gas Chromatography and Mass Spectrometry

Purpose: In the previous experiment, organic extracts of the extracellular media from control and elicitor-treated cells were examined by TLC. That analysis provided a qualitative analysis for changes in the extracellular components, but did not give any information about the relative abundance nor the identity of the compounds. Gas chromatography is a sensitive method for the separation and quantitative analysis of compounds that can be volatilized. Coupled with Mass spectrometry, GC-MS can provide key information to elucidate chemical structures while requiring only minute amounts (ng to µg) of sample. Of course, other techniques such as NMR can provide more definitive identification, especially with regards to stereo isomers, but these methods often require very large amounts of purified compound (mg) for analysis. The objectives for this laboratory are: 1. to establish a standard curve relating GC detection to absolute amounts of hexadecane (a standard provided); 2. Quantify the amount of any new compounds appearing in the media of elicitor-treated cells; 3. Use GC-MS data to predict a structure for the new compound(s) appearing in the extracts of the elicitor-treated cells; and 4. propose a biochemical pathway for the synthesis of these compound(s).

Equipment:
- Gas Chromatograph
- GC-MS data sheets
- 10 µg/ml hexadecane in hexane
- hexane

Protocol

Group A

1. Make solutions of hexadecane at 2, 10 and 50 ng/µl.
2. Inject 0.5 µl samples of hexadecane solutions into GC
3. Plot peak areas versus amounts of hexadecane

Group B

1. Resuspend chloroform extracts with 200 µl of an external standard (hexadecane)
2. Inject 0.5 µl of samples
3. Calculate and plot the amount of new compound(s) present in the extracts from control and elicitor-treated cells.

Protocol VI

Measuring De Novo Synthesis Rates of a Metabolite

Purpose: The observation of extracellular sesquiterpenes produced by plants in response to pathogen challenge suggests that the cells somehow control this process. This process can be regulated at several levels. One way is that the cells synthesize constitutively and sequester the sesquiterpenes within the cell and only release these compounds upon challenge with a pathogen. Another possibility is that the biosynthetic machinery responsible for the synthesis of sesquiterpenes is "activated" in response to pathogen challenge, resulting in the synthesis and secretion of the sesquiterpenes. There are several ways one could test for these possibilities. Measuring the *in vivo* synthesis rate of sesquiterpenes before and after pathogen challenge is one way to determine if the sesquiterpene biosynthetic capacity of the cells changes.

Equipment:
- 4 ml cell cultures at various stages within the induction trt
- Büchner funnels and Miracloth
- vacuum flasks
- 50/15 ml collection tubes
- equipment for chloroform extractions

Protocol

1. Set-up 2 x 4 ml of cells at various stages of induction treatment (This needs to be done Monday evening and Tuesday morning prior to Tuesday afternoon lab)
2. Add 25 µl of 14C-acetate (equivalent to 250,000 dpm) to the cell cultures and continue incubation for a minimum of 2 hours. Don't forget to count an aliquot of the label added to the cultures.
3. Harvest and collect cells and media. Add 10 ml water to each flask before collecting sample. Cell sample should be labeled and frozen (-20^{o}C) until later laboratory.
4. Extract media sample with chloroform (10 ml sample, twice extracted with 20 ml chloroform).
5. Rotoevaporate the samples to dryness.
6. Final resuspension of media extract should be 100 µl. Determine the amount of radioactivity in a 10 µl aliquot.
7. Run 20 to 50 µl aliquots of the chloroform extracts on silica TLC plates in cyclohexane:acetone solvent, scrape zone corresponding to capsidiol into a scintillation vial, and determine dpm incorporated into capsidiol.

Protocol VII

Measuring De Novo Synthesis Rates of an Intracellular Metabolite

Purpose: In a previous laboratory, we pulse-labeled cells with radioactive acetate to determine if the elicitor-inducible, extracellular sesquiterpenes were synthesized de novo. In this laboratory, we will determine if the de novo synthesis rates of sterols changes in response to elicitor-treatment. Would you expect the sterol biosynthesis rate to change or remain the same? Describe in mechanistic terms how the biochemical pathway for sesquiterpenes and sterols might be regulated to account for both of these possibilities.

Equipment:
- shaker
- large test tubes
- 500 ml side-arm flasks
- glass pipettes

Solutions:
- 10 mM NaOH in MeOH
- MeOH
- $CHCl_3$
- EtOH:Acetone (1:1)
- acetone:diethyl ether (1:2)
- 10 mM cholesterol in EtOH:Acetone
- 2% digitonin in 50% EtOH

Protocol

1. Add 2 ml 10 mM NaOH in MeOH to 0.8-1 gm cells and shake at 150 rpm for 2 hrs.
2. Carefully collect MeOH extract using a 1 ml pipetteman.
3. To the cellular residue, add 0.4 ml H_2O, 1.0 ml $CHCl_3$, and 1.0 ml MeOH, shake for 30 min.
4. Collect $CHCl_3$:MeOH extract using a 1 ml pipetteman.
5. To the cellular residue, add 1.0 ml $CHCl_3$ and 1.0 ml MeOH, shake for 30 min.
6. Collect $CHCl_3$:MeOH extract using a 1 ml pipetteman.
7. Resuspend cellular residue with 1.0 ml H_2O and count 0.25 ml of the sample.
8. Pool organic extracts, add 2 ml of $CHCl_3$ and 2 ml H_2O, mix, centrifuge 1,000 to 2,000 rpm of 5 min.
9. Remove lower organic phase to conical collection flask, and roto-evaporate until dryness.
10. Resuspend residue with 1 ml EtOH: acetone, determine dpm in 200 µl aliquot
11. Mix 400 µl of sample with 200 µl of 2% digitonin in 50% EtOH. If a precipitate is not obvious, add 10 µl of 10 mM cholesterol as a carrier (What is meant by carrier?). Let precipitate form for at least 1 hour (or until next lab. period).

12. Collect precipitate by centrifugation for 5 min. Wash pellet twice with acetone:ether, remembering to centrifuge between washes.
13. Resuspend pellets with EtOH:acetone (400 µl), transfer to scintillation vials, and determine dpm.

Protocol VIII

Enzyme Assay for Sesquiterpene Cyclase

In previous experiments, we observed that cell cultures pulse-labeled with radioactive acetate differentially incorporated the radioactivity into sterols and sesquiterpenes, depending on elicitor-treatment of the cell cultures. From a proposed biochemical pathway for these two metabolites, we inferred that the branch point enzymes might be controlling the flow of carbon into sterols and sesquiterpenes. If this were the case, then the sesquiterpene cyclase, the enzyme dedicating carbon to sesquiterpene biosynthesis, should be absent in control cells and present in elicitor-treated cells. In this laboratory, we will learn how to prepare cell-free extracts and to measure the sesquiterpene cyclase enzyme activity.

1. Prepare homogenization buffer ; 100 mL

1M K.P.buffer(7.0)	8 mL
glycerol	20 mL
100 mM Meta bisulfate (sodium meta sulfate)	10 mL
200 mM MgCL2	7.4 mL
ß-ME	100 µL
PVP	1g
Na ascorbic acid	0.2g

2. Grind samples(0.5g) with 2 mL homo. buffer + a small amount of insoluble PVPP.

3. Transfer homogenate to an eppendorf tube and centrifuge at maximum speed for 5 min and save supernatant for assay.

4. Assay

-3H-FPP; 0.1 µL/50 µL assay -5 pmoles (1-200,000 dpm)
-Cold FPP; 0.5 µL/50 µL assay - 1.84 nmoles

Master Mix; for 10 samples
Hot FPP 0.1 x10 = 1 µL
Cold FPP 0.5 x10 = 5 µL
Rx. buffer 38 x10 =380 µL

Reaction buffer;

	5 mL
250 mM Tris 7.0	1.25 mL/ 1M Tris 7.0(FW 121)
50 mM $MgCL_2$	1.25 mL/ 0.2M $MgCL_2$
	2.5 mL/H_2O

Assays;(Total volume = 50 µL)

	blank	A	B
sample	0	2	5 µL
Rx. buffer	10	8	5 µL
Master Mix.	40	40	40 µL

incubate for 30 min. at room temperature to 35^{o}C.

5. Add 150 µL hexane , vortex briefly and spin for 10 sec.

6. Remove 100µL hexane phase to another eppendorf tube containing a small amount of silica gel (230-400 Mesh)

7. Centrifuge for 10 sec.

8. Transfer 50μL to scintillation vial, determine radioactivity and calculate enzyme activity (nmoles of product formed/hr)

Protocol IX

Protein Determination Using Bradford Dye binding Assay

In the last lab, the sesquiterpene cyclase enzyme activity in cells collected at various times after elicitor-treatment was determined. Such determinations require the homogenization of cell samples, but there was no way of knowing whether each sample was homogenized to the same extent or if protein was extracted with the same efficiency. To correct for variation in extraction efficiency, enzyme activity data is often expressed on a per mg of protein basis. This serves to normalize data between samples and allows for easier comparison of data between experiments.

1. Prepare a standard curve using BSA as the standard protein of known concentration.

FOR EXAMPLE: range should be from 0.01 to 10 µg protein

	blank	1	2	3	4
BSA (x µg/µl)	0	2.5	5	10	25 µl
H_2O	800	797.5	795	790	775 µl
Dye	200	200	200	200	200 µl

Use the blank sample to zero the ABS function of the spectrophotometer to 595 nm. Then read experimental samples.

2. Repeat the above procedure except use the supernatant samples you generated in the last laboratory period. Try 5 and 10 µl samples to begin with.

3. Calculate the protein concentration (µg/µl) for the supernatant samples using the standard curve.

4. Using the specific activity of FPP used for the enzyme assays and the protein concentrations determined in this laboratory, calculate the level of cyclase enzyme activity per sample in terms of nmoles of product formed per mg protein • hour. Plot these values against time after elicitor addition.

Protocol X

PARTIAL PURIFICATION OF THE SESQUITERPENE CYCLASE

Observations up to this point have indicated that the cells ability to produce sesquiterpenes requires an activation of the isoprenoid biosynthetic pathway, as indicated by the in vivo labeling studies and measurements of the cyclase enzyme activity. In order to differentiate between various mechanisms that could be responsible for regulating the cyclase enzyme activity, one needs to evaluate the kinetic parameters of the cyclase enzyme and ultimately, to be able to measure the absolute level of cyclase enzyme protein (using immunological means). Both of these determinations require purified cyclase protein.

Typical means for purifying a protein from a mixture such as a cellular homogenate rely on a protein's size, charge, hydrophobicity, isoelectric point and affinity for various ligands. In the experiment below, groups will separate the cyclase protein based on the cyclase's ionic charge using Q-sepharose, an anion exchange resin.

Preparation of columns:

Make sure the columns run – run water through the columns. Make sure it has a good flow rate (>5 ml/min)

Q-Sepharose (2-4 cm column)

1. This resin has been swollen and equilibrate in the running buffer.

2. Pour and rinse the column with running buffer. You will only need a column bed of approximately 5 ml of resin.

Preparation of cyclase sample:

1. Grind samples (4 g) with 2 mL Homo. buffer (10 mM Tris, pH7.5, 15 mM $MgCl_2$, 5 mM mercaptoethanol, 10 mM ascorbate, 10 mM sodium metabisulfide).

2. Decant into eppendorf tube and spin 14,000 rpm for 5 min. Remove the supernatant to a clean eppendorf tube. Don't forget to save an aliquot of the supernatant for enzyme activity assays and protein determinations.

Running the ion-exchange Column:

1. Load 0.5-1 ml of sample directly onto the column bed. Let it draw into the column, then continue to add running buffer (gently, do not disturb the column bed).

2. Wash column with 10 ml running buffer and collect wash solution.

3. Elute column with the following KCL solutions and collect 1 ml fractions, all in running buffer:

i. 2 ml 50 mM KCl
ii. 2 ml 100 mM KCl
iii. 2 ml 200 mM KCl

iv. 2 ml 500 mM KCl
v. 2 ml 2 M KCl

Enzyme assay:

Determine cyclase activity in the initial supernatant and various fractions.

-3H-FPP; 0.1µL/50 uL assay -5 pmoles (100,000 dpm)
-Cold FPP; 0.5µL/50 uL assay - 0.5 µg (molecular wt. 433 g/mole)

Master Mix; for 30 assays
Hot FPP 0.1 x30 = 3 µL
Cold FPP 0.5 x30 = 15 µL
Rx. buffer 38 x30 =1,140 µL

Reaction buffer; 5 mL
250 mM Tris 7.0 1.25 mL/ 1M Tris 7.0
50 mM $MgCL_2$ 1.25 mL/ 0.2M $MgCL_2$
2.5 mL/H_2O

1. Assays;(Total volume = 50 uL)

	blank	supernatant/fraction
sample	0	10 µL
Rx. buffer	10	0 µL
Master Mix.	40	40µL

incubate for 15 min. at room temp.

2. Add 150 uL hexane , vortex briefly and spin for 10 sec.

3. Remove 100µL hexane phase to another eppendorf tube containing a small amount of silica gel(230-400 Mesh)

4. Centrifuge for 10 sec.

5. Transfer 50µL to scintillation vial, determine radioactivity and calculate enzyme activity (nmoles of product formed/hr)

6. Determine protein amounts in the initial supernatant and each fraction using the Bradford dye assay

Protocol XI

Determination of Km and Vmax for sesquiterpene cyclase

To accurately compare the enzyme activity in one cell-free extract to another, one must measure the enzyme activities at substrate concentrations above the Km. The Vmax is another characteristic of an enzyme which influences ones ability to detect enzyme activity in an in vitro assay. In this exercise, you will be given a purified sesquiterpene cyclase preparation, and are to determine these physical constants for the enzyme.

1. Prepare reagents:

enzyme: prepare dilution of 1/100 with reaction buffer

FPP (cold)(1 µg/µl, 433 gm/mole): prepare dilutions of 1/10; 1/100; 1/1,000; 1/10,000 with reaction buffer

^{3}H-FPP (2.5 pmole/µl): prepare a dilution 1/100 with reaction buffer

Reaction buffer:	5 mL
250 mM Tris 7.0	1.25 mL/ 1M Tris 7.0(FW121)
50 mM $MgCL_2$	1.25 mL/ 0.2M $MgCL_2$
	2.5 mL/H_2O

2. Assays(Total volume = 50 µL)

	BG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
enzyme	0	10	10	10	10	10	10	10	10	10	10	10	10	10
cold FPP														
1/10	10	10	3	1										
1/100				10	3	1								
1/1000							10	3	1					
1/10000										10	3	1		
3H-FPP	10	10	10	10	10	10	10	10	10	10	10	10	10	10

Reaction buffer to 50 µl per sample

3. Incubate for 30 min. at room temperature

4. Add 150 uL hexane , vortex briefly and spin for 10 sec.

5. Remove 100µL hexane phase to another eppendorf tube containing a small amount of silica gel(230-400 Mesh)

6. Centrifuge for 10 sec.

7. Transfer 50µL to scintillation vial, determine radioactivity and calculate enzyme activity (nmoles of product formed/hr) for each sample. Determine Km and Vmax.

Protocol XII

Induction Time Course of Sesquiterpene Cyclase Activity in Elicitor-Treated Cells

A previous experiment determined that control cells did not contain sesquiterpene cyclase activity but elicitor-treated cells did. As a means of further characterizing the response of the elicitor-treated cells, you are to measure the cyclase activity in cells that have been treated with elicitor for various lengths of time. After determining the induction pattern for the cyclase enzyme activity, predict what the induction pattern for capsidiol accumulation should be. Describe mechanisms that could account for discrepancies between the observed pattern of capsidiol accumulation to that expected. Describe molecular mechanism(s) that may be operative in cells to explain the induction pattern for the cyclase enzyme activity.

1. Prepare homogenization buffer ; 100 mL

1M K.P.buffer(7.0)	8 mL
glycerol	20 mL
100mM Meta bisulfate (sodium meta sulfate)	10 mL
200mM MgCL2	7.4 mL
BME	100 uL
PVP	1g
Na ascorbic acid	0.2g

2. Grind samples(0.5g) with 1 to 2 mL Homo. buffer

3. Filter through 2 layers of nylon(optional), spin 8000 rpm for 5 min and save supernatant for assay.

4. Assay

-3H-FPP; 0.1µL/50 uL assay -2.5 pmoles ($4.4x10^5$ dpm)
-Cold FPP; 0.5µL/50 uL assay - 0.694 nmoles

Master Mix; for 15 samples
Hot FPP 0.1 x15 = 1.5 µL
Cold FPP 0.5 x15 = 7.5 µL
Rx. buffer 39 x15 =585 µL

Reaction buffer;

	5mL
250mM Tris7.0	1.25mL/ 1M Tris 7.0(FW121)
50mM $MgCL_2$	1.25mL/ 0.2M $MgCL_2$
	2.5mL/H_2O

Assays;(Total volume=50uL)

	blank	A	B
sample	0	5	10 µL
Rx. buffer	10	5	0 µL
Master Mix.	40	40	40µL

incubate for 30min. at 35^oC

5. Add 150uL hexane , vortex briefly and spin for 10sec.

6. Remove 100µL hexane phase to another eppendorf tube containing a small amount of silica gel(230-400Mesh)

7. Centrifuge for 10 sec.

8. Transfer 50µL to scintillation vial, determine radioactivity and calculate enzyme activity (nmoles of product formed/hr)

9. Determine protein concentrations in all the supernatant samples.

Protocol XIII

Immunodetection of the Sesquiterpene Cyclase Protein

Your previous experiments have shown that tobacco cell cultures respond to fungal elicitor treatment by the synthesis and secretion of anti microbial sesquiterpenoids, and that the synthesis of these compounds is correlated with an induction of a sesquiterpene cyclase enzyme activity. The next quest is to investigate the mechanisms controlling the induction or activation of this enzyme. Two possibilities to consider are: 1, the enzyme is always present, but can be in an inactive and active state; or 2, the enzyme protein is absent from control cells, but present in elicitor-treated cells. One way to distinguish between these two possibilities is to measure the absolute amount of the protein present in the control and induced cells.

The technique used to make such measurements is referred to as Western blotting or immunodetection. In essence, protein samples are separated on polyacrylamide gels contain SDS where proteins are separated on the basis of size, then those proteins are transferred from the gel to a nylon membrane using electrophoresis again, and finally the cyclase protein is detected on the nylon membrane using antibodies that react specifically with the cyclase protein.

Gel Electrophoresis

1. Preparing a SDS polyacrylamide gel.

A. The separating gel 11%

30% acrylamide	3.5 ml
4X running buffer	2.5 ml
water	3.4 ml
TEMED	20 µl
	mix and degas
10% ammonium persulfate	50 µl

mix and add to glass plate sandwich, fill to 1 cm below teeth of comb, and overlay with isopropanol. When separating gel is polymerized, remove isopropanol, rinse interface with water, and carefully dry with Whatmann paper

B. The stacking gel 5%

30% acrylamide	855 µl
4X stacking buffer	1.25 ml
water	3.0 ml
TEMED	10 µl
	mix and degas
10% ammonium persulfate	25 µl

Fill glass sandwich and add comb.

2. While the stacking gel is polymerizing, prepare the samples to be run on the gel. A preferred sample is prepared by mixing 50 µg protein in 25 µl with 15 µl of a 2X loading buffer. Samples are heat denatured at 95oC for 3 to 5 min.

3. When the gel has polymerized, remove the comb, rinse wells with water and assemble the apparatus for electrophoresis. Add 1X tank buffer to the gel and buffer reservoirs, and then load samples.

4. Electrophoresis at 32 mAmps (constant current) until the voltage reaches 150 volts. Then run the gel at 175 Volts (constant voltage) until the tracking dye reaches the leading edge of the gel.

Transferring Protein from a Gel to a Nitrocellulose Membrane

5. When gel is finished running, disassemble the apparatus and prepare the transblotter for protein transfer to nitrocellulose. Have ready before the gel is finished running the following cut to exactly the size of the gel: 1 piece of nitrocellulose and 4 pieces of Whatmann 3mm paper

6. Assembling the transfer sandwich. In order of bottom to top, 2 pieces of 3mm soaked with transfer soln. 1 piece of nitrocellulose soaked in transfer soln , the gel, and 2 pieces of 3mm soaked in transfer soln.

7. Transfer the proteins from the gel to the nitrocellulose membrane by electrophoresis at 25 volts (constant voltage) overnight.

Immunodetection of Protein

8. After transfer of the proteins to the membrane, disassemble the transfer apparatus and place the membrane in blocking solution (5% dried milk in a Tris-buffered saline solution), and agitate for a minimum of 30 min. Add antibody at 1/1000 dilution and continue incubation overnight.

1x TBS: for 1 liter	2.4 gm Tris 29 gm NaCl, pH 7.5

9. Wash blot 5 times with 1x TBS

10. Add 25 ml of blocking solution (5% dried milk in 1x TBS), add 25 µl of goat anti-mouse antibody conjugated with alkaline phosphatase (AP), and continue incubation for 1 hour with gentle agitation.

11. Wash blot 5 times with 1x TBS.

12. Prepare AP substrate

a. dissolve 1 mg 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 200 µl dimethyl formamide (DMF)

b. dissolve 10 mg nitro blue tetrazolium (NTB) in 70 µl DMF and add 3x 10 µl water while mixing

c. Combine BCIP, NBT and 1.5 ml 10x AP buffer, bring to 15 ml with water

10x AP buffer: for 100 ml	12.1 gm Tris 5.8 gm NaCl 1 gm $MgCl_2$, pH 9.5

13. Wash blot 1 time with 1x AP buffer

14. Add AP substrate to blot and incubate 10 to 30 min for color development. Stop by washing the blot with water.

Protocol XIV

ISOLATION OF GENOMIC DNA

The previous experiments have shown that tobacco cell cultures respond to fungal elicitor treatment by the synthesis and secretion of antimicrobial sesquiterpenoids, and that the synthesis of these compounds is correlated with an induction of a sesquiterpene cyclase enzyme activity, and in the last experiment, that the induction of cyclase enzyme activity was correlated with the induction of the cyclase protein. How could the level of the cyclase protein be regulated like this? One possibility is that somehow elicitor treatment induces transcription of the cyclase gene, which results in elevated levels of the cyclase mRNA, which is translated and results in the accumulation of the cyclase protein. An alternative explanation is that the cyclase mRNA is always present (constitutive transcription of the cyclase gene) and that elicitor-treatment induces enhanced translation of the cyclase mRNA. One way to distinguish between these two possibilites is to measure the actual amount of the cyclase mRNA. This is conveniently done using a technique known as RT-PCR, reverse transcription polymerase chain reaction, which can measure the relative abundance of a single mRNA species within a complex mixture. Before such an experiment can be done, however, several preparatory experiments must be done to verify the method. These include designing PCR primers and demonstrating that the primers will specifically amplify a cyclase gene from genomic DNA.

The next few protocols detail the steps necessary to validate the cyclase specific PCR primers.

1. Powder freshly harvested young leaves in liquid nitrogen

2. Transfer 100 mg of tissue into an eppi tube and homogenize with 0.5 ml of extraction buffer

3. Add 0.5 ml phenol:chloroform:isoamyl alcohol (25:24:1), vortex and spin in microfuge for 5 min.

4. Remove aqueous phase (upper layer) to clean eppi tube. Add1 µl of 10 mg /ml RNAse A and incubate 20 min at room temperature

5. Extract the sample with phenol (add equal volume of phenol, vortex, spin for 5 min)

6. Remove aqueous phase to clean eppi tube.

7. Extract sample with chloroform (add equal volume of chloroform, vortex, spin for 5 min)

8. Remove 250 µl of the aqueous layer (upper layer) to a clean eppi tube, and overlay with 250 µl isopropanol. Spool the precipitated DNA – gently stir the mixture with a yellow pipette tip. The genomic DNA will come out of solution as stringy white material.

9. Before the isopropanol has totally mixed with the aqueous layer, remove the clotted DNA with a pipette, taking as little isopropanol as possible, and transfer the DNA to an eppi tube containing 1 mL of 70% EtOH.

10. Repeat the 70% EtOH wash.

11. Transfer the DNA to an eppendorf tube, spin briefly, withdraw as much liquid as possible, then use a hair dryer to dry the DNA.

12. Add 0.1 to 1 ml of 1X TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) and let the DNA dissolve until the next lab period at 4^{o}C.

Extraction buffer:

100 mM Tris pH 8.0
20 mM EDTA
0.5 M NaCl
0.5 % SDS
0.5 % ß-mercaptoethanol

NEXT LAB PERIOD

9. Remove a 10 µl aliquot of the resuspended gDNA to 600 µl of H_20 and determine its absorbance at 260, 280 and 300 nm.

10. Assuming 1 Abs unit at 260 nm is equivalent to 50 µg of DNA/ml, calculate the concentration of your DNA sample

Protocol XV

Amplification of a Cyclase Gene Fragment

The goal of this laboratory is to amplify out a fragment of the cyclase gene from genomic DNA using the polymerase chain reaction. Based on sequence information of the isolated cyclase protein, you designed oligo primers and are now to use these primers to isolate a fragement of the cyclase gene. The next couple of labs are determine if the PCR reactions worked, to clone the amplified PCR products and to verify that the cloned DNAs correspond to the cyclase gene.

Polymerase Chain Reaction

1. Prepare a 1:20 dilution of template DNA(e.g. genomic DNA) and dilute primers to a concentration of 100 to 500 µg per mL

2. Combine the following in a 500 µL eppendorf tube:

	A	B	C
DNA template (500 ng)	2.5 µl	2.5 µl	2.5 µl
primer 1	1 µl	0	1 µl
primer 2	0	1 µl	1 µl
2.5 mM dNTPs	5 µl	5 µl	5 µl
10x buffer	2.5 µl	2.5 µl	2.5 µl
50 mM MgCl2	0.75 µl	0.75 µl	0.75 µl
water	adjust volume to 25 µl final		
Taq DNA polymerase	1µl	1µl	1µl

3. Overlay with 50 µl paraffin oil (viscosity 125/135)

4. Add a few drops of paraffin oil to wells to be used in thermocycler heat block and insert tubes

5. A typical PCR program follows, however, optimum conditions must be determined empirically depending on your template, primers, etc.

PCR 50 program:

step1: 92C 1'30"
step2: 50C 2'
step3: 72C 2'
step4: 30 times
step5: 72C 10'
step6: 4C indefinitely
step7: End

6. Check 5 µl samples on a 1% agarose gel in the next lab period.

Preparing a gel

1. Weigh out 0.25 gm of agarose and transfer to 125 erlenmyer flask.

2. Add 25 ml of 1X TBE (this is a buffer salt solution)

3. Heat in a microwave oven, med to low power for 1-3 min. Mix the agarose solution half way through the heating cycle. Make sure all the agarose is in solution before proceeding to the next step. Add 1µl of a 10mg/ml ethidium bromide solution.

4. Pour the melted agarose into the gel molding tray. Make sure the ends of the tray are sealed and that a comb (wide tooth) is in position.

5. Allow the gel to set for 20 to 30 min. Then submerge the gel in the electrophoresis tank containing 1X TBE buffer.

Loading the gel

1. Mix 5 µl of PCR reaction product with 5 µl H_2O and 2 µl of loading dye (contains glycerol and a tracking dye).

2. Load 10 µl aliquots of the samples into a well. You should also load a molecular weight standard - a sample containing DNA molecules of different, but known sizes.

Electrophoresis

1. Connect the gel box electrical leads to the power supply. Black to black and red to red.

2. Turn on the power and adjust the voltage to 150 volts. A typical run takes approximately 20-30 min. You can tell if your gel is running properly if the blue tracking dye migrates into the gel.

3. View the gel on the UV light box and take a picture of your gel. Using the gun-camera, place the hood over the UV light, turn on the UV light and pull the trigger. Pull your exposure from the camera, wait 60 seconds, then reveal the picture.

Protocol XVI

CLONING THE CYCLASE PCR FRAGMENT

Any fragment of DNA can be conserved, amplified and manipulated if it is cloned into an appropriate plasmid vector. In the following experiments, the cyclase PCR fragment will be ligated into a plasmid vector, recombinant DNA molecules will be selected on the basis of a gene disruption phenotype and the cloned DNA characterized by restriction mapping. The method we will be using is known as TA cloning and is based on unique feature of Taq polymerases. The Taq polymerases used in PCR often add a single deoxyadenosine to the 3'-ends of an amplified DNA fragment in a template-independent fashion. This means the PCR fragments have one extra 3'-A overhang, which can be used to facilitate cloning into a linearized plasmid having a single 3'-T overhang.

Ligation of the PCR fragment

1. Pipetting and aliquoting of solutions, mix in separate eppi tube

		A	B
DNA samples	PCR fragment	1.0 µl	-
	pGEM vector	1.0 µl	1.0 µl
Ligase Buffer		4.0 µl	4.0 µl
water		1.0 µl	2.0 µl
Ligase		1.0 µl	1.0 µl

3. Incubate the reactions for 1 hour at room temperature.

Transformation of bacteria

1. Add 100 µl of competent cells to the remaining 5 µl of the ligation reactions. As a positive control for the transformation, transform bacteria with undigested pGEM plasmid as well (use 1 µl plasmid, 100 µl of cells)

2. Incubate the reactions for 10-30 minutes on ice.

3. While the tubes are incubating, obtain petri plates (1 LB and 1 LB+amp for each ligation/transformation), label them accordingly and spread 200 µl of XGAL indicator solution and 100 µl of IPTG inducer solution onto each plate. Let the plates air dry (open) in the transfer hood.

4. Heat shock the cells. Remove all three tubes from the ice and immediately incubate them at 37-42^{O}C for 2 minutes.

5. Add 200 to 400 µl of liquid LB media to each tube, mix and allow cells to recover for 1 to 60 minutes at room temperature with periodic gentle shaking. The longer you can allow the cells to recover the better, but if time is short, simply proceed to step 6.

6. Spread 200 µl of each sample onto the appropriate petri plates.

7. Invert all the plates and place them in a 37^{O}C incubator overnight.

Picking colonies for mini-preps

1. Score the plates for growth and blue/white selection the following day (plates can be put into the refrigerator for a few days before scoring them if necessary).

2. Pick 2 single colonies from the selection plate and transfer the bacteria to sterile incubation tubes containing liquid LB +amp media. Incubate the tubes in a shaking incubator overnight at 37^{O}C. Which colonies did you choose and why?

Isolation of plasmid DNA

1. Spin down 1 ml of each bacterial culture in a separate eppi tube using an eppendorf centrifuge (3-5 min).

2. Remove the clear supernatant with a pipetman and discard. If the supernatant is not clear, then the bacteria have not be centrifuged long enough. Just spin again.

3. Add 100 µl of ice cold soln I (glucose, Tris)(resuspends the cells), vortex and put on ice 3-5 min.

4. Add 200 µl of soln II (SDS, NaOH)(lysis of the cells, denatures proteins and chromosomal DNA), close the tops and rapidly invert the tubes several times. Incubate the samples on ice for 5 min.

5. Add 150 µl of soln III (KOAc)(precipitates denatured proteins and chromosomal DNA and other junk), close the tops and rapidly invert the tubes several times. Incubate the samples on ice for 5 min.

6. Spin the samples in the microfuge for 5 min. The precipitate smears along the side of the tube.

7. Remove 400 µl of the supernatant to a clean eppi tube and add 800 µl of ethanol. Mix and incubate on ice for 5-10.

8. Spin the sample in the microfuge for 5 min to pellet the plasmid DNA. Carefully remove as much of the supernatant as you can using a pipetman.

9. Add another 400 µl of ethanol, mix and spin 5 min. Carefully remove as much of the supernatant as you can. Invert tubes and let them air dry for 10-15 min. Note: it is very important to evaporate off all the ethanol, ethanol is poisonous to restriction enzymes.

10. Resuspend the DNA pellets with 80 µl of 1XTE.

<u>Digestion of the DNA Sample with restriction enzymes</u>

1. Pipetting and aliquoting of solutions

		eppi tubes	
		1	2
DNA sample		8 µl	8 µl
Reaction Buffer		1 µl	1 µl
Restriction Enzyme	EcoR1	1 µl	-
H_2O		-	1 µl

2. Incubate the reactions for 30-120 minutes at 37^{o}C.

3. Add 2 µl of Tracking Dye to each sample

(Optional: Freeze the samples at -20^{O}C (freezer compartment of your refrigerator) until the next class period.)

4. Examining the DNA digestion fragments by gel electrophoresis and determine the fragment sizes in base pairs

Protocol XVII

DEVELOPING A RESTRICTION MAP FOR THE CYCLASE CLONE

In the last experiment you observed that the digestion of a recombinant plasmid with the restriction enzyme EcoR1 released the cyclase DNA inserts from the plasmid vectors. You were to have determined the sizes of the insert fragments. Now you are to carry out additional restriction enzyme digestions with enzymes that have a single restriction site within the insert DNA, observe the pattern of DNA fragments resulting from these digestions by agarose gel electrophoresis and to generate a restriction map indicating the location of each restriction site along the two insert DNAs.

Digestion of the DNA Sample with restriction enzymes

1. Pipetting and aliquoting of solutions

		eppi tubes			
		1	2	3	4
DNA sample		8 µl	8 µl	8 µl	8 µl
Reaction Buffer (React 2)		1 µl	1 µl	1 µl	1 µl
Restriction Enzyme	EcoR1	0.5µl	0.5µl	0.5µl	0.5µl
	EcoRV		0.5µl		
	Pst1			0.5µl	
	HindIII				0.5µl

2. Incubate the reactions for 30-120 minutes at 37^{o}C.

3. Add 2 µl of Tracking Dye to each sample

4. Examining the DNA digestion fragments by gel electrophoresis (protocol XV)

Protocol XVIII

DNA SEQUENCING

There are many ways for verifying that a cloned DNA fragment corresponds to a particular gene. One relies on the ability of the cloned DNA when expressed in bacteria to generate a new protein of known function. This is most often done with enzymes. However, this method is not always feasible because of technical reasons (Can you think of few?). A second means is to complement a genetic mutant (Can you explain this?). A third is to demonstrate that the DNA sequence of a cloned fragment predicts a polypeptide that is identical to the one of interest. In the current experiment, you are to determine the DNA sequence of the fragment cloned in a previous experiment, and to determine if this DNA fragment is a segment of a sesquiterpene cyclase gene.

DNA Sequencing: We do not have time for each group to sequence DNA clones manually, but instead will use automated sequencing to obtain the sequence for at least one clone per group. You will then examine the DNA sequences using a variety of computer programs and sites, as well as a manual inspection/interpretation.

DNA sequence below needs to be analyzed in 2 ways.

First, you want to know if the gene segment isolated in class actually encodes for a sesquiterpene cyclase. Do do this you will use the data bases available at the National Center for Biotechnology Information, or ncbi. Find this web site and familarize yourself with it. You can search life science literature, look at any number of genome sequencing projects, or determine if the DNA fragment you isolated is similar to anything reported in the literature up to now. Use the Blast sequence comparison program at the ncbi site for this. There are at least two options for the Blast sequence comparison – what are they and find a way to display and discuss the results of these comparisons.

Second, present an organizational map (intron/exon organization, indicating open reading frames and direction of translation) of the DNA fragment you have cloned, and predict the sequence of the mRNA that would arise from this DNA fragment. Report the sizes for both the DNA and predicted RNA fragments. You might want to consult the ORF finder program within the NCBI site for assistance.

Protocol XIX

ISOLATION OF TOTAL RNA

A coordinated induction of cyclase enzyme activity and the absolute level of the cyclase protein was previously observed. That experiment suggested that the level of cyclase protein was due to either a transient induction in the synthesis rate of the cyclase protein, a transient suppression in the degradation rate of the cyclase protein, or some combination of the two. Since an induction in the synthesis rate implies an increased level of cyclase mRNA associated with polysomes, the current objective is to measure the level of the cyclase mRNA in control and elicitor treated cells using the RT-PCR, reverse transcription-polymerase chain reaction, technique. Such experiments rely the isolation of high quality RNA, which is first converted to single-stranded DNA by reverse transcription, then amplification of the cyclase mRNA using specific primers in a PCR assay. In this laboratory period, you are to isolate total RNA from cell culture samples provided.

NOTE: RNA preparations are very labile, especially to RNases which can contaminate almost all materials and solutions. All materials and solutions (expect organic solutions) should be autoclaved prior to use, and gloves should be worn at all times during the isolation procedure.

Trizol RNA Extraction

1. Grind 0.5 g of tissue to a fine powder in liquid nitrogen with a mortar and pestle.

2. Transfer the sample to a 1.5 ml eppendorf tube containing 750 µl of Trizol (reagent for extraction of RNA from cells, contains detergent and phenol). Mix the sample as it thaws.

3. Incubate the mix at room temperature for 5 minutes. Add 150 µL of chloroform, and vortex for 30 seconds. Incubate at room temperature for 3 minutes. Centrifuge at room temperature 11,500 rpms for 15 minutes to separate the phases. The RNA is in the aqueous phase.

4. Transfer the aqueous phase to a new 1.5 mL tube. Add equal volume (approx. 375 µL) of isopropanol and vortex briefly. Incubate at room temperature for 10 minutes. Centrifuge at 11,500 rpms for 10 minutes to pellet the RNA.

5. Remove the supernatant with a pipetman being careful not to disturb the pellet. Wash the pellet with 500 µL of room temperature 70% ethanol. Centrifuge 5 minutes to collect the pellet. Repeat this 70% ethanol wash, then 100% ethanol wash.

6. Remove all of the supernatant with a pipetman, again being careful not to disturb the pellet. Dry the pellet for 5 to 10 minutes in a 70°C heating block.

7. Dissolve the pellet in 20 µL of sterile, RNase-free 1XTE buffer by pipeting and place in a 70°C heating block for 10 minutes. Place the RNA samples on ice.

8. Determine the concentration of the RNA by adding 5 µL of an RNA sample to 495 µL of H_2O, and reading the absorbance at 260 nm. Assuming 40 µg of RNA per ml has an Abs. 260 = 1.0, calculate the concentration of your RNA samples.

9. If the RNA samples are not at least 2.5 µg/µl, ethanol precipitate 10 to 15 µg aliquots of each RNA sample in preparation for the next lab period. Add 1/10 vol. 3 M NaAcetate to 1 vol. of RNA sample, plus 2 vol of ethanol.

Protocol XX

QUALITY CONTROL ANALYSIS OF RNA BY GEL ELECTROPHORESIS

In the previous laboratory period you isolated total RNA from control and elicitor induced cell cultures. Now, you are to size separate the RNA species via gel electrophoresis and visualize the quality of the RNA by staining with ethidium bromide. Good quality RNA will containing ribosomal RNA plus RNA species that vary in size from a few 100 nucleotides to a few 1,000 nucleotides. Low quality RNA will be degraded and migrate as small fragments.

NOTE: RNA preparations are very labile, especially to RNases which can contaminate almost all materials and solutions. All materials and solutions (expect organic solutions) should be autoclaved prior to use, and gloves should be worn at all times during the isolation procedure.

1. Preparation of a formaldehyde gel.

Mix 4 ml of 10X MOPS buffer
0.4 g of agarose
26 ml of H_2O

Melt agarose in microwave oven, then add 1 µl ethidium bromide, 10 ml formaldehyde. **THIS SHOULD BE DONE IN THE HOOD!**

Pour gel and allow it to solidify in the hood.

Immerse gel in 1X MOPS running buffer

2. Preparation of RNA samples.

Calculate the volume of sample necessary for 5 to 10 µg RNA

Mix X µl of sample (equivalent of 5 to 10 µg RNA)
(adjust volume to 4 µl total with H_2O)
10 µl formamide
3.4 µl formaldehyde
2 µl 10X MOPS buffer

Heat samples at 60oC for 10 to 15 min, cool samples at room temperature

Add 5 µl tracking dye (THIS IS RNA TRACKING DYE, NOT DNA TRACKING DYE)

3. Load samples onto gels and electrophoresis at 150 V for 30 to 60 min.

4. Wash the gel twice with large excess of 25 mM sodium phosphate buffer, pH 6.5 for 15 to 30 min. Photograph gel under UV light

Protocol XXI

MEASURING THE CYCLASE mRNA USING AN RT-PCR ASSAY

In order to measure the level of the sesquiterpene cyclase mRNA in control versus elicitor-treated cells, an RT-PCR amplification of the cyclase mRNA in the various samples will be performed. This is an indirect assay which relies first on the conversion of the cyclase mRNA in any sample to single-stranded DNA, then the amplification of a region of the cyclase mRNA (now ssDNA). Both of these steps must be quantitative and of equal efficiency in order to have a valid comparison (What do you think is meant by the terms quantitative and efficiency here?). Finally, you will compare the "amount of cyclase mRNA" in each of the samples by running the PCR reaction products out on a gel and examining the intensity of the bands that migrate to a predicted position (size) on the gel.

Reverse transcription (RT) Reaction

1. Combine in a sterile 0.5 mL eppendorf tube

RNA	x µl (5 µg total RNA)
Oligo dT primer	1 µl (20 pmols of primer)
Water	x µl to a total of 12 µl

2. Heat to 70oC for 10 min.

3. Tap spin at full speed in microfuge and allow the sample to equilibrate to room temperature

4. Add

RNAse inhibitor	1 µl
5xRT buffer	4 µl
100 mM DTT	2 µl
10 mM dNTPs	1 µl
RTase	1 µl

Incubate at 42oC for 1 hr

Polymerase Chain Reaction (PCR) assay

1st strand cDNA template	3 µl
Forward primer	1 µl (50 pmols)
Reverse primer	1 µl (50 pmols)
10x PCR buffer	5 µl
10 mM dNTPs	2 µl
50 mM MgCl2	1.5 µl
Taq polymerase	1 µl (1 unit of activity)
Water	to 50 µl

5. A typical PCR program follows, however, optimum conditions must be determined empirically depending on your template, primers, etc.

PCR 50 program:

step1: 92C 1'30"
step2: 50C 2'

step3: 72C 2'
step4: repeat steps 1-3 28 times
step5: 72C 10'
step6: 4C indefinitely
step7: End

6. Check 5 µl samples on a 1% agarose gel in the next lab period.