Molecular Biology of Plants

A Laboratory Course Manual

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OVERVIEW

INTRODUCTION

This course is intended for people with a molecular biology background who wish to become familiar with plants as experimental organisms. Our goal is to familiarize the student with some of the current research and techniques in the field of plant molecular biology, and also to introduce a modest amount of classical botany and plant physiology. We hope to show some of what makes plant life unique and different from other systems.

Among the many interesting experimental systems in the plant kingdom, we have chosen to focus the laboratory work on Nicotiana tabacum (tobacco) and Zea mays (maize). This choice is made because these materials are used in the instructors' own laboratories, because they represent both monocots and dicots, and because they allow us to cover both cell culture and whole plant genetics. Several other species are used for occasional experiments. You should feel free to take with you any of the strains or cultures used during the course.

Plant experiments often take a long time to finish. The consequences of this on the laboratory schedule are: (i) experiments are grouped with respect to timing, rather than by relatedness of topic; (ii) many days there will be a main experiment to perform plus several continuing experiments to monitor; (iii) some experiments may not get finished during the time of the course, although most of the critical steps will have been performed. On the next two pages we give a lab flow chart, and an outline grouping related experiments, in order to lessen the confusion. Protocols are given in this manual in the chronological order in which they are to be initiated.

Each day will begin at 9:00 AM with a 3 hour lecture/seminar held in the second floor library of McClintock Building (brick and stucco building directly down the hill behind the Blackford Cafeteria). After lunch, about 1:30 PM, the laboratory sessions will begin in the top floor laboratory of Delbruck Building (the gray and green shingle building on Bungtown road just beyond the Gazebo). In the evenings we will either continue with the lab, have discussions related to the experiments, or have additional talks.

This is the fourth year of the Plant Course at Cold Spring Harbor Laboratory. The first two years (1981, 1982) the instructors were John Bedbrook and Fred Ausubel; the third year (1983) they were John Bedbrook, Russell Malmberg, and Ian Sussex. The first 3 years of the course were supported by a grant from the National Science Foundation, and this grant has been renewed for 3 more years (NSF-PCM-8316292). A major factor in the renewal of the grant (and the course) was the comments of the students of the first 3 years. These comments also influenced the current choice of experiments and seminar speakers. We strongly encourage you to submit course criticisms to us when it is over.

We thank Regina Schwarz (Delbruck Building), Mike Ockler (Art) and Nancy Ford and Judy Cuddihy (Publications) for helping us put this manual together; we thank Gerry LoFranco (Delbruck) for her help before and during the course.

The experimental protocols given in this book have been contributed by a variety of workers in the plant sciences. We have tried to attribute the origin of each procedure where possible; and we would like to apologize to any contributor or originator of a technique whom we have inadvertantly not identified. We are especially grateful to Burle Gengenbach, Ron Phillips, Andy Wang, Steve Dellaporta, Paul Chomet, and Don Miles for their work in designing and leading specific laboratory sessions during the 1984 course, and the Maureen Hanson for writing the introduction to plant cell culture appendix.

The plant course would not have been possible without the work contributed by the laboratory assistants who helped plan and prepare the laboratory

sessions: Margaret Boylan, Susan Brown, Barbara Dunn, Jonathon Jones, Jean McIndoo, Rod Reidel, Vinni Schoene, Jane Smith, Stephen Smith, Kit Steinbeck, John Waldron, and Mike Zarowitz.

CHRONOLOGICAL LABORATORY FLOW CHART

Primary Experiment	Secondary and Continued Experiments	
F 8: Anatomy & Development		
S 9: Anatomy & Development		
S10: Tobacco Cell Culture		
M11: Maize Embryogenesis	LT Selection	
T12: Grafting Tomato	Crown Galls	
W13: Tobacco leaf protoplasts isolation and fusion		
R14: Maize genetics	Maize minipreps	Seeds to Culture
F15: Maize genetics	Field day / pollination	
S17: Tobacco culture RNA		
M18: Co-cultivation	Tobacco RNA	
T19: Maize cytogenetics	In situ hybridization	
W20: Maize cytogenetics		Co-cultivation
R21: Tobacco leaf protoplast	Liposome preparation and fusion	
F22: Maize mt DNA	CAT assay	Suspen. Transfer
S23: Maize genome DNA	Maize mt DNA	Co-cultivation
S24: Nitrate reductase assay	SDS Protein Extract	Maize genome DNA
M25: Maize photosynthesis		
T26: Maize photosynthesis	Harvest Bean Embryos	
W27: Bean embryo rocket	Evaluate cultures	

EXPERIMENTS GROUPED BY TOPICS

PROTOCOLS

PLANT MORPHOLOGY AND ANATOMY LABORATORY

Introduction

The study of plant (and animal) structure has been influenced by biologists having a comparative or evolutionary outlook. Thus there has developed a "type" system for thinking about the relationship between the structures of different kinds of organisms. There is considered to be a normal type of organism, organ, tissue, etc., from which various specializations can occur. These specializations can be considered to be:

1. Modifications of the normal type with no evident special function.

2. Adaptations to particular evolutionary/ecological situations. In plants many of these adaptation are to special environments, and many are concerned with the water economy of the plant. These may be surface modifications or alterations in surface/volume relationships or physiological changes.

3. Aberrations where the normal development and structure are altered as a result of interactions with some other organisms.

Each of the 3 principal organs of the plant, stem, leaf and root, may be specialized from the normal type in any of these three ways. This lab is planned to explain the so-called normal type of structure and some of its specializations.

A. Study of a "normal type" of plant and some modifications.

1. Normal type. Take one of the tomato plants that are available and use it as an example of a normal type of plant. Be careful to remove it with minimum disturbance to the root system. The following is a list of information that is useful to have about a plant and forms the basis for studies about its development. This information will also be used for examining other plants and determining in what way their structure and development differs from the normal type.

Stem How are leaves arranged at the nodes, 1 or more per node?

What is the phyllotaxy, opposite or alternate?

Are axillary buds visible? Is their growth inhibited or not?

Are internodes elongated or not (rosette plant).

What is the distribution of elongation of internodes? What is the plants "architecture?")

Leaf Is the leaf simple or compound?

Is it differentiated into blade, midrib, and petiole?

What is its symmetry, dorsoventral, radial, or bilateral?

Does leaf shape change with position on the plant?

Are the leaf surfaces smooth or hair-covered?

Is the leaf venation parallel or net?

Root How are lateral roots arranged on a root, two, three, etc. rows?

Are lateral roots determinate or not?

Does the primary root persist or not?

Do any, or all, roots have cambial activity?

2. Modifications. Examine some of the plants in this collection and identify in what way the stem, leaf, or root is modified in its development from what you would consider to be normal. The modifications here do not seem to be adaptations or aberrations and simply indicate the range of structural diversity that might be expected to be encountered in large groups of organisms.

3. Adaptations. Make the same kinds of analysis as you did above for the plants in this group. Remember that many adaptations are for water economy, others are for storage, others are for light gathering or shedding, others are for predation or protection.

4. Aberrations. Determine how the normal development of the plant has been altered by the interaction of the plant's developmental system with the invading organisms in each of the cases in this collection.

B. Anatomy.

As an example of internal structure and the particular pattern that is made, examine the lignified cells of the xylem in the leaf and stem.

1. Stem.

Cut razor blade hand-sections transversally and longitudinally at the internode and node of the tomato plant. Identify the different lignified cell types of the xylem. Locate the primary vascular tissue (consisting of xylem and phloem); is it a continuous band or in separate bundles? Is there evidence of cambial activity producing secondary tissue? Observe the vascular connections between the stem and leaf in the nodal sections. Compare your sections with the diagrams of stems and identify as many structures as possible.

Procedure for Making Hand-Sections

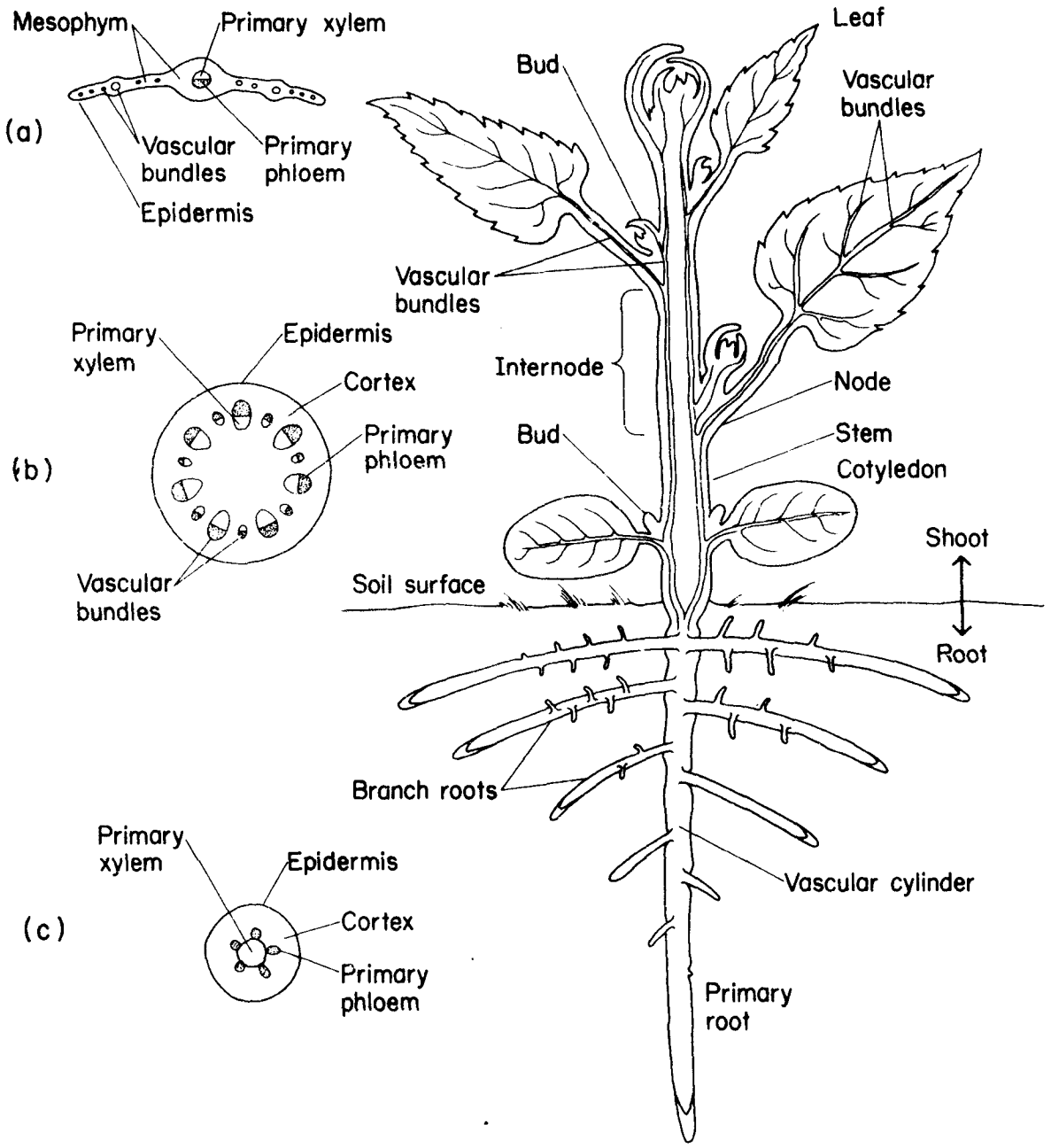
a. Cut a section of stem from the tomato plant in an internodal area. The part to be sectioned must be held firmly between your thumb and index finger. Make a preliminary cut at a right angle to the long axis of the part approximately 1/2 to 1/4 inches from the end of your thumb and finger. This and subsequent sections should be made by cutting toward you with a slicing motion (as opposed to a chopping motion) of the razor blade, making use of as much blade surface as possible.

b. Make a series of sections (slices), trying to make each as thin as possible. Place each section in a petri dish of 50% ethanol which is used to both kill and fix the cytoplasm of each cell. Although the ideal hand-section should be of the entire part (an entire cross section), keep even those sections which are not entire--frequently they will offer the thinnest pieces of tissue. Make approximately 10 sections. Leave the sections in the ethanol for 4-5 min.

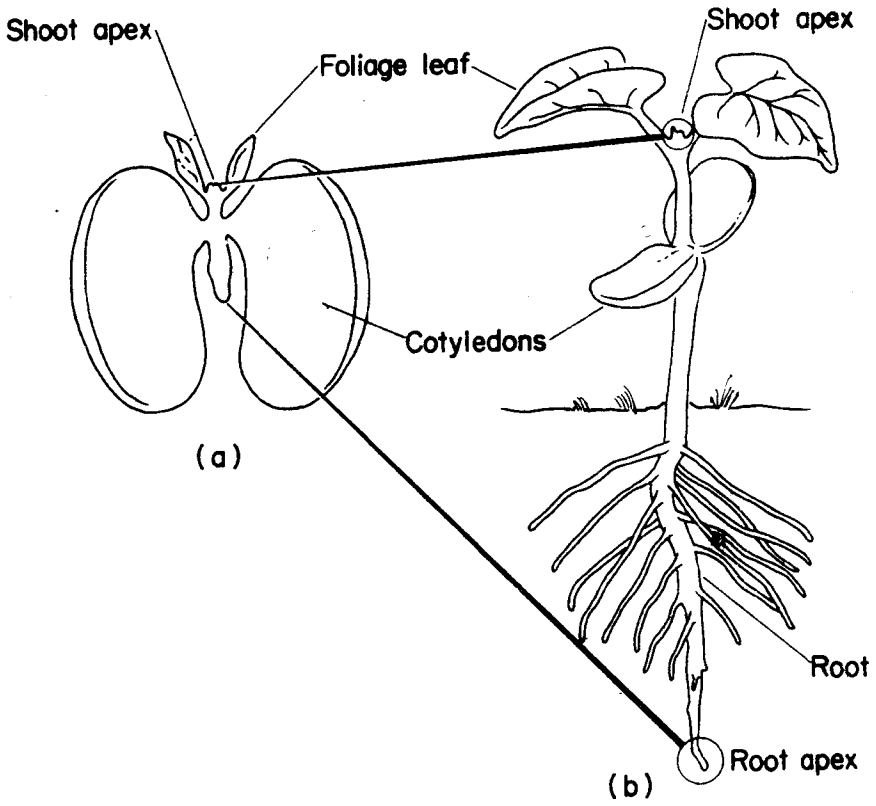
C. Meristems

The shoot apical meristem of Lupinus albus (White lupin, lupini bean) is one of the easiest to dissect and observe. Get some of the lupin seedlings and, paying attention to the phyllotaxy, remove leaves with a fine pair of forceps to reveal the shoot apical meristem. Use a dissecting microscope, and increase the magnification as needed. The shoot apical meristem is a bright green hemisphere between the youngest leaf primordia. What are its approximate dimensions? What is the phyllotaxy? When do leaflets become evident on the young leaf primordia? What is the direction of leaflet initiation? When do hairs first appear on leaf primordia? Where do they first appear?

As time permits make comparable studies of other materials that will be available. Especially dissect other meristems.



Typical Dicotyledonous Plant



Apical Merisems in the Seed and Seedling

EMBRYOS, MERISTEMS, AND EPIDERMAL HAIRS

Embryos

Maize. Get an ear of maize, remove the husks, and look at the attachment of the silk (style) to the kernal. Break the ear in half and remove individual kernels (fruit) from the ear. Identify the upper surface by the presence of the embryo. Use a sharp scalpel to make a V-shaped cut in the fruit wall around the embryo. Lift up the flap of tissue to expose the embryo. Lift out the embryo onto a microscope slide and observe it under a stereomicroscope. Look on the upper surface of the embryo for the root-shoot axis, and identify the large boat-shaped scutellum (equivalent to the cotyledon of the dicot embryo).

Bean The pod is the bean fruit. Identify the stigma and style at the distal end of the pod, and the sepals at the near end. Open up the pod by splitting it longitudinally to reveal the developing seeds. Pull one seed off and identify the micropyle (the hole that the pollen tube grew through to fertilize the egg). It is located near the attachment point of the seed to the pod. Split open the seed coat and remove the embryo. Identify the two cotyledons, and the embryo axis with the shoot apex, and primary leaves, and the root pole at the opposite end of the axis.

Crucifer. Split the pod (=fruit) lengthwise to reveal the developing seeds. Remove a seed and open it using sharp forceps. Remove the developing embryo. By removing embryos from pods at different positions along the stem of the plant you can see a developmental succession of the embryos.

Meristems

Lupin. Get some of the germinated seedlings of lupin. Hold them vertical and remove successively younger leaves that are arranged in a spiral phyllotactic sequence. When you cannot remove any smaller leaves put the shoot tip under the stereoscope and, keeping it vertical, remove younger leaves until you get to the shoot apical meristem. Note the shape changes as the leaves develop. The youngest are simple in outline, then leaflet primordia begin to develop and hairs emerge from the leaf surfaces.

Cauliflower. Break a piece of the white head off the cauliflower and observe it under the stereomicroscope. The whole surface is a mass of vegetative meristems surrounded by leaf primordia. At a later growth stage these meristems will be converted into flower meristems.

Rhododendron. Remove developing bud scales from the apical bud, at first without using the microscope, then as you get to smaller bud scale under the microscope, holding the shoot vertically. The meristem is surrounded by small developing leaf primordia.

Hairs

Tomato. In tomato several mutants are known for hair type. Get a wildtype tomato plant and slice off thin epidermal sections of the stem and leaf to identify the number of different hair types that are present. Then look at each of the four mutants to determine how they differ from the wild type.

CELL CULTURE TRANSFERS

Experiments Performed In The Hood

A. Liquid Suspension Cultures
B. Callus Cultures
C. Shoot Cultures
D. Seed Sterilzation
E. Culture Initiation
F. Regeneration From Leaves

Experiments Performed At Benches

G. Sterile Plants To Soil (demonstration)
H. Anther Culture
I. Staining Cultures for Viability
J. Staining Cultures for Chromosomes

Introduction

These protocols are designed to introduce you to many of the standard techniques of plant cell, tissue, and organ culture. This topic includes a wide variety of practices (little theory), whose common denominator is that the material is sterile. Plant cultures generally are grown on simple, defined media (see the Supplement). Plant cultures grow slowly, so that almost any contaminant will out-grow them; hence all work is done in laminar flow hoods with filtered air. Before beginning work, you should:

(1) Turn on the hood about an hour ahead of time.

(2) Clean hood with an aqueous disenfectant cleaning solution (dilute benzalkonium chloride is useful).

(3) Surface sterilize by rubbing the surface with ethanol.

Some useful points to practice are:

(1) Be aware of the direction of airflow, and try not to position objects between the onrushing air, and the sterile material.

(2) Never carry anything open around the room. Always keep petri plates either in their original bag or wrapped with parafilm.

(3) Use sterile disposable plastic pipets for manipulations, but do not mouth pipet. Always open the pipet bag only in the hood, then gently shake them out until one emerges that can be removed without touching others. Reseal the bag when done.

(4) Be wary of drips of media. These can become paths for contaminants to enter the sterile cultures. Wipe drips from flasks, and flame the rims.

(5) Periodically autoclave metal forceps and scalpels, perhaps once per week.

The laboratory we are using has 4 laminar flow hoods for sterile work, 3 up on the top floor, and 1 down below near the kitchen area. Each of these has room for 2 people. Each hood must therefore be used alternately by 2 lab groups. The second group of experiments should be performed when a hood is not available.

Liquid Suspension Cultures

This experiment is intended to be coordinated with the nitrate reductase assay experiment. Each lab group will choose one of 3 media to maintain its liquid suspension cultures, which will then be harvested to test the effects of media on nitrate reductase activity. The media are all based on MS1 (see supplement):

normal MS1
MS1 with no ammonia, only nitrate
MS1 with no nitrate, but with glutamine

The suspension cultures will be a line of Nicotiana tabacum Xanthi named NTX-282. You will need to subculture them every 6 days, judging the necessary dilution based on their rate of growth. This will allow for 2 days of growth prior to harvesting for the nitrate reductase assay.

Maintenance

To subculture a liquid suspension culture, simply take a flask with medium, and a flask containing cells. Flame the rims. Pour the medium into the cells, swirl, and pour the 1:1 mixture back into the empty flask. Wipe any drips with a Kimwipe, and flame the rims. If you repeat this procedure you end up with a 3:1 split. Generally 1:1 and 3:1 are the usual dilutions, although some cultures can readily withstand much greater dilution.

Suspension cultures require constant shaking. They can sit in a hood for manipulation for about a half an hour without damage. If you have your cultures out longer than that, you should swirl them every 10 min.

Experiments that require growth curves in liquid suspension cultures can be performed in a variety of ways. Some finely divided cultures can be monitored by spectrophotometer or Klett meter. Another alternative is to pour the liquid suspensions into a graduated centrifuge tube, and then let them settle to the bottom for 15 to 30 min. The settled cell volume is then read, and the culture returned to the flask. Settled cell volumes may be harmful to some cultures, so the best practice is to pick the shortest settling time that gives good values, and to only measure every 2 to 3 days.

Initiation

To initiate a suspension culture, pick a plate of rapidly growing, friable callus. Inoculate about a half a plate full of callus into a flask containing medium. Shake the suspension culture as in maintenance. After a week, check the culture. It may be possible to subculture it by a 1:1 split, or it may need more time to break apart and begin growth. Avoid subculturing it too dilute. Every week as you subculture it, the larger clumps will gradually disappear (if it is tobacco). Some researchers prefer to filter out the larger clumps through sterile cheesecloth.

Plating

We will actually perform a plating during the co-cultivation experiment. Pour a suspension culture into a graduated 50 ml centrifuge tube, and let settle 15 to 20 min. Remove the supernatant. Add an equal volume of melted, lukewarm, agar medium. Mix, and then pipet 10 ml onto a standard media petri plate. Quickly swirl the plate to make sure the agar-cell slurry is spread evenly. Let the plate sit for 30 min at room temperature to be sure it is hard, then wrap with parafilm and incubate in the dark.