Plant Growth and Development
Botany 137/Fall 2003

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Syllabus
Botany 137/Fall 2003

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Office hours: M from 12:00 noon to 1:00pm.; W from 12:00 noon to 3:00pm.

Course description:

This course discusses the molecular and genetic control of the formation of an adult plant and its different organs and systems. Lectures will discuss the combined use of morphology, physiology, cell biology and genetics with the new emerging molecular techniques that have shaped plant development in recent years. Special techniques of molecular biology and genetics will be discussed as needed. Student participation during lecture and laboratory sessions will be strongly encouraged.

Prerequisites:

You should have taken general biology courses (BioSci1A and 1B) and genetics (BioSc 140A).

Objectives:

1- To introduce students to basic concepts in plant development.
2- To introduce students to techniques and methodologies used in the study of plant development.
3- To encourage critical thinking during problem solving in plant development.
4- To introduce students to scientific literature in plant development.

Reading material:

Reading material will include chapters from the textbook Molecular Genetics of Plant Development by Stephen H. Howell, Cambridge University Press 1998. Additional books relevant to plant growth and development will be on reserve in the library for consultation. In addition, selected papers from the scientific literature will be assigned and thoroughly discussed.

NOTE: This is a "web enhanced" course and as such, all materials will be available through Blackboard to registered students.

Examinations and Grading:

Midterm 1	20%
Midterm 2	20%
Final (accumulative)	20%
Lab Reports	30%
Participation in scientific paper discussions	10%

Materials to be evaluated during the midterms and final exam will include 1) the material discussed during classes and the indicated chapters from the textbook, and 2) the material from the scientific papers discussed and handouts. No make-up exams will be allowed, unless serious personal calamity or illness can be documented. The final grade will be a letter grade representing the points obtained during the semester. The conversion from "number grade" to "letter grade" will be based on the following schedule: A=90-100; B=80-89; C=70-79; D=60-69; and F<60.

Laboratory reports should be handed in at the specified dates (see Laboratories in Plant Development section). In order to write up a lab report (see guidelines to write a report), attendance to the lab sessions is essential: if you do not attend a lab session, no lab report will be accepted from you and will result in 0 (zero) points for that particular laboratory. Late lab reports will deduct 10 points/day late (over a total of 100 points) of your grade for that lab. Lab reports over 4 days late will not be accepted and will result in a 0 (zero) grade.

Course policies:

The lecture and laboratory rooms should be considered sanctuaries for the pursuit of knowledge. Those who enter them should be committed to learning and to respect other's ideas and opinions. Undesirable behavior in the class or laboratory will lead to dismissal or even expulsion from the class. Undesirable behavior includes (but is not limited to), harassment of any kind, disrupting class when arriving late, reading newspapers when in lecture or the lab, and continuously talking at inappropriate times during class or lab sessions.

Cheating (at any level) is an intolerable behavior that has no place in any scientific, educational or social activity. Cheating will result in a failing grade in the course and further actions taken by the University leading to expulsion, suspension or probation.

If you have special needs as addressed by the Americans with Disabilities Act (ADA) and need course materials in alternative formats, notify me immediately. Reasonable efforts will be made to accommodate your special needs.

Tentative Outline of Classes (subject to change).

Textbook: Molecular Genetics of Plant Development by Stephen H. Howell, 1998.

Week 1: Aug. 25 and 27.
- Welcome.
- What is plant development? Its tools? (Chapter 1).

Week 2: Sep. 3.
- Model organisms: Maize and *Arabidopsis* (Chapter 1).

Week 3: Sep. 8 and 10.
- Plant hormones (*).

Week 4: Sep. 15 and 17.
- Cell lineage and positional information (Chapter 2).
- Male and female gametophyte development (Chapter 9).

Week 5: Sep. 22 and **24**.
- Fertilization (*).
- **MIDTERM EXAM 1**

Week 6: Sep. 29 and Oct. 1.
- Embryogenesis I (Chapters 3).
- Embryogenesis II (Chapters 3).

Week 7: Oct. 6 and 8.
- Seedling development: Photomorphogenesis (Chapter 4).

Week 8: Oct. 13 and 15.
- The shoot development (Chapter 5).

Week 9: Oct. 20 and 22.
- Leaf development (Chapter 6)

Weeks 10: Oct. 27 and 29.
- Flower development I (Chapter 8).

Week 11: Nov. 3 and **5**.
- Flower development II: sex determination (*).
- **MIDTERM EXAM 2**

Week 12: Nov. 10 and 12.
- Pollination I (Chapter 10).
- Pollination II (Chapter 10).

Week 13: Nov. 17 and 19.
- Seed and fruit development (Chapter 11).

Week 14: Nov. 24.
- Root development (Chapter 12).

Week 15: Dec. 1 and 3.
- Vascular development (Chapter 13).

Week 16: Dec. 8 and 10.
- Plant genetic engineering (*).

Week 17: Dec. 15 (11:00 am to 1:00 pm).
- **FINAL EXAM**

(*) Handouts for these lectures will be provided in class.

General Safety Practices in the laboratory for Plant growth and Development.

In order to maximize our learning experience without threatening our health or that of other people around us we should be able to observe the following rules:

1. **NO** food or drinks are allowed in the laboratory.

2. You should wear protective clothing (lab coat) and gloves, and work in chemical hoods when handling hazardous chemicals in the lab.

3. Do not assume anything when you are not sure about proper handling of chemicals or solutions. Do not hesitate asking me.

4. Follow the following instructions for the disposal of pipette tips, microcentrifuge tubes, sharps (needles, razor blades) and glass (Pasteur pipettes, slides and cover slips, and broken glass):

- Collect used pipette tips and microcentrifuge tubes in a small container on your bench. When you are finish with your work dispose of this items in the **"biohazards waste"** container.
- Dispose of any sharp objects (needles, razor blades) immediately after use into the **"sharps waste"** container.
- Dispose of any glass object (Pasteur pipettes, slides and cover slips, and broken glass) immediately after use into the **"glass waste"** container.
- All the containers are localized next to the laboratory exit door.

If you need any further assistance with safety issues in the laboratory, do not hesitate contacting me.

Alejandro Calderon-Urrea, Ph. D.
Associate Professor of Developmental Genetics
Department of Biology

Laboratory Report Guidelines

A laboratory report is the way you have to express what you have learned in the laboratory. It is also the first step in the long training process that will allow you to write publishable scientific papers. Whether you are interested in pursuing a scientific career or in working in other professional activities, the experience you get from writing laboratory reports will be invaluable. For the purposes of this course Lab reports should be 7 pages (maximum) or less (but no less than 3 pages) and should be in the format of a scientific paper including the following sections:

Title (10 points):

The title should be less than fifteen (15) words and reflect the factual content of the laboratory report. I have given very general subjects for each laboratory (for example, Laboratory #1: Chlorophyll/Carbohydrates in Plant Tissues). You should produce a title for the lab report based on the experiences in the lab and the main questions addressed.

Abstract (10 points):

This is a concise presentation of the question addressed, the methods used, the results and your main conclusions. It is normally a paragraph of approximately 100-200 words. This is normally the section you write last, even though it is located at the beginning of the lab report.

Introduction (10 points):

This is normally a background statement to introduce the readers to the subject you are addressing and its significance. You should use references to present your background statements. At the end of this section you should indicate the purpose of the laboratory (objective or objectives).

Materials and Methods (10 points):

This is a list of the material (with suppliers) and a description of the methods used. *For the purposes of this class you will get full credit for this section if you indicate "as described in the Botany 137 Lab Manual"*. However, do not forget to cite the manual in the References section.

Results (20 points):

In this section you should present the facts of your lab experiences and explain them. Data are presented in tables, graphs, drawings, photographs *and descriptions*. The explanations, in paragraph form, should refer to the supporting material to indicate trends and important observations. Also in this section, you should be able to present conclusions based on your observations.

Discussion (30 points):

This is the fun part of the report and where your imagination will help you connect facts. In this section you should state each of your conclusions (based on your results) and discuss them in light of other studies published in the literature. In this section you should address the questions posed on each of the Laboratory Manuals.

Literature cited (10 points):

This is a listing of all the literature you cited throughout the report. The format is very rigid and every scientific journal has its own rules. Whatever the format you use in your lab reports, you must be consistent. You can use as template citation of references in journals such as "The Plant Cell", "Plant Physiology", "Cell", "Development" or "Developmental Biology". When making reference to a specific web site, write in parenthesis the URL address for the site directly in the body of your report and not in this section.

NOTE: When writing the lab. reports use the following formatting instructions: 1) use ONE inch margins on all sides of the page; 2) use one, or one and half (1.5 inches) as line spacing. Decide on which line spacing to use depending on the amount of information you have to present. LAB REPORTS USING TWO LINE SPACING WILL BE RETURNED WITHOUT GRADING, AND ALL THE RULES FOR TURNING IN LATE LAB REPORTS WILL BE APPLIED. 3) Use font size 12 and a common font type such as Times, Times New Roman, Helvetica, Garamond, or Courier New.

Laboratories in Plant Development (subject to change).

Laboratory #1: Writing the Lab report: Chlorophyll/Carbohydrates in Plant Tissues.

Week 1: Aug. 25.

- Welcome and orientation.
- Lab activity and "Writing the Lab report".

Lab. #1 lab report due date: Sep. 8 (revision date Sep. 3).

Laboratory #2: Expression of foreign genes into plant cells: the use of *Agrobacterium tumefaciens.*

Week 1: Sep. 8.

- Experiment set up and co-cultivation.

Week 2: Sep. 15.

- Explant transfer into shoot induction media.
- Transgenic plants.

Sep. 22.

- (Scientific paper discussion #1).

Week 3: Nov. 10.

- GUS assay.

Lab. #2 report due date: Dec. 1.

Laboratory #3: Morphogenesis in tobacco leaf tissue.

Week 1: Sep. 29.

- Collection and culture of tobacco leaf tissue.

Week 2: Oct. 6.

- Data collection.

- (Scientific paper discussion#2).

Week 3: Oct. 13.

- Final data collection and discussion.

Lab. #3 report due date: Oct. 20.

Laboratory #4: Regenerating abilities of shoot apical meristem (SAM).

Week 1: Oct. 20.

- Micro-dissection and culturing of SAM from *Coleus* plants.

Week 2: Oct 27.

- Data collection.

- (Scientific paper discussion#3).

Week 3: Nov. 3.

- Final data collection and discussion.

Lab. #4 report due date: Nov. 10.

Laboratory #5: The Effects of Different Light Wavelengths on Germinating Corn Embryos.

Week 1: Nov. 10.

- Micro-dissection and culturing maize embryos.

Week 2: Nov. 17.

- Data collection.

Lab. #5 report due date: Nov. 24.

Nov. 24.

- (Scientific paper discussion#4).

Laboratory #1.
Writing the Lab report: Chlorophyll/Carbohydrates in Plant Tissues.
From:Botany 10 lab manual
Department of Biology
California State University, Fresno
Lab. report due date: Sep. 8

I. Introduction:

Photosynthesis occurs in two stages. The first is the photochemical capture of light energy and its temporary storage in high-energy chemical bonds. This step takes place only when chlorophyl is present. The second stage uses the energy temporarily stored in the high-energy bonds of ATP and NADPH to accumulate organic compounds in the plant. The simplest biochemical expression of the synthesis of organic compounds from carbon dioxide (CO_2) by photosynthesis is given by the following equation.

$$\text{Light} + CO_2 + H_2O \text{ ---> } (C\ H_2O)n + O_2.$$

The organic product of photosynthesis is given as $(C\ H_2O)n$ which is the basic component of carbohydrate. In putting six of these units together (that is, n=6), the simple sugars (usually glucose or fructose) are produced. Combining glucose and fructose gives sucrose (n=12) which is the most abundant sugar found in nature. Sucrose is the ordinary table sugar that is used in soft drinks and candy bars. Depending on the nature of the bonding between the simple sugars, long strings of simple sugars result in starch and cellulose. In other words, thanks to photosynthesis we have French fries to eat (starch), cotton clothes to wear (cellulose), and wood pencils and paper for doing homework (cellulose)!

II. Objectives:

In the absence of light there is no energy source for photosynthesis and there is no assimilation of carbon dioxide. The failure to find sugars and starch in the absence of chlorophyll is the basis of this experiment.

III. Procedures:

- Students will observe the pigmentation of the Coleus plant laves.
- Harvest leaves for the iodine test. Put one leaf into a glass petri dish.
- Cover the leaf with 70% ethanol and cover. Boil the leaf in a microwave for one minute.
- Add iodine solution and incubate at room temperature for 2 minutes. Wash the iodine solution and wash the leaf several times with water.

IV. Discussion questions:

What is the coloration pattern of the *Coleous* leaf?
What is the coloration pattern after staining for the presence of starch?
How are these two patterns related?

USEFUL REFERENCES

On reserve in the library:
1) Lab reports by two Genetics 172 students.
2) Madore. 1989. Carbohydrate metabolism in photosynthetic and non-photosynthetic tissues of variegated leaves of *Coleous blumei* Brnth. Plant Physiol. 93: 617-622.
3) Fisher. 1985. Morphology and anatomy of the leaf of *Coleous blumei* Amer. J. Bot. 72: 392-406.

Laboratory #2.
Expression of foreign genes into plant cells: the use of *Agrobacterium tumefaciens*.
Lab report due date: Dec. 1
(The introduction section was modified from: http://helios.bto.ed.ac.uk/bto/microbes/crown.htm)

I. INTRODUCTION:

Biology of *Agrobacterium tumefaciens*:

Agrobacterium tumefaciens causes **crown gall** disease of a wide range of dicotyledonous (broad-leaved) plants, especially members of the rose family such as apple, pear, peach, cherry, almond, raspberry and roses. A separate strain, termed biovar 3, causes crown gall of grapevine.

The disease gains its name from the large tumour-like swellings (galls) that typically occur at the crown of the plant, just above soil level. Although it reduces the marketability of nursery stock, it usually does not cause serious damage to older plants. Nevertheless, this disease is one of the most widely known, because of its remarkable biology. Basically, the bacterium transfers part of its DNA to the plant, and this DNA integrates into the plant's genome, causing the production of tumours and associated changes in plant metabolism.

The unique mode of action of *A. tumefaciens* has enabled this bacterium to be used as a tool in **plant breeding**. Any desired genes, such as insecticidal toxin genes (see *Bacillus thuringiensis*) or herbicide-resistance genes, can be engineered into the bacterial DNA and thereby inserted into the plant genome. The use of *Agrobacterium* not only shortens the conventional plant breeding process, but also allows entirely new (non-plant) genes to be engineered into crops.

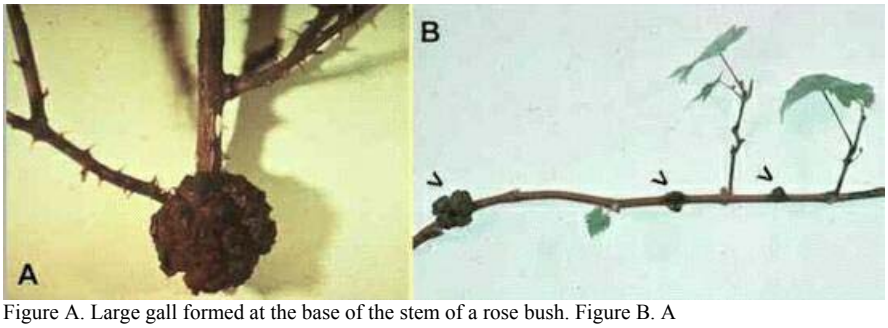


Figure A. Large gall formed at the base of the stem of a rose bush. Figure B. A series of galls (arrowheads) along a branch of a grapevine.

The story of *Agrobacterium* goes even further than this, making it one of the most interesting and significant bacteria for detailed study. For example, there is a highly effective **biological control** system for this disease - one of the first and most successful examples of biological control of plant disease.

The bacterium and its plasmids:

A. tumefaciens is a Gram-negative, non-sporing, motile, rod-shaped bacterium, closely related to *Rhizobium* which forms nitrogen-fixing nodules on clover and other leguminous plants. Strains of *Agrobacterium* are classified in three **biovars** based on their utilisation of different carbohydrates and other biochemical tests. The differences between biovars are determined by genes on the single circle of chromosomal DNA. Biovar differences are not particularly relevant to the pathogenicity of *A. tumefaciens*, except in one respect: biovar 3 is found worldwide as the pathogen of gravevines. But this is almost certainly because biovar 3 has been spread around the world in vegetative cuttings of vines, not by natural mechanisms.

Most of the genes involved in crown gall disease are not borne on the chromosome of *A. tumefaciens* but on a large **plasmid**, termed the ***Ti* (tumour-inducing)** plasmid. In the same way, most of the genes that enable *Rhizobium* strains to produce nitrogen-fixing nodules are contained on a large plasmid termed the ***Sym*** (symbiotic) **plasmid**. Thus, the characteristic biology of these two bacteria is a function mainly of their plasmids, not of the bacterial chromosome.

[A plasmid is a circle of DNA separate from the chromosome, capable of replicating independently in the cell and of being transferred from one bacterial cell to another by conjugation. Plasmids encode non-essential functions, in the sense that a bacterium can grow normally in culture even if the plasmid is lost.]

The central role of plasmids in these bacteria can be shown easily by "curing" of strains. If the bacterium is grown near its maximum temperature (about 30oC in the case of *Agrobacterium* or *Rhizobium*) then the plasmid is lost and pathogenicity (of *Agrobacterium*) or nodule-forming ability (of *Rhizobium*) also is lost. However, loss of the plasmid does not affect bacterial growth in culture - the plasmid-free strains are entirely functional bacteria.

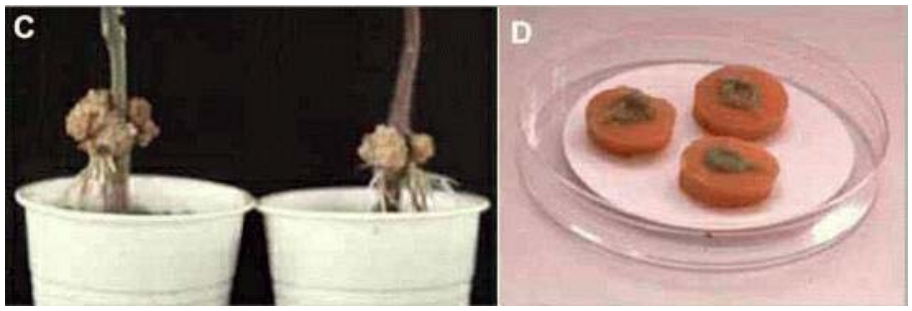
In laboratory conditions it is also possible to cure *Agrobacterium* or *Rhizobium* and then introduce the plasmid of the other organism. Introduction of the *Ti* plasmid into *Rhizobium* causes this to form galls; introduction of the *Sym* plasmid into *Agrobacterium* causes it to form nodule-like structures, although they are not fully functional.

Studies such as these raise many interesting and challenging questions about the nature of bacteria. For example, what does the name of a bacterial species or genus really mean, if the organism can change so drastically by loss or gain of a non-essential plasmid? And how much gene exchange occurs my means of plasmids and other mobile genetic elements within natural populations?

The infection process:

Agrobacterium tumeaciens is found commonly on and around root surfaces - the region termed the **rhizosphere** - where it seems to survive by using nutrients that leak from the root tissues. But it infects only through wound sites, either naturally occurring or caused by transplanting of seedlings and nursery stock. This requirement for wounds can be demonstrated easily in laboratory conditions. For example, **Figure C** shows the bases of two young tomato plants where a drop of *A. tumefaciens* bacterial suspension was placed on the stem and a pin prick was then made into the stem at this point. The photograph was taken 5 weeks later. **Figure D** shows another laboratory assay, where bacterial suspension was added to the surface of freshly cut carrot disks. After 2 weeks the young galls (green-coloured) developed from the meristematic tissues around the central vascular system.

In natural conditions, the motile cells of *A. tumefaciens* are attracted to wound sites by **chemotaxis**. This is partly a response to the release of sugars and other common root components, and it is found even in plasmid-cured strains. However, strains that contain the *Ti* plasmid respond even more strongly, because they recognise wound phenolic compounds such as **acetosyringone** (**Figure F**) which are strongly attractive at even very low concentrations (10-7 Molar). Thus, one of the functions of the *Ti* plasmid is to code for additional, specific chemotactic receptors that are inserted in the bacterial membrane and enable the bacterium to recognise wound sites.



Acetosyringone plays a further role in the infection process, because at higher concentrations (about 10-5 to 10-4 Molar) than those that cause chemotaxis it activates the virulence genes (**Vir** genes) on the *Ti* plasmid (see **Figure G**). These genes coordinate the infection process and, in particular:

* lead to the production of proteins (**permeases**) that are inserted in the bacterial cell membrane for uptake of compounds (**opines**) that will be produced by the tumours (see later);
* cause the production of an **endonuclease** - a restriction enzyme - that excises part of the *Ti* plasmid termed the **T-DNA** (transferred DNA).

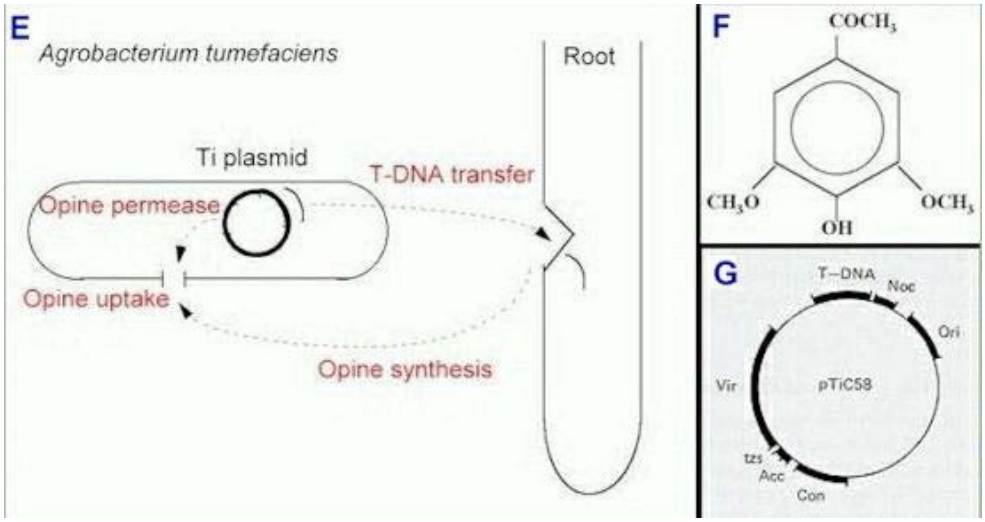


Figure E. Overview of infection of a plant wound site by *Agrobacterium tumefaciens*. The *Ti* plasmid codes for a nutrient-uptake protein (opine permease) that inserts in the bacterial cell membrane. The plasmid also copies and excises part of its DNA, which enters the plant cells and causes them to produce opines.

Figure F. Structure of acetosyringone.

Figure G. Diagram of some major regions of the *Ti* plasmid of *A. tumefaciens* strain C58. **T-DNA** = transferred DNA; **Noc** = nopaline catabolising genes; **Ori** = origin of replication of the plasmid; **Con** = region governing conjugative transfer of the plasmid to other *Agrobacterium* strains; **Acc** = agrocinopine catabolising genes; **tzs** = transzeatin synthesis; **Vir** = virulence genes.

As shown diagrammatically in Figure E, the excised T-DNA is released by the bacterium and enters the plant cells, where it integrates into the plant chromosomes and dictates the functioning of those cells. The actual mechanism of transfer is still unclear, but it seems to require a conditioning process, perhaps mediated by the production of **cytokinins** (plant hormones) by the bacterium. The **tzs (transzeatin) gene** on the Ti plasmid codes for the hormone (Fig. G).

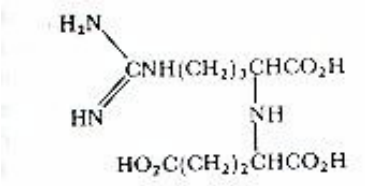
It is important to note that only a small part of the plasmid (the T-DNA) enters the plant; the rest of the plasmid remains in the bacterium to serve further roles.

When integrated into the plant genome, the genes on the **T-DNA** code for:

* **production of cytokinins**
* **production of indoleacetic acid**
* **synthesis and release of novel plant metabolites -** the **opines** and **agrocinopines**.

The plant hormones upset the normal balance of cell growth, leading to the production of galls and thus to a nutrient-rich environment for the bacteria. The opines are unique amino acid derivatives, different from normal plant products, and the agrocinopines similarly are unique phosphorylated sugar derivatives. All these compounds can be used by the bacterium as the sole carbon and energy source, and because they are absent from normal plants they provide *Agrobacterium* with a unique food source that other bacteria cannot use.

Different strains of *A. tumefaciens* contain different types of *Ti* plasmid which code for production of different types of opines. One of the most common types of *Ti* plasmid (found in strain C58 of *A. tumefaciens*; Fig. G) codes for production of **nopaline** (structure shown below), and for **agrocinopine A**. The part of the plasmid that remains in the bacterium codes for the uptake and catabolism of these compounds (the **Noc** gene and the **Acc** gene shown in Fig. G). The other common type of *Ti* plasmid codes for synthesis of **octopine** and **agropine**. The significance of this difference will be clear when we discuss biological control of crown gall.



Nopaline

To end this account of the disease process, we should return to a question raised earlier: **how much genetic exchange occurs between bacteria in natural conditions?**

When *Agrobacterium* is isolated from the root surface of plants in natural or cropping environments, the majority of strains (90% or more) are found to be non-pathogenic - even when the isolations are made from diseased plants. These non-pathogenic strains have traditionally been called by the species name *Agrobacterium radiobacter*. So we must conclude that *Agrobacterium* is essentially a rhizosphere inhabitant, and only a small proportion of strains are pathogenic (containing the *Ti* plasmid). Incidentally, the same is true of *Rhizobium* - most strains isolated from the root zone are incapable of nodulating plants.

In many ways this makes sense biologically: the bacterium is, basically, a rhizosphere inhabitant because pathogenic strains of *Agrobacterium* could only respond rapidly to wound sites if there were an established population in the root zone. But the *Ti* plasmid is a **conjugative plasmid** - it can be transferred from one cell to another, under the control of the **Con** region (Figure G). In laboratory conditions, this conjugative transfer is strongly promoted by the presence of nopaline, so it seems that the pathogenic strain creates the conditions (nopaline production from infected wound sites) that enable it to transfer its plasmid to other strains in the rhizosphere.

Genetic engineering of plants with A. tumefaciens:

A. tumefaciens has been used extensively for genetic engineering of plants. This is achieved by engineering selected genes into the T-DNA of the bacterial plasmid in laboratory conditions so that they become integrated into the plant chromosomes when the T-DNA is transferred. However, several alternative methods also are used for genetic engineering of plants, including the use of small DNA-coated microprojectiles which are fired into plant cells. A few of the commercial applications of these technologies are shown in table 1.

Tabla 1: Some commercial releases of transgenic plants (*)

Crop and release date	Name	Company	Novel properties
Tomato (1994)	**Flavr Savr**	Calgene	Vine-ripened flavour, shelf life
Tomato (1995)		Zeneca	Consistency of tomato paste
Cotton Potato Maize (1996-97)	**Bollgard** **NewLeaf** **YieldGuard**	Monsanto	*Bacillus thuringiensis* toxin for insect resistance
Soybean Canola (rape) Cotton (1995-96)	**Roundup Ready**	Monsanto	Glyphosate herbicide resistance

(*)RG Birch (1997) Plant transformation: problems and strategies for practical application. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**, 297-326.

The transgenic tomatoes do not express the gene for **polygalacturonase**, an enzyme that degrades pectin, leading to softening of the fruit tissues. As a result, the tomatoes can be left on the plant for longer to accumulate flavour components and they also give a better consistency of tomato pastes.

Several crop plants have been engineered to express the **insecticidal toxin gene** of *Bacillus thuringiensis* (see Profile on *Bacillus thuringiensis*), so that insects attempting to eat these plants are killed. This is highly successful, but has the potential disadvantage that continuous exposure of insects to the toxin will select for the development of toxin resistance. Cropping strategies must be devised to "manage" this.

Several crops also have been engineered for resistance to herbicides such as **glyphosate**, so that the herbicide can be used for weed control without damaging the crop. The best-known examples are the "Roundup Ready" crop plants marketed by Monsanto.

Other transgenic strategies being explored or commercialised include:

* Engineering for virus resistance by incorporation of viral coat protein genes or antisense RNA.
* Engineering for resistance to fungal pathogens, by enhanced expression of fungal wall-degrading enzymes (chitinase and glucanases).
* Engineering of plants so that, during a late stage in the development of their seeds, they express a gene that renders the seeds sterile. This "terminator technology" ensures that growers cannot save some of their own seed for use in the following year, but must buy fresh seed from the commercial seed producer.

The basis of *Agrobacterium*-mediated genetic engineering is that the T-DNA of *A. tumefaciens* is excised and integrates into the plant genome as part of the natural infection process by this bacterium. So, any foreign DNA inserted into the T-DNA will also be integrated.

However, the only essential parts of the T-DNA are its two small (25 base pair) **border repeats**, at least one of which is needed for plant transformation. So the T-DNA is engineered to remove the genes coding for plant hormones, and a length of DNA is inserted that contains a selectable marker (e.g. an antibiotic-resistance gene; usually **kanamycin** resistance). This length of DNA must also contain a **restriction site** - a site with a specific sequence of nucleotides where a restriction enzyme will cut the DNA. For example, the restriction enzyme *Bam*H1 cuts DNA wherever the nucleotide sequence GGATCC occurs (with CCTAGG on the complementary DNA strand). It leaves overlap sites, termed "sticky ends", so that any other piece of DNA cut with the same enzyme can be inserted into this site.

The transformation of plants requires:

* an *Agrobacterium* cell to act as the vehicle for a transforming plasmid.
* a *Ti* plasmid with functional *Vir* genes (see Fig. G) for recognising plant signals and for excising the T-DNA
* the T-DNA with appropriate deletions and gene inserts.

However, the T-DNA does not need to be on the same plasmid as the *Vir* genes, so it is often convenient to construct a smaller, self-replicating plasmid containing the T-DNA and to place this in *Agrobacterium* cells with a "disarmed" *Ti* plasmid (a binary vector system).

Plant transformation can be achieved by incubating *Agrobacterium* with plant leaf disks in liquid medium. Then the bacterium is killed with an antibiotic, the non-transformed cells are killed by addition of hygromycin, and the remaining cells (which survive hygromycin treatment because they have the resistance gene) are used to regenerate plants.

II. OBJECTIVES:

- To understand the process of transgenic plant production using *Agrobacterium*.
- To provide an open discussion forum to address concerns about the use of transgenic plants in biotechnology.

III. PRODUCTION OF TOBACCO TRANSGENIC PLANTS AND ASSAY FOR THE INTRODUCED TRANSGENES

Week #1:

Procedures:

Cocultivation of tobacco leaf discs with Agrobacterium

Steps 1-4 wil be done for you by the instructor:

1. Grow overnight culture of *Agrobacterium* strain carrying a plasmid construct bearing the gene(s) of interest on its T-DNA. We will use *A. tumefaciens* C58C1::pGV2260::pCAMBIA1301. The plasmid in these bacteria carries the *uidA* gene that codes for β-glucuronidase enzyme (screenable marker) and *hptII* gene which codes for hygromycin resistance (selectable marker).
2. Centrifuge the bacterial suspension for about 10 minutes (3,000 g)
3. Decant the supernatant
4. Resuspend the bacterial pellet in the same volume of half-strength Murashige and Skoog salt solution
5. Pipette the suspension into a sterile petri plate (Work aseptically !)
6. Carefully and aseptically excise the explants from green-house grown plants:
7. Collect several young tobacco leaves.
8. Surface sterilize them using 25% bleach+tween 20 for 5 minutes.
9. Rinse two times (with sterile dH_2O).

10. Working on the bench, and under aseptic conditions demonstrated by the instructor, cut the leaves into 2x2 or 3x3 mm (the wounding releases phenolic compounds which induce the *vir* genes located on the Ti plasmid and thus initiate events leading to gene transfer). Sterilize your instruments by dipping them in alcohol between each manipulation and flaming them.
11. Place the cut explants in the bacterial suspension and let explants soak for ten minutes
12. Using a sterile filter paper, blot dry the explants
13. Place the explants on MS medium (with no antibiotics or growth hormones), wrap the plates with Parafilm, and incubate at 21°C for 1 week.

Observations to make:
Take notes of how many explants you start with so we can calculate indices of contamination and transgenic plant production.

Culture media:
MS medium: is Murashige and Skoog medium (Sigma, Co.) supplemented with 2% sucrose and solidified with Agargel (Sigma, Co.).
Shoot Induction Medium (SIM): MS medium+3mg/L BAP(6-benzylaminopurine)+1mg/L IAA (Indole 3-acetic acid).

***Agrobacterium tumefaciens* strains:**
A) A control strain: Strain with no Ti plasmid, only *Vir* genes (C58C1::pGV2260).
B) A C58C1 Strain (C58C1::pGV2260::pCAMBIA1301) containing two genes to be introduced to the plant: the *hptII* gene (resistance to the antibiotic hygromycin) and the *uidA* gene (gene for the β-glucuronidase enzyme).
NOTE: The plasmid that contains the Vir genes is called pGV2260, and the one containing the T-region is pCAMBIA1301.

Week #2

Procedures:

Induction of transgenic shoots

1. At the end of this co-cultivation period (1 week), suspend and shake the explants in an antibiotic solution (Cefotaxime) to remove the bacteria.
2. Transfer the explants onto a regeneration medium (Shoot Induction Medium) containing bacteriostatic antibiotics (Cefotaxime), and a selective agent (hygromycin).
3. Transfer explants into fresh media every 3 weeks, or as needed due to bacterial contamination. You should plan on making THREE TRANSFERS (Yes, that is 9 weeks) before you are able to see little tobacco plants (plantles).

Week #3

Procedures:

Testing for the presence of the introduced genes

1. Test a part of the surviving plantlets (along with control plantles) for GUS expression (GUS is the "street" name of the β-glucuronidase enzyme) by GUS histochemical assay. Place the explants in a well plate and dispense 200-300 µl of X-Gluc buffer. Incubate the samples at 37C for 2 hours (overnight work best. So you only will be able to check your experiment a week later).

IV. THE LAB REPORT

1. How many shoots per explant did you get after incubation in the shoot induction media of explants inoculated with the control strain (C58C1::pGV2260)?
2. How many shoots per explant did you get after incubation in the shoot induction media of explants inoculated with the strain carrying the plasmid vector pCAMBIA1301 (C58C1::pGV2260::pCAMBIA1301)?
3. What is the difference between 1) and 2) telling you?
4. How many explants tested positive for GUS? If less than the total number of explants you tested, why do you think this difference occurs?
5. Is the production of transgenic plants using *Agrobacterium* 100% efficient?
6. Discuss pros and cons for society of transgenic plants technology.

V. RESOURCES AND USEFUL REFERENCES

Thess articles are on reserve in the library:

1) P. Christou. 1996. Transformation technology. Trends in Plant Science **1**;423-431.
2) Ye X, Al-Babili S, Kloti A, Zhang J, Lucca P, Beyer P, Potrykus I. Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science. 2000 Jan 14;287(5451):303-5.
3) J. Zupan and P. Zambryski. 1997. The Agrobacterium DNA trasfer complex. Critical reviews in plant sciences 16;279-295. (Only the first four pages of this paper are relevant to our discussion).

4) de la Riva, Gonzalez-Cabrera, Vazquez-Padron and Ayra-Pardo. 1998. Agrobacterium tumefaciens: a natural tool for plant transformation. EJB Electronic Journal of Biotechnology, 1; 1-16.

Further reading (you can find them in the library):

Biology of *Agrobacterium*, and infection behaviour:

1) DC Sigee (1993) *Bacterial Plant Pathology: Cell and Molecular Aspects*. Cambridge University Press. Library call number: SB734 .S54 1993.

Plant transformation:

There are many texts and reviews covering this field, including:

2) D Grierson (ed.) (1991) *Plant Genetic Engineering*. Blackie, Glasgow. Library call number: SB123.57 .P57 1991.

3) RG Birch (1997) Plant transformation: problems and strategies for practical application. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**, 297-326. Library call number: QK1 .A48 v.48.

Websites:

1) **Genetic Engineering News**: a major source of information on genetic engineering in agriculture and in drug development, etc.: http://www.genengnews.com/main.htm

2) **Excellent site from the United States Department of Agriculture** giving many links to Plant Genetic Engineering, including the use of a natural insecticidal gene from *Bacillus thuringiensis* (Bt):
http://www.nal.usda.gov/bic/BTTOX/bttoxin.htm

3) **On regulatory issues concerning the release of genetically engineered crops:**
http://www.ndsu.nodak.edu/instruct/mcclean/plsc731/examples/crop.htm

4) Below is an excellent "public information" site explaining how the "Terminator" technology works [this technology enables seed-producing companies to engineer crops so that they kill their own seeds in the second generation, thus making it impossible for farmers to save and replant the seeds]:
http://www.bio.indiana.edu/people/terminator.html

5) **Use of antibiotic-resistance "marker" genes in Genetically Modified Crops:**
http://www.foe.co.uk/camps/foodbio/brief/anti4a.htm

6) A site from **Gene Exchange** - "Union of Concerned Scientists" in the USA: http://www.ucsusa.org/publications/pubs-home.html#Gene

Laboratory #3.
Plant Cell Totipotency and Morphogenesis in Tobacco Leaf Tissue.
Lab report due date: Oct. 20

I. Introduction:

In contrast to other organisms, plants have the capacity to regenerate new organisms from a single somatic cell. This trait is totipotency. To a large extent this is controlled by cellular hormone balance. Plant tissues may respond to changes in hormone balance by changing cellular fate (i.e., callus gives rise to shoots or roots, stems give rise to roots or embryos, leaves give rise to shoots and roots). The two hormones that appear to have the greatest impact on cellular fate have direct effects on the cell cycle. These are auxins and cytokinins.

In this laboratory exercise we will assess the effect of the balance of auxins and cytokinins on organ determination, or cellular fate.

II. Objective:

- To understand the role of auxins and cytokinins during organogenesis in tobacco.

Week #1:

III. Procedures:

- Collect several young tobacco leaves.
- Surface sterilize them using 25% bleach+tween 20 for 5 minutes.
- Rinse two times (with sterile dH_2O).
- Working on the bench, and under aseptic conditions demonstrated by the instructor, cut the leaves into 3-5 mm^2. Sterilize your instruments by dipping them in alcohol between each manipulation and flaming them. Put the leaf pieces into container containing the different culture medium. Shut the lids of the containers. Store jars at room temperature away from direct sunlight.
- Culture media.

We will use a basal culture medium supplemented with hormones at different concentrations [basal medium is MS+2% sucrose, solidified with Agargel (Sigma, Co.)]:

Medium A: basal medium without hormones (control).
Medium B: basal medium+3mg/L BAP (6-benzylaminopurine).
Medium C: basal medium+6mg/L BAP.
Medium D: basal medium+3mg/L BAP+1mg/L IAA (Indole 3-acetic acid).
Medium E: basal medium+6mg/L BAP+1mg/L IAA.
Medium F: basal medium+6mg/L BAP+3mg/L IAA.
Medium G: basal medium+3mg/L BAP+3mg/L IAA.
Medium H: basal medium+1mg/L IAA.
Medium I: basal medium+2mg/L IAA.

Week #2 and #3

Observe the following:

- The development of the explants and take notes on a weekly basis for 3 weeks.
- The color, texture, and expansion of the leaf disks.
- The timing and type of morphogenetic development.
- Describe and count the number of organs that develop.

IV. Questions to address in the lab report

1- Which medium is the best for shoot production? For root production? Why?
2- Is callus necessary for organogenesis?
3- What is the relationship between cytokinins and auxins in root and shoot production?
4- Discuss possible applications of direct organogenesis in the biothechnology industry.

REFERENCES

On reserve in the library:
1) Patterns in Plant development, 2nd ed. 1989, by T.A. Steeves and I.M. Sussex.
2) Plant Cell Culture Technology, Botanical Monographs (vol. 23). 1986, by M.M. Yeoman.
3) Clark, S. 1997. Organ formation at the vegetative shoot meristem. The Plant Cell 9; 1067-1076.

Laboratory #4.
Regeneration Abilities of the Shoot Apical Meristem (SAM).
Lab report due date: Nov. 10

Introduction:

Micropropagation is an important alternative to more conventional methods of plant propagation. It involves production of plants from very small plant parts (e.g. buds, nodes, leaf segments, root segments etc.), grown aseptically (free from any microorganism) in a container where the environment and nutrition can be controlled (*in vitro* conditions). The resultant plants are genetically identical to parent plants.

Micropropagation is possible due to the plant regeneration abilities of meristems developing at different position throughout the plant. The most apical of the meristems in a plant is called the Shoot Apical Meristem and it continuously produce organs (leaves, etc.) and more meristems during the life of the plant. The meristems below the apical meristem are normally formed just above the place where leaves are initiated and are called axillary meristems. Depending on the plant, axillary meristems are inhibited to varying extents by a process called "apical dominance" (discuss apical dominance in your lab report).

Objectives:

- To study the regeneration abilities of plant meristems.
- Introduce student to a technique for *in vitro* plant meristem culture.

Week #1:

Procedures:

- Collect several shoots from Coleus plants (at least five per student).

- Surface sterilize them using 25% bleach+tween 20 for 5 minutes.

- Working on the bench, and under aseptic conditions demonstrated by the instructor, dissect the meristems using a pair of forceps and a scalpel (do not touch the plant material with your hands). Also sterilize your instruments by dipping them in alcohol between each manipulation and flaming them. Small meristems, 1-3 mm long with a few leaves can be cut and transferred to agar medium. Put the shoots into container containing culture medium. Shut the lids of the containers. Store jars at room temperature away from direct sunlight.

-Culture media.
We will use the same basal culture medium supplemented with a hormone at different concentrations [basal medium is MS+2% sucrose, solidified with Agargel (Sigma, Co.)]:
Medium A: basal medium without hormone (control).
Medium B: basal medium+ 2mg/L BAP (6-benzylaminopurine).
Medium C: basal medium+ 5mg/L BAP.

Week #2 and #3

Make observations of the sate of development of the meristems.

QUESTIONS TO ADDRESS IN THE LAB REPORT

- Can the meristems develop in the control medium? Explain and discuss your results in light of what is known.
- According to your results what could be a possible function for BAP?
- What is apical dominance?
- What is somaclonal variation?
- Discuss possible applications of the technique of micropropagation.

REFERENCES

On reserve in the library:
1) Patterns in Plant development, 2nd ed. 1989, by T.A. Steeves and I.M. Sussex.
2) Plant Cell Culture Technology, Botanical Monographs (vol. 23). 1986, by M.M. Yeoman.

Laboratory #5.
The Effects of Different Light Wavelengths on Germinating Corn Embryos
By:

Denise K. Case*, Natalie Vieira*, Lucy Flores*, Jan Mateer* and
Alejandro Calderon-Urrea.
Department of Biology, California State University, Fresno
(*) Undergraduate students, Botany 137, fall 1998.

Lab. report due date: Nov. 24

I. Introduction

Light is essential for photosynthesis, a process by which light energy is used to convert water and carbon dioxide to sugar and other carbohydrates used for food as well as regulation of physiological processes. The sun radiates light in all the colors of the rainbow, which appears white to humans. Pigments, or light-harvesting units of plants, absorb certain colors of the spectrum for photosynthesis and reflect the rest.

II. Objectives:

In this experiment, excised corn embryos will be used to determine the effects of different light wavelengths on early plant growth and development.

III. Procedures:

Since bacteria, molds and fungi can grow on the rich agar medium used for embryo culture, it is important to use sterile technique in this experiment. Corn embryos are completely enclosed in the seed tissues and are sterile as long as the outer seed coat is intact. If the seed coat and tissues are removed aseptically, the embryo can be isolated free of contaminating microorganisms.

All instruments (spatulas, forceps, razor blades or scalpels) used to remove embryos should be sterilized. This is done by dipping in ethanol and flaming. Store the sterilized instruments between pieces of paper towel. You should also sterilize your fingertips since you will be manipulating the corn kernel during removal of the embryo. Fingers can be sterilized by dipping in ethanol. **It is not necessary to flame them!**

- Break an ear of corn in half so that you can remove individual kernels from the center of the ear of corn. Kernels must be removed with the base intact to maintain the sterility of the embryo. Remove kernels from approximately the same area of the ear to obtain embryos of the same size. Dip the kernels briefly in ethanol and set them aside under a paper towel to dry.
- Place a kernel of corn on a piece of paper towel with one of its flat sides up. Look for a small protuberance from the surface at the top of the kernel. The embryo is located on the same side as this structure.
- With the embryo side up, make an inverted V-shaped cut into the seed with a sterile scalpel or razor blade. Peel back the seed coat to expose the embryo.
- Using sterile forceps or spatula, carefully remove the embryo and place it rounded side down on the agar surface of a petri plate and immediately cover the plate.
- Place embryos in groups of six in 4 sterile basal media plates (one plate per treatment. See treatments bellow).
- Seal each plate with a thin strip of parafilm so the agar will not dry out. All cultures will be incubated at room temperature.
- Place one plate in each of the following treatments: fluorescent white light, red light, green light and no light. Red and green lights are produced by wrapping fluorescent lights with red and green colored cellophane respectively. The plates grown in the dark are wrapped with aluminum foil and placed in a dry drawer or carton box away from any source of light.
- Th embryos are grown at room temperature under these conditions for a period of two weeks.
- After incubation measure (in cm) shoot growth, root growth, and shoot (leaves) coloration. Measure the amount of root and shoot growth for each of the emerging shoots and calculate the average growth. Graph your results as histogram bars. Give numerical values to shoot coloration for graphing purposes.

IV. Questions to address in the lab report

- Under what type of light the corn embryos grew better? Explain why. What do you think is happening?
- How does the growth in each of the treatments differ from the control?

USEFUL REFERENCES

On reserve in the library:
1) Yamamoto Y.Y., Matsui M., Ang L.-H. and Deng X.-W. Role of a COP1 interactive protein in mediating light-regulated gene expression in Arabidopsis. The Plant Cell **10**; 1083-1084.

List of Scientific Papers to Discuss.

These papers deal with very specific issues on each of the general fields indicated. When reading these articles try to focus on answering the following general questions:

1) What is the basic question being addressed?
2) What are the tools and methodologies used to answer the question?
3) What are the results?
4) What did the scientists conclude based on their results? Are there any speculations?
5) What would you do differently to address the same question? What would be the next question to address, and how would you address it?

A cautionary note: Do not be discouraged if you are unable to understand the language of the paper, or if you are unable to answer all the questions above. Just do your best!! It is very difficult to read and understand the scientific literature. At the end of the course, however, you will realize that it is just a matter of practice. The papers will be discussed during the indicated laboratory sessions. You should be ready to answer questions relevant to the paper in discussion.

Discussion 1: Sep. 22.

Genetic Engineering in Plants:

Background papers:

These papers will not be discussed specifically but if you read them you will have a better understanding of plant genetic transformation and the social issues associated with it.

1) J. Zhu, *et al.* 2000. The basis of crown gall tumorigenesis. Journal of Bacteriology **182**; 3885-3895.
2) Hellens, *et al.* 2000. A guide to *Agrobacterium* binary Ti vectors. Trends in Plant Science **5;** 446-451.
3) D. Shibata, and Y.-G. Liu. 2000. *Agrobacterium*-mediated plant transformation with large DNA fragments. Trends in Plant Science **5**; 354-357.
4) L.R. Herrera-Estrella. 2000. Genetically modified crops and developing countries. Plant Physiology **124**; 923-925.
5) R.S. Hails. 2000. Genetically modified plants-the debate continues. Trends in Ecology and Evolution **10**; 14-18.
6) J. Gewolb. 2002. Plant scientist see big potential in tiny plastids. Science **295**; 258-259.R.

Discussion paper:

This paper will be discussed at length, so be prepared to answer questions regarding the paper.

Ye X, Al-Babili S, Kloti A, Zhang J, Lucca P, Beyer P, Potrykus I. 2000. Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm.
Science; **287** (5451):303-305.

Discussion 2: Oct. 6.

Hormones:

Background papers:

D. R. McCarty, and J. Chory. 2000. Conservation and innovation in plant signaling pathways. Cell **103**; 201-209.

Discussion paper:

Y. Yin, *et al.* 2002. BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. Cell **109**; 181-191.

Discussion 3: Oct. 27.

Morphogenesis:

Background papers:

J.C. Fletcher. 2002. Coordination of stem cell proliferation and cell fate decisions in the angiosperm shoot apical meristem. BioEssays **24**; 27-37.

Discussion paper:

E. Rojo. *et al.* 2002. CLV3 is localized to the extracellular space, where it activates the Arabidopsis CLAVATA stem signaling pathway. The Plant Cell **14**; 969-977.

Discussion 4: Nov. 24.

Photomorphogenesis:

Background papers:

C. Schwechheimer, and X.-W. Deng. 20020 The COP/DET/FUS proteins-regulators of eukaryotic growth and development. Seminars in Cell and Developmental Biology 11; 495-503.

Discussion paper:

M. Holm, *et al.* 2002. Two interacting bZIP proteins are direct targets of COP1-mediated control of light-dependant gene expression in *Arabidopsis*. Genes and Development **16**; 1247-1259.