## ***Arabidopsis* Lab Manual**

## Genetics Project Laboratory

Spring 2010

Instructor: Dr. Lawrence Hobbie

Teaching assistant:

## This lab notebook belongs to:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

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### The name of the enhancer mutant is: ­\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_Table of Contents

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### Biology 224 Laboratory, Spring 2010

The laboratory class will meet in Science 124, Monday and Wednesday, 3:50-6:20 pm

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### Overview of Course

### Goals of the Course

In this genetics project lab course, you will carry out four experiments during the semester. These experiments are similar to those carried out by many geneticists. Through this in-depth concentration on just a few experiments, I hope that you will gain the following:

1. an understanding of the genetic approach to biological problems and of the theory and methods of molecular genetic mapping
2. an understanding of the key features of experimental design
3. an understanding of the importance of proper statistical analysis of experimental results
4. a knowledge of the organization of the scientific literature and scientific databases
5. an improved ability to give presentations on scientific subjects
6. an appreciation for the challenges and rewards of research

### Experimental Material and Projects

In all the experiments that you will work on, you will use the small weed *Arabidopsis thaliana*, a relative of mustard and broccoli, which not coincidentally is the plant that I use in my own research. *Arabidopsis* is widely employed in laboratory studies of almost every aspect of plant biology. In the first three experiments, you will characterize mutant plants with altered responses to the plant hormone auxin using genetic and physiological approaches. In the fourth experiment, you will use molecular biology techniques to determine the chromosomal position of a mutation. You will need to be organized, efficient, and aware of what you are doing for which project at all times.

### General Organization of the Laboratory

Most lab periods will begin with an introductory lecture. You will work in pairs during the lab. During the first few weeks of the semester, the instructor and TA will guide you through the techniques that you will need to learn as you carry out Experiments 1 and 2 simultaneously. You will then work more independently to complete Experiment 3 (an experiment of your own design) during the next month or so, concluding with presentations of your results. Then, for the last few weeks of the semester, we will concentrate on Experiment 4.

To encourage you to come to lab prepared, you are required to submit before each of the first 15 lab periods, via Moodle, a brief summary, in your own words, of what you will be doing in lab and a brief explanation of why you are doing it. Copying directly from the lab manual is not acceptable for this prelab summary: the proper approach is to read the entire description of the lab, and then write in your own words your understanding of what you will be doing and why. This prelab description must be at least one paragraph (three sentences) long.

The procedures you are to follow in the experiments are described in detail in this lab manual. You should keep your lab manual in a one-inch three-ring binder with pockets inside the cover. The lab manual will also serve as your lab notebook, where you must record the details and dates for the experiments that you do in lab; you will find that the more complete your lab notebook, the easier it will be for you to complete your lab reports. You will need to submit the relevant pages from your lab notebook along with your lab reports, so keep your lab notebook up-to-date, accurate, and complete. This is especially important for Experiment 3, your independent project.

The due dates for the various assignments (other than prelabs) are as follows:

|  |  |
| --- | --- |
| **Assignment** | **Due Date** |
| Lab report on Experiment 1 | Feb. 12 |
| Lab report on Experiment 2 | Feb. 22 |
| Presentation of plan for Experiment 3 | Feb. 24 |
| Abstract submission deadline  | March 13 |
| Rough draft of poster | April 5 |
| Practice presentation of poster | April 7 |
| Poster presentation of Experiment 3 results | April 12 |
| Lab report on Experiment 4 | May 10 |

Each group will give a short presentation on its plans for characterizing the mutant that it has been assigned. The second will be at the end of the semester, when each group will give a presentation of its results from the genetics project in the form of a poster at a poster session.

The final grade for the project lab course will be determined as follows:

5% Prelab summaries

15% Lab report on Experiment 1

15% Lab report on Experiment 2

10% Presentation of plans for Experiment 3

25% Poster presentation of results of Experiment 3

15% Lab report on Experiment 3

15% Lab report on Experiment 4

Policies on accessibility, absences, and late work are identical to those for Biology 222.

### Keys to success in genetics lab

* Follow directions.
* Be organized: prepare for lab by reading over the lab manual and thinking about what you did previously, so that you know what you’re doing before you come to lab.
* Keep good notes of what you do in lab: every time you come to lab, write down in your lab notebook the key details of what you have done.
* Be meticulous: perform an experiment carefully and without rushing so it will be done correctly!
* Plan to complete what you need to for the week.
* Consult with the instructor or TA with any questions.

### Safety in the Laboratory

Because there is the possibility for accidents and danger in every laboratory, you must pay careful attention to the instructor’s and TA’s directions with regard to laboratory safety. Protective equipment such as safety goggles and gloves will be available and should be used when so directed by the instructor. The instructor will point out and explain the use of safety equipment such as an eyewash, a safety shower, and a fire blanket. The laboratory can be kept a safe place by using common sense and following directions.

#### Laboratory Rules

**1. No eating, drinking, or smoking in the laboratory.**

**2. Wear safety glasses or goggles** for any procedure in which your eyes could be endangered, such as handling a hot solution or hazardous chemicals or viewing UV light.

3. **Wear protective gloves** for any exercise involving hazardous chemicals, such as ethidium bromide or hot liquids.

4. Report any injury to the instructor or TA immediately.

**5. Closed-top non-mesh shoes and long pants** are recommended attire in the lab. Long hair should be tied back.

**6. Keep the laboratory clean.** Discard your trash in the wastebaskets before you leave. Broken glassware or glass items such as slides go in the broken glass containers only. Do NOT put other kinds of trash into the broken glass container. **Don't mix glass and non-glass items in the trash.** For pipet tips, tubes, etc. there will be special waste containers.

**7. Discard waste liquids in the proper container, as designated by the instructor.** Some hazardous wastes must be kept separate during disposal.

**8. Rinse out dirty glassware and then place it in the areas designated by the instructors.**

9. Return and put away all equipment and supplies when finished.

**10. Listen carefully** to the instructor’s directions regarding any hazards in the lab.

11. The instructor or a TA must be present for you to work in the lab; never work alone.

12. Wash your hands before leaving the laboratory.

**Biology 224 Lab Schedule, Spring 2010**

|  |  |  |  |
| --- | --- | --- | --- |
| **Date** | **Expt 1: Analysis of inheritance** | **Expt 2:** **Analysis of root auxin response** |  |
| Jan. 25 (M) | IntroductionReview of pipettor useSterilize & plate seeds |  |
| Jan. 27 (W) | Plates out to growth chamber | IntroductionSterilize & plate seeds Calculations for making plates |
| Jan. 29 (F)  |  | Plates out to growth chamber |
| Feb. 1 (M) |  | Make plates |
| Feb. 3 (W) | Analyze seedlings & put to dirt | Transfer seedlings to hormone plates |
| Feb. 8 (M) | Discuss results | Measure seedlings; enter & analyze data | **Expt 3:** **Student-designed experiment** |
| Feb. 10 (W) | Measure seedlingsEnter data | Discuss results | Introduction; begin literature search |
| Feb. 12 (F) | **1st lab report due** |  |  |
| Feb. 15 (M) |  |  | Literature search  |
| Feb. 17 (W) |  | Literature search & discuss project |
| Feb. 22 (M) | **2nd lab report due** | Project planning |
| Feb. 24 (W) |  | **Project presentations** |
| March 1 (M) | Work on project |
| March 3 (W) | Work on project |
| March 8 (M) | Work on project |
| March 10 (W) | Work on project |
|  |  | **Expt 3:** **Student-designed experiment** |
| March 13 (S) | **Deadline for abstract submission to Adelphi Research Conference** |
| March 13-21 | **Spring Break** |
| March 22 (M) |  | Work on project |
| March 24 (W) | Work on project |
| March 29 (M) | Work on project |
| March 31 (W) | Work on project |
| April 5 (M) | **Rough draft of poster due** |
| April 7 (W) | **Final poster preparation & practice presentations** |
| April 12 (M) | **Adelphi Research Conference poster presentations** |
| April 14 (W) | Review, revise, practice presentations |
| April 16 (F) | **Experiment 4: Molecular mapping** |  |  |
| April 19 (M) | DNA isolation; set up PCRs |  |
| April 21 (W) | Run gels |
| April 26 (M) | Discuss results; set up PCRs |
| April 28 (W) | Run gels |
| May 3(M) | Set up PCRs |
| May 5 (W) | Run gels |
| May 10 (M) | **3rd lab report due** |
| May 11 (T) | Finals start |

**Genetics Project Descriptions**

An encouraging note

This introduction contains lots of information and will therefore likely be confusing the first time you read it and the first time it is explained to you. Do not be discouraged in the beginning! The project will be explained to you more than once. If you re-read these project descriptions several times during the semester, you will find that you understand more each time you do. You will, I hope, find it rewarding to feel that you understand such a complex project as this one.

### Introduction to the Projects: Auxin

In this project you will use the genetic approach to a biological problem. The genetic approach enables researchers to identify genes important for a biological process of interest, and to understand the roles of these genes (these are important steps in fully understanding how the biological process works). One biological process that scientists are using the genetic approach to understand is the control of plant growth by the hormone auxin. Auxins are small molecules that are involved in almost every aspect of plant growth and development; they cause plant cells to divide, elongate, or differentiate. The structures of four molecules with auxin activity are shown at right: the major naturally occurring auxin, indole-3-acetic acid (IAA); a related natural auxin, indole-3-butyric acid (IBA), which is widely used in horticulture in compounds that promote root development in cuttings; and two synthetic auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthalene acetic acid (1-NAA).

 

Fig.1. Natural and synthetic auxins.

**Why Study Auxin?**

Auxin was identified in the 1930s, yet we still do not fully understand how a plant cell synthesizes, metabolizes, recognizes, responds to, and transports auxin. A genetic approach has been very successful in identifying at least some of the genes involved in auxin physiology. Because of the importance of auxin in plant growth and development, a better understanding of auxin’s biosynthesis, transport, and response might enable plant scientists to develop agriculturally-important crop plants with improved yield or desirable growth characteristics.

**The Genetic Approach**

The first step in the genetic approach is to isolate mutants that are altered in the process of interest. Many mutants that are altered in their response to auxin have already been isolated and characterized by researchers around the world. One straightforward way to isolate such mutants relies on the finding that although low concentrations of auxin are necessary for plant growth, high concentrations actually inhibit growth. (This growth inhibition at high concentrations is the basis for the action of a number of herbicides, such as the synthetic auxin 2,4-D.) In a screen that has been widely used to identify auxin mutants, seeds are germinated on growth medium that contains a concentration of auxin sufficient to inhibit the growth of wild-type seedlings. Mutant seedlings that are less responsive to auxin are able to elongate their roots on the auxin-containing medium and can be easily identified. A series of *auxin-resistant* (*axr*) mutants have been thus selected and characterized, including the *axr4* mutant that was the starting point for this project (Hobbie and Estelle, 1995). This mutant was also isolated independently for defects in root gravity response (“gravitropism”; Simmons et al., 1995). The *axr4* mutants were shown by Yamamoto and Yamamoto (1999) to have interesting differences in their responses to different types of auxins that suggested what type of pathway might be affected by the *axr4*. The *AXR4* gene was recently cloned and the sequence of the AXR4 protein determined (Dharmasiri et al., 2006). However, in this case (as happens fairly frequently), characterization of the gene at the molecular level did not reveal the biochemical function of the protein. The central question that the project you will work on seeks to answer in the long term is: *What is the biochemical function of the AXR4 gene?*

One approach often pursued in such a situation is to extend the genetic approach to try to identify other genes in the same pathway or interacting pathways. If such related genes can be identified, perhaps enough may already be known or can be discovered, about their function to infer the function of the starting gene. For example, if related genes encode enzymes that affect lipids, that may indicate that the starting gene is also involved in lipid synthesis or metabolism in some way. One way to identify these interacting genes is to look for second mutations, called modifier mutations, that alter the phenotype produced by the starting mutation. One type of modifier mutation is an enhancer mutation, which when combined with the starting mutation produces a stronger phenotype. A second type of modifier mutation is a suppressor mutation, which when combined with the starting mutation, produces a weaker or even wild-type phenotype. We have isolated a number of putative enhancers of *axr4*, one of which you will analyze during this lab (if you don’t know the definition of “putative”, look it up!). The approach used to isolate the enhancers is described in more detail below.

The second step in the genetic approach is to characterize the mutants phenotypically and genotypically. This characterization helps us to understand the normal role of the mutated gene. As part of the genetic characterization, you will determine, in Experiment 1, if the enhancer mutation is dominant or recessive. You will also, in the same experiment, be able to determine if the enhancer mutation itself (when separated from *axr4*) confers auxin resistance. To do so, you will analyze the auxin resistance of F2 seedlings resulting from a cross between the enhancer mutant (which also contains the *axr4* mutation, i.e. *aaee*) and a wild type plant (*AAEE*). The wild-type and mutant alleles of both *AXR4* and the *ENHANCER* genes will segregate or assort independently in the F2 generation, giving rise to at least three and potentially four different phenotypic classes. After determining the proportion of seedlings in each class, you will use the chi-squared test to test the hypothesis that you generate.

To begin the phenotypic characterization, you will carry out Experiment 2, in which you will measure the auxin response of the plants that contain both the enhancer mutation and the *axr4* mutation (we will use the shorthand designation of *aaee* for the genotype of these plants: *a* for the *axr4* mutation, and *e* for the *enhancer* mutation). You will do this by analyzing the growth of roots in these plants in the absence and presence of different types of exogenous auxin. You will then design and perform one other experiment (Experiment 3) to determine how your mutants may be altered in their growth, morphology, or physiology.

The third step in the genetic approach is to identify and study at the molecular level the gene that is mutated. You will begin this process in Experiment 4 by attempting to determine an approximate map position for the enhancer mutation, i.e. where is this mutation located on the chromosomal map of *Arabidopsis*? You will isolate DNA from plants and then characterize the DNA with the techniques of polymerase chain reaction and gel electrophoresis (mapping is explained in more detail below).

**Identification of the Enhancer Mutant: Step 1, Mutagenesis**

Mutations naturally occur at very low rates because organisms have evolved the ability to prevent, detect, and correct mutations to ensure the stability of the genetic material. Therefore, to increase the frequency of mutations to a detectable level, seeds from *axr4-3*  *Arabidopsis* were soaked in the mutagenic chemical ethylmethane sulfonate (EMS), which generally produces single basepair changes in the DNA sequence. Note that EMS produces mutations randomly—there is as yet no way in plants to cause mutations in a specific gene of interest. This seed that has been directly mutagenized is called the “M1” generation. In a heavily-mutagenized M1 populationof sufficient size, there will be seeds with mutations in essentially every gene in the entire plant (although each seed probably has only 5-20 mutations). Mutations produced in the M1 seed will almost always be heterozygous. This heterozygosity means that only dominant mutations can be identified by their mutant phenotype in the M1 generation. To be able to identify recessive mutations, M1 seed was grown up into plants and the M1 plants allowed to self-fertilize, producing what is called the M2 seed. The M2 seed carries the same mutations that were present in the M1 generation, but in some of the M2 seed a particular mutation will have become homozygous. This homozygosity enables recessive mutations to be detected by their mutant phenotype, and explains why M2 seed is generally used for mutant screens and selections.

**Step 2, Selection**

The M2 seeds were sterilized, spread on plates containing a high concentration of auxin (0.4 µM 2,4-D), and then screened a week or more later for seedlings whose roots elongated more than those of control *axr4* seedlings (which themselves elongated

more than those of wild-type seedlings on this medium; Fig. 2).

These seedlings with enhanced auxin resistance are the ones of interest to us: they are likely to contain mutations in genes that affect similar functions as those in which *AXR4* is involved. These seedlings were transferred to soil and grown up. The M2 plants self-fertilized to give the M3 generation. The M3 seeds were re-tested, and those plants which still showed an enhanced auxin-resistant phenotype (i.e. greater than *axr4*) were planted. Plants from the M3 generation were back-crossed to wild-type plants and, in the F2, the plants with the enhanced auxin-resistant phenotype were again identified (back-crossing is done to eliminate the other irrelevant mutations produced by the EMS that were present in the M2 and M3 lines). These lines are termed “1X backcrossed”. All students will be characterizing the same enhancer mutant this semester.

putative M2 mutants

*axr4-3* seeds

EMS treatment

M1 seed

self-fertilize

M2 seed

select long roots on 4x10-7 M 2,4-D

M3 seed

self-fertilize

re-test for auxin resistant root elongation

verified M3 mutants

cross to wild-type Columbia

F1 seeds

F2 seeds

self-fertilize

Analyze ratio

Identify enhancer mutant

F2 verified enhancer

self-fertilize

1X backcrossed enhancer, F3

additional re-testing and backcrossing

Figure 3. Identification and testing of *axr4* enhancer mutants.

**Determining the Inheritance Pattern and Phenotype of the Enhancer Mutation**

In Experiment 1, you will determine the inheritance pattern of the enhancer mutation by comparing growth on auxin of wild-type seedlings, *axr4*, enhancer mutant seedlings, and F2 seedlings from a cross between wild type and enhancer mutant. You will analyze the results of this experiment using the chi-squared test. You will also determine whether the enhancer is itself auxin resistant or auxin 

wt *axr4* enhancer

Fig, 2. Seedlings grown on 0.4 μM 2,4-D

Fig, 3. Outline of enhancer isolation procedure

enhancer mutation is less likely to have been already studied by other scientists, as many auxin-resistant mutations have been previously characterized.

**Phenotypic Characterization of Your Mutant**

During the first weeks of the semester, you will be guided in carrying out one phenotypic experiment (Experiment 2) to determine how the enhancer mutation affects root growth in the absence and presence of exogenous auxin. This experience will help you to design your own experiment to examine the enhancer mutant phenotype (Experiment 3). You will need to do research in the scientific literature to learn more about the topic, see what other researchers have done, and come up with an hypothesis to test. In consultation with the instructor, you will design an experiment, and then will present your plan to your classmates. During the middle of the semester you will carry out this experiment (Experiment 3), whose results you will analyze and present in a poster at the Adelphi Research Conference. You must perform the experiment at least twice to demonstrate that your results are reproducible.

A note about gene symbols in *Arabidopsis*

The scientists who study *Arabidopsis* have agreed on certain conventions or rules for naming gene symbols in this plant. Here are some of the most important rules:

1. Mutant alleles of genes are given a three letter designation that is written in lower case italics, e.g. *axr* for “auxin-resistant”. This is true whether the mutation is dominant or recessive. Multiple mutations that result in the same phenotype can be given different names, e.g. *aux* for “auxin-resistant”, or have a number added, e.g. *axr4* is a mutation in a different gene from *axr1, axr2, axr3* etc.

2. Wild-type alleles of genes use upper-case italics, e.g. *AXR4*.

3. The protein product of a gene is written in upper-case, no italics, e.g. AXR4 refers to the protein product of the *AXR4* gene.

4. Different mutant alleles in the same gene are distinguished by a hyphen followed by a number, e.g. *axr4-1* and *axr4-2* are different mutations both in the *AXR4* gene.

Note that in this lab manual the abbreviations *a* for *axr4* and *e* for the *enhancer* are used.

## **References**

Dharmasiri S, Swarup R, Mockaitis K, Dharmasiri N, Singh SK, Kowalchyk M, Marchant A, Mills S, Sandberg G, Bennett MJ, Estelle M. 2006. AXR4 is required for localization of the auxin influx facilitator AUX1. *Science* **312**,1218-20.

Hobbie L, Estelle M. 1995. The *axr4* auxin-resistant mutants of *Arabidopsis thaliana* define a gene important for root gravitropism and lateral root initiation. *Plant Journal* **7**, 211-220.

## Lukowitz W, Gillmor CS, and Scheible W-R. 2000. Positional cloning in Arabidopsis. Why it feels good to have a genome initiative working for you. *Plant Physiology* **123**, 795-805.

## Simmons C, Migliaccio F, Masson P, Caspar T, Soll D. 1995. A novel root gravitropism mutant of *Arabidopsis thaliana* exhibiting altered auxin physiology. *Physiologia Plantarum* **93**, 790-798.

## Woodward AW, Bartel B. 2005. Auxin: regulation, action, and interaction. *Annals of Botany* **95**, 707-35.

Yamamoto M, Yamamoto KT. 1999. [Effects of natural and synthetic auxins on the gravitropic growth habit of roots in two auxin-resistant mutants of Arabidopsis, *axr1* and *axr4*: evidence for defects in the auxin influx mechanism of axr4.](http://www.ncbi.nlm.nih.gov/pubmed/11543173?itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum&ordinalpos=28) J. Plant Res. **112**, 391-396.

## **First Lab, Monday, Jan. 25**

## Overview of first class

Introduction to genetics lab, safety, and to the genetics project

Introduction to calculations for making plates

Review of micropipettor use

Begin characterization of mutant (Experiment 1):

Sterilize and plate seeds for inheritance analysis (Step 1 of Experiment 1)

## Notes for first class

We will begin with an overview of safety procedures for this laboratory class (see laboratory introduction).

You should be sure that you know who the other students at your lab table are. Introduce yourself!

Today you will begin the phenotypic characterization of your *Arabidopsis* mutant by sterilizing mutant seeds along with control *axr4-3* and wild-type seeds and spreading them on plates. In a future lab period you will transfer them to plates containing plant hormone that you will prepare, and then you will analyze their root growth.

**Use of Micropipettors**

Micropipettors are expensive precision instruments that must be used properly. Please pay attention during the instructor’s description of how to use them, and read the instructions below carefully!

Pipettors are named according to the largest volume that they can hold: a P10 (gray top button) can pipet no more than 10 µL, a P100 (yellow top button) no more than 100 µL, and a P1000 (blue top button) no more than 1000 µL (1 mL). Note that the different sized pipettors use different pipet tips. For the highest accuracy in pipetting, use the smallest size pipettor that can pipet the proper volume, e.g. 10 µL will be most accurately pipetted using a P10, not a P100, and 100 µL will be most accurately pipetted using a P100, not a P1000.

**•Never** rotate the volume adjuster above or below the limits of the pipettor (below 0 or above the upper volume range).

**•Never** immerse the barrel of the pipettor in liquid or use the pipettor without a disposable tip in place.

**•Never** let the plunger of the pipettor snap back after depressing it, as the plunger could be damaged and aerosols could enter the pipet barrel.

**•Never** lay down the pipettor with fluid in the attached tip, as fluid could enter the barrel and damage the pipettor.

**• Never** drop or flame the pipettor, as this will damage it.

**•** Be sure to **set the volume correctly**. Setting the volume incorrectly is a very common source of errors when pipetting! If you have questions about the proper setting, ask the instructor or TA.

**• Be sure** that you can see the liquid entering and exiting the pipet tip as you pipet. This precaution will help you to pipet correctly and accurately.

**•** If your pipettor is not working properly, or you have questions, consult the instructor or TA.

**Pipetting Directions**

1. Rotate the volume adjustor on the pipettor to the desired setting.

2. Firmly attach a proper-sized pipet tip on the end of the pipettor:

a. You will be provided with tips in dispenser boxes.

b. The proper way to remove a tip is not to pick it up with your fingers, which could contaminate the other tips in the box, but to insert the thin tip of the pipettor into the pipet tip as the tip sits in the dispenser box, give a little twist while pressing down to seat the pipet tip firmly on the pipettor, and then remove the tip from the box.

c. Once the pipet tip is out of the box, you can also give it an extra twist onto the pipettor with your hand if you are careful not to contaminate the pipet tip.

3. Pick up the microcentrifuge tube out of which you will be pipetting. To reduce contamination, hold the body of the tube, not the lid. Hold the tube up nearly at eye level rather than leaving it in the rack; in this way you can properly position the tip in the liquid, and observe the changes in liquid level in the pipet tip. Also, do not have another person hold the tube for you.

4. Practice depressing the plunger on the pipettor.

a. You should be able to feel two stop positions: depressing to the first position measures the desired volume, depressing to the second position is used to “blow out” the last remaining drop of liquid.

b. Do not depress to the second stop when measuring the volume, or you will get a bigger volume of liquid than you intended.

5. To withdraw a sample from the tube:

a. Hold the pipettor nearly vertical;

b. Depress the plunger to the first stop and hold. Dip tip into the solution to be pipetted, and slowly raise the plunger to pull fluid into the tip. If you raise the plunger very quickly, you risk getting air bubbles into your sample (especially a problem when using the P1000) as well as creating aerosols, which with some samples could be hazardous.

c. Slide pipet tip out along inside wall of tube, to dislodge droplets stuck to the tip. Check that there is no air space at the end of the tube.

If something goes wrong along the way, and you have not touched the pipet tip to anything else, you can always put the liquid back in the tube and re-pipet it.

6. To expel the sample into a second tube:

a. Be sure you are holding the second tube so you can see it also!

b. Touch the pipet tip to the inside wall of the tube, to create a capillary effect, and slowly depress the plunger to the first stop to expel the sample. Depress the plunger to the second stop to blow out the last bit of liquid. Hold the plunger in the depressed position.

c. Slide the pipet out of the tube with the plunger depressed to avoid getting liquid back into tip.

d. Remove the tip manually, or eject it with the second (tip-ejection) button, into a container designated for that purpose, either a beaker or a plastic tub. Do not eject the tip into the sink.

7. To prevent cross-contamination of reagents, use a new tip for each transfer. The only exception is if you are adding the same reagent to a series of tubes in such a way that you will not contaminate your original stock solution. For example, if you are putting the same volume of water into a series of clean tubes that have nothing else in them, you can use the same tip for all the transfers.

**Experiment 1**

This lab manual is organized primarily by experiment rather than by days. Refer to the schedule of tasks in the front of the lab manual to be sure you know what to do on each day. Today you should carry out Step 1 in the description of Experiment 1 that follows

**Experiment 1**

**Analyzing the Inheritance and Auxin Response of the Enhancer Mutation**

In this experiment you will determine the inheritance of the enhancer mutation (i.e. dominant or recessive) and if this mutation by itself makes a plant auxin resistant. We know that the enhancer mutation together with the *axr4-3* mutation (genotype *aaee*) makes plants auxin resistant. Here we are interested in whether the enhancer mutation without the *axr4-3* mutation makes plants auxin-sensitive or auxin-resistant. The plants with only the enhancer mutation will have the genotype *AAee*. You will be able to determine these two characteristics by analyzing the F2­ seed from a cross between the mutant and wild type (a “backcross”). The F2 seedlings will also be used as part of Experiment 4 to map the enhancer mutation. After analyzing the F2 seedlings, you will put some of them into dirt and in the future we might use them as part of our mapping population.

Experiment 1 has the following steps:

Step 1, Day 0 (Mon., Jan. 25.): Sterilize seed and spread on plates containing auxin (4 x 10-7 M 2,4-D). Cold treat.

Step 2, Day 2 (Weds., Jan. 27.): Put plates with seeds into growth chamber.

Step 3, Day 9 (Weds., Feb. 3, after 7 days of growth): Analyze seedlings.

**Step 1: Seed sterilization and plating (Mon., Jan. 25)**

During the first lab period on this experiment you and your lab partner will sterilize and plate the following seeds:

* the WM20.2 enhancer mutant, backcrossed 1X to wild type Col (the enhancer is in the Wassilewskija ecotype)
* Columbia (abbreviated “wtCol”): a wild-type control
* Wassilewskija (abbreviated “wtWs”): a wild-type control
* *axr4-3*: the parental line for the enhancer mutant, also a control
* F2 seeds, produced by crossing the enhancer mutant to one the Columbia wild type.

Because it is good laboratory practice to keep a detailed laboratory notebook, record the detailed information about the genotype of the seeds in the space provided for “Notes on Today’s Procedure” at the end of this protocol.

You will spread these seeds on plates that contain, in addition to growth medium, a synthetic form of auxin called “2,4-D” at a rather high concentration, 4 x 10-7 M. These plates are abbreviated “4D”.

**Step 1: Sterilize the seeds and spread them on plates.**

To sterilize the seed, follow these steps:

1. If the seed is not already in tubes, transfer some seed to a 1.5 mL microcentrifuge tube that you have labeled, preferably on the lid, with the identity of the seed.

2.a. Add, to each tube, 1 mL of the provided sterilizing solution using the large pipet tips and the 1000 µL pipettors (the largest ones, with the blue buttons).

 What do you have your pipettor set on?\_\_\_\_\_\_\_\_\_\_\_

b. Close the lid of the tube and shake to suspend the tubes in the solution; you may need to flick the bottom of the tube with your finger to loosen the seeds.

c. Incubate the seeds in the sterilizing solution for 15 minutes. (The sterilizing solution consists of 30% bleach, 0.02% TritonX-100 (a detergent), and water).

CAUTION: The sterilizing solution contains bleach, which is hazardous if it gets in your eyes and causes spots if it gets on your clothing. Wear safety glasses and use special caution not to get any solution on your clothes.

3. While the seeds are incubating, label the plates on which you will spread the seeds. For Experiment 1, you will use “4D” plates containing a high concentration of the synthetic auxin 2,4-D. Be sure to label the plates on the bottom with your initials, the date, and the identifying information (genotype) of the seeds. Also label the lid of each plate with your initials so you will be able to find your plates easily in the growth chamber.

4. Also while the seeds are incubating, you should melt the top agar (0.7% agar in water) in the bottle provided. To do so, put it in the microwave with the top loose but covering the mouth of the bottle and then heat on low power (20% power) for 5-10 minutes until the agar is melted—do not let the agar boil over! If it does, clean up the microwave. Once it is melted, you can put it in a 55 degree water bath if available to keep it from solidifying.

5. After the 15 minute incubation, give the tubes a very brief spin in the microcentrifuge: just press and hold the “quick spin” button for about 5 seconds. Get in the habit of placing your tubes in the microcentrifuge with the hinges facing the outside.

If possible, you would perform the subsequent steps in a sterile (“laminar flow”) hood such as the ones available in the instructor’s lab, but if you work carefully you should be able to maintain adequate sterility of the seeds while working at your lab bench.

6.a. Remove the tubes from the microcentrifuge.

b. Carefully open the lid of the tube.

c. Use the same pipettor and type of tip (alternatively, you may use a sterile Pasteur pipet with pipet bulb) to remove as much of the sterilizing solution as possible without getting any seeds into the pipet tip.

d. Discard the sterilizing solution into a liquid waste beaker.

If you do get a few seeds into the pipet tip by accident, gently return them to the tube (some of the sterilizing solution will go along with them). Let the seeds settle for about 15 seconds, then try again to pick up the sterilizing solution without getting any seeds.

e. Touch only the outside of the tube; do not touch the inside of the lid, as this may compromise the sterility.

f. If the pipet tip has not touched anything other than the sterilizing solution and the inside of the tubes, and there are no seeds stuck inside it, you can use the same pipet tip for another tube. Otherwise change pipet tips after each tube.

7.a. Use a new pipet tip to place 1 mL of sterile distilled water into each tube. Add it against the side of the tubes. Don’t squirt in the water so hard that the seeds splash out!

b. Close the lids and shake the tubes to resuspend the seeds. You may need to “flick” the bottom of the tubes with your finger. Proceed immediately to the next step.

c. Now, repeat the spin (step 4), removal of water (step 5), and addition of more water (step 6a) three more times. This will make a total of three complete washes. The seeds will end up sitting in water.

If necessary, you can stop the procedure here: close the lids of the tubes and store them in a rack in the refrigerator for not more than a week before spreading them on plates. However, it is best if you continue the procedure to spread the seeds on plates.

#### To spread seeds on plates

8. Use a Pasteur pipet and pipet bulb, or a P1000 pipettor and pipet tip, to suck up the seeds from the tube and then to squirt them onto the appropriate labeled plate. The instructor will describe what kind of plate and how many plates to use. For Experiment 1, use one “4D” plates for each tube of seeds; be sure to record this in the space for “Notes on Today’s Procedure.” It is all right to pick up some or all of the water in the tubes along with the seeds. Try to open the lid of the plate as briefly as possible when distributing the seeds.

9. Pipet 3 mL of melted top agar from the bottle onto the plate on top of (or, if the agar is hot, around) the seeds, quickly close the lid of the plate, and then tip or shake the plate gently in all directions to distribute the seeds as evenly as possible over the surface of the plate. Let the plate sit for at least minutes to allow the agar to harden. It is important to spread the seeds evenly; if they are too crowded, they will not grow well and it will be difficult to pull seedlings off the plate for the next step.

Caution: If you pick up the plate too soon and shake it, you may dislodge the top agar, which will never again be able to adhere properly to the plate. So, test to see if the top agar is solidified by first tipping the plate gently.

10. When the top agar is solidified, wrap surgical (“micropore”) tape around the edge of the plate so that it seals the gap between the lid and the bottom of the plate. Use rubber bands to hold your plates together.

11. Place the stack of plates on a tray. This tray will be covered with plastic wrap and placed in the refrigerator. This cold treatment (called “stratification”) ensures good germination of the seeds.

The cold treatment can be carried out either when the seeds are in the tubes of water or when the seeds are on the plates. The stratification should last least two days, and can be as long as about two weeks, but not forever (eventually *Arabidopsis* seeds will start to germinate on their own, even in the cold and the dark).

## Notes on today’s procedure

## In this section of each procedure you should record briefly what you did each day in lab. Be especially sure to record important information that is not already in the lab manual such as detailed information on the seeds you sterilized (the ID labels and generations, if available), the type of plates that you used, the dates that you do things, and anything unusual that happens or that you observe (for example, suppose that the label on one of your tubes is hard to read; you think it says “43”, but it could be “48”. Make a note of it—if you have confusing results later on, maybe your lab notebook will help you explain them).

**Notes on today’s procedure**

#### Experiment 1 continued

#### Step 2: Moving the plates to the growth chamber (Weds. Jan. 27)

When the plates have been cold-treated for two days, they should be placed into the growth chamber. Unwrap the plates and put them in a single layer flat on the shelf.

You or someone in your group is responsible for making sure that the plates are moved—the instructor or TA may do this for you if you arrange it. Be sure you arrange it so that it happens, and record the date when this is done.

**Notes on today’s procedure**

## **Step 3: Analyzing the F2 seedlings (Weds. Feb. 3)**

1. First you must determine the root length of the parental wild-type Wassilewskija and Columbia, mutant *axr4-3*, and WM20.2 enhancermutant seedlings. Use forceps to remove 10 seedlings from each plate (do so gently, placing the forceps under the cotyledons and lifting up rather than squeezing the hypocotyl) and stretch them out on a clear place on the plate or on a separate blank plate so the roots can be easily measured. The root is measured from the bottom of the hypocotyl to the root tip. You can identify the root-hypocotyl junction by the presence of the brown seed coat or the appearance of root hairs, which begin just at the root side of this junction. Record the root lengths here, in mm, and find the mean and range.

Date:

Growth medium used:\_\_\_\_\_\_\_\_\_\_\_\_ Age of seedlings:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Table 1A. Root lengths of wild-type Wassilewskija seedlings, in mm

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  |  |  |  |  |

Average =

Range =

Table 1B. Root lengths of wild-type Columbia seedlings, in mm

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  |  |  |  |  |

Average =

Range =

Table 1C. Root lengths of *axr4-3* seedlings, in mm

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  |  |  |  |  |

Average =

Range =

2. Repeat the above analysis for the WM20.2 enhancer mutant seedlings. Mutant\_\_\_\_\_\_\_\_ Generation\_\_\_\_\_

Table 1D. Root lengths of enhancer mutant seedlings, in mm

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  |  |  |  |  |

Average =

Range =

3. Now you will analyze the F2 seedlings. Each person should analyze one plate of F2 seedlings. Be careful to keep track of which one you are analyzing, and keep the seedlings separate on clearly-labeled plates!

a. First inspect the plate: do you see more than one type of seedling based on phenotype?

(You should! If not, consult the instructor: there may be a problem with your F2 population). If you do, then continue on with the analysis.

b. Remove, one by one, all of the seedlings from the F2 plate and separate according to root length on blank plates.

c. By comparison with the controls, you should be able to separate your seedlings into different size classes. You can expect to see seedlings that resemble the wild type, the *axr4­*, and the enhancer mutant. To be considered an enhancer, the seedling should have roots clearly longer than the *axr4-3* control seedlings. Count the number in each class and record the numbers in Table 1E. Measure the longest and shortest seedlings of each class and record the lengths also in Table 1E. Note that you do NOT have to measure every F2 seedling!

Cross Analyzed (include the mutant name & number of backcrosses): \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Table 1E. Analysis of F2 from cross ofenhancer to wtLer

|  |  |  |
| --- | --- | --- |
| Phenotype | Number in Class | Root size (range) |
|  |  |  |
|  |  |  |
|  |  |  |
|  |  |  |

Note: Usually you would find only three different root size phenotypes. A 4th line is given in the table in case there are more than three classes.

Information on the chi-squared test is given below; you will need to read and complete the data analysis section below for your lab report.

4. The seedlings with a particular phenotype from the enhancer x Col F2 population may be used in the future to map the chromosomal location of the mutation, i.e. they are the “mapping population”. In general, the least abundant class of seedlings is used in mapping, for reasons to be explained later. Consult with the instructor to determine which seedlings to use, and make a note of it.

Use these seedlings:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Ask the instructor to verify your analysis of the F2 before you put the seedlings into dirt.

These seedlings must be placed into dirt and grown up so that we can collect tissue from which to isolate DNA (in two weeks) and so that the F3 seed can be collected (in two months or so…). The instructor will demonstrate how to transplant seedlings to dirt. Be sure that you label your plants with complete information: the date, your initials, the genotype, and phenotype.

You should also place into dirt at least two of each type of control plants, as directed by the instructor.

##### Putting seedlings in dirt

Use the pots or flats of wetted dirt provided, or prepare them yourself as directed.

a. Prepare a label for the seedlings: on a labeling stake provided, write the cross and the phenotype of the seedlings, your initials, and the date.

b. Transfer a seedling from a plate to the dirt by grasping the root tip gently with forceps and pulling the root down into the dirt, then packing the dirt around the seedling.

Put in the dirt first the two seedlings whose phenotype you are the most confident of, and mark these with a special label or stick so you will be able to identify them later.

c. Place the label in the dirt.

d. Clean up, wash & dry the forceps and other equipment that you have used, return items to their proper places, and wash your hands.

Record the total number of F2 seedlings that you put to dirt here:\_\_\_\_\_\_\_\_\_\_

Notes on today’s procedure

**The Chi-square(d) Test**

**Introduction**

The chi-squared test is a statistical test used to determine to what extent a set of data supports a particular hypothesis. It is especially widely used in genetics, where it provides answers to questions such as these:

* Does this trait behave as if it is controlled by a single gene?
* Could a trait be controlled by multiple genes?
* Are two genes linked or do they segregate independently?

Note that the chi-squared test, like any statistical analysis, can show that your date support or fails to support an hypothesis, but cannot absolutely prove or disprove an hypothesis.

The results of chi-squared analysis should be presented in papers (and lab reports!) describing the genetic characterization of mutants.

**Method**

To apply the chi-squared test, follow these steps:

1. Based on the data and the type of cross, propose a genetic hypothesis that could explain the results. For example, if you are following a single trait in a cross of two heterozygotes and see 100 individuals with one form of the trait and 44 with the alternate form of the trait, you might consider this approximately a 3:1 segregation and therefore hypothesize that the trait is controlled by a single recessive allele. The null hypothesis would be that the trait is not controlled by a single recessive allele. If you are analyzing the F2 of a dihybrid cross for two traits, you might see a segregation ratio of 94: 33: 25: 8, and postulate that the two traits are controlled by two unlinked genes each with a dominant and recessive allele (giving a 9:3:3:1 ratio). When testing if two genes are linked, one usually tests the null hypothesis, that the two genes are not linked. In the F2 analysis of the enhancer, your hypothesis should include whether the enhancer is dominant or recessive and whether it is (by itself) auxin-resistant or auxin-sensitive.

2. Based on the hypothesis you proposed, make a prediction for the ratio of different classes of progeny expected in the cross. For example, if you are analyzing the F2 of a dihybrid cross for two traits, you can predict that you should see a ratio of 9:3:3:1. Be sure that you know what kind of cross was done! Two unlinked genes segregating in a test cross (AaBb x aabb) will give 1:1:1:1 instead of a 9:3:3:1 ratio.

3. Based on the predicted ratio and the actual total number of progeny in your data set, calculate the expected numbers of individuals for the different classes of progeny. The expected numbers of progeny in each class is calculated by taking the proportion predicted for each class and multiplying by the total. For example, if you predict a 9:3:3:1 ratio (i.e. 9/16, 3/16, 3/16, 1/16) in the four phenotypic classes, and there were 160 total progeny analyzed, then the expected number in the largest class is 9/16 x 160 = 90, the expected number in each of the next largest classes is 3/16 x 160 = 30, and the expected number in the smallest class is 1/16 x 160 = 10. Always use the actual total number of progeny that you observed to calculate the expected values; it is OK to have fractional values for the expected numbers, although it usually will not make a difference if you round off.

4. Apply the chi-squared formula: χ2 = Σ (O-E)2

E

This formula states that, for each class of progeny, subtract the expected from the observed, square the result, divide by the expected, and then add these values for all the classes of progeny. The sum is the chi-squared value. A straightforward way to calculate chi-squared values is with a table such as the one shown here.

Table 1F. Example of Chi-Squared Analysis

|  |  |  |  |
| --- | --- | --- | --- |
| Class | Observed (O) | Expected (E) | (O-E)2/E |
| Phenotype A B | 94 | 90 | (94-90)2/90 = 0.18 |
| Phenotype A b | 33 | 30 | (33-30)2/30 = 0.3 |
| Phenotype a B | 25 | 30 | (25-30)2/30 = 0.83 |
| Phenotype a b | 8 | 10 | (8-10)2/10 = 0.4 |
| Total | 160 | 160 | χ2 = 1.71 |

5. Interpret the chi-squared value. The calculated chi-squared value and the degrees of freedom present in a cross are found in a chi-squared table, which gives a probability (*p*) value. This *p* value tells us the likelihood that the difference between the observed and expected values is due to chance.

* A low *p* value (less than the chosen level of significance, usually 0.05) indicates that it is unlikely that the difference between observed and expected is due to chance, and therefore it is likely that this difference is meaningful. In this case we say that there is a “statistically significant difference between the observed data and the prediction”, and the data do NOT support the hypothesis that was used to give rise to the expected values.
* A high *p* value (greater than the chosen level of significance, usually 0.05) shows that statistically there is no significant difference between the observed and expected (predicted) values, i.e. just by chance the degree of difference that we observe would occur more than 5% of the time. In this case we say that the data DO support the hypothesis that was used to produce the expected values.

The degrees of freedom (df) present in a cross equals the number of classes of progeny minus one. Essentially this tells us the number of categories that are independent of each other. If we have four classes of progeny and we know the numbers in three of the classes and the total number of progeny, then the number in the fourth class is determined. Thus, for four classes of progeny, the numbers in three of them are independent of each other, and the degrees of freedom is three.

Knowing the degrees of freedom, we find where our calculated chi-squared value will fall in the table. In the case of this example, there are four classes of progeny, and so df = 3. Therefore we look in the third line of the table for the chi-squared value, and see that our chi-squared value of 1.71 falls between 0.584 and 2.366, the chi-squared values in the columns headed by 0.90 and 0.50 respectively. The column headings are the *p* values; therefore we can say that our p value falls between 0.90 and 0.50, which is written 0.90>*p*>0.50, or simply *p*>0.5. As the *p* value is not less than 0.05, these data do not show a significant deviation from that expected by chance from a 9:3:3:1 ratio, and the data therefore do support the hypothesis that there are two unlinked recessive alleles controlling these two traits.

When presenting the results of a chi-squared test, you need to state the hypothesis, the total chi-squared value, the degrees of freedom, and the *p* value. So, for the example above, we would present the results as follows:

“For a 9:3:3:1 ratio, the chi-squared value with 3 degrees of freedom = 1.71, *p* > 0.5, thus the hypothesis is supported.”

**Additional notes**

1. Be cautious in your interpretation! Remember that a *p* value can support but not prove your hypothesis.

2. You can test multiple hypotheses with the same set of data; just be sure to state the specific hypothesis that you are testing in a given case.

**Chi-Squared Table**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **↓df** | ***p=*0.90** | **0.50** | **0.25** | **0.10** | **0.05** | **0.025** | **0.01** | **0.005** |
| **1** | 0.016 | 0.455 | 1.32 | 2.706 | 3.841 | 5.024 | 6.635 | 7.879 |
| **2** | 0.211 | 1.386 | 2.77 | 4.605 | 5.991 | 7.378 | 9.210 | 10.597 |
| **3** | 0.584 | 2.366 | 4.11 | 6.251 | 7.815 | 9.348 | 11.345 | 12.838 |
| **4** | 1.064 | 3.357 | 5.39 | 7.779 | 9.488 | 11.143 | 13.277 | 14.860 |
| **5** | 1.610 | 4.351 | 6.63 | 9.236 | 11.070 | 12.833 | 15.086 | 16.750 |

The top row is the *p* values, the left-hand column is the degrees of freedom, and the numbers in the body of the table are the chi-squared values that correspond to a given degrees of freedom and *p* value.

**Analysis of Data from F2 Seedlings**

1. From the data in Table 1E, formulate an hypothesis about the genetic behavior and auxin resistance of the enhancer mutation in this cross: is it dominant, semidominant, or recessive? Is the enhancer by itself auxin sensitive (like wild type) or auxin resistant? Write your hypothesis here.

**Hypothesis:** The enhancer mutation is inherited in a ­­­­­­­­­­­­­­­­­­­­­­­­\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ fashion and in a plant that does not contain the *axr4* mutation results in auxin \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_.

2. Based on this hypothesis, what is the predicted ratio of progeny in the different phenotypic classes? (It will be discussed in class how to use your hypothesis to make a prediction).

**Prediction:**

3. Test your hypothesis using the chi-squared test by filling in Table 1G.

|  |  |  |  |
| --- | --- | --- | --- |
| **Table 1G: for cross of enhancer to wtCol** | # F2 seedlings observed | # F2 seedlings expected | (O-E)2E |
| phenotypic class 1 |  |  |  |
| phenotypic class 2 |  |  |  |
| phenotypic class 3 |  |  |  |
| phenotypic class 4, if observed |  |  |  |
| **Total** |  |  |  |

4. How many degrees of freedom are there in this cross? Use this number and the total chi-squared value obtained from Table 1G to find, in the “Chi-Squared Table” above, the *p* value for your results and your hypothesis.

What is the *p* value obtained by this test? \_\_\_\_\_\_\_\_\_\_\_

Does this support or fail to support your hypothesis?

If your *p* value doesn’t support your hypothesis, it may mean several things:

a. Your hypothesis is incorrect (the obvious choice): for example, you thought the mutation was dominant, but it was actually recessive.

b. One of your “hidden” assumptions is incorrect. For example, it could be that your scoring was inaccurate (whereas you assumed that you could tell the difference between the different phenotypes); it could be that one of the classes of progeny has reduced viability (whereas you were assuming that the genotype did not affect viability); or it could be that someone used the wrong packet of seed (whereas you trustingly assumed that whatever the instructor gave you must be correct).

5. If you can, write your conclusion about the mode of inheritance and auxin resistance or auxin sensitivity of the enhancer mutation that you are studying:

**Conclusions**

#### General Directions for Lab Reports

The most important considerations for your lab reports are that they be clearly written and in your own words. Your goal is to show that you understand what you did and why you did it, and convey it clearly to the reader. You do not need to write out a detailed account of every step you performed in this lab (after all, this is in the lab manual, which of course you were following religiously). You do need to demonstrate that you understand the overall plan of the lab. Do not repeat word-for-word what is written in the lab manual!

Lab reports should be written in the past tense; you are reporting what was done, not what you will do.

A note on references and citations

lab reports will almost always include citations to sources of information that you use such as books, Web sites, this manual, and, most important, published scientific articles in peer-reviewed journals (you should understand what “peer-reviewed” means). You are especially likely to have such citations in your introduction and discussion sections, where you are giving background information and comparing your results to those previously published.

References should be cited in the text after the information that comes from them. All the references should be summarized in a list of references on a separate page at the end.

*In-text citation style*

There are two main styles for in-text citations in science, the “citation-sequence” and the “name-year” styles. Please use the “name-year” style. Detailed information about how to use this citation style, and also about how to cite on-line sources in your references, is given at http://www.bedfordstmartins.com/online/cite8.html

The “name-year” style for in-text citations works as follows. If you used information from a paper by Wendy Chen and David Singh that was published in 2004, you would cite it in the text in either of two ways: “Chen and Singh (2004) showed that auxin was important for lateral root growth” or: “Auxin has been found to be important for lateral root growth (Chen and Singh, 2004).” You should not give page numbers or first initials. If there are three or more authors, then an in-text citation of the article uses the first author’s name and the abbreviation “et al.” in place of the rest of the names. For example, “Auxin has been found to be important for lateral root growth (Chen et al., 2004).

*References style*

In the list of references cited at the end, all the authors are listed. There are a number of formats used for giving the relevant information in a list of references cited; it seems as if each journal has its own format. You should follow exactly the format used in the “References” section at the end of the Introduction, e.g.:

## Lukowitz W, Gillmor CS, and Scheible W-R. 2000. Positional cloning in Arabidopsis. Why it feels good to have a genome initiative working for you. *Plant Physiology* **123**, 795-805.

A consistent format for citing a book is as follows:

Author AA, Author BB, Author CC. Date. Title. Editor AA. City: Publisher.

Also, there is a proper format to be used when citing Web sites. Please use the format given at this Web site: http://www.bedfordstmartins.com/online/cite8.html

In particular, look at the section entitled “1. World Wide Website”.

If you have any questions about references, ask the instructor.

**Writing the first lab report, on Experiment 1 (due February 12)**

This lab report should include the following sections:

1. Introduction (at least one page)

Clearly describe the background and goals of the lab. Briefly discuss why we are studying the enhancer and how the enhancer mutant was produced and isolated. What were you trying to learn about the enhancer? What hypothesis were you trying to test?

2. Methods

Give an outline of the procedures followed and describe the main steps in the procedures, e.g. “Sterilized seeds and spread on Ats plates containing 0.7% agar, 1% sucrose, and 0.4 µM 2,4-D”. However, you do not need to describe in detail experimental procedures that were given in the lab handout. Instead, you can state “according to the procedures in the lab manual”. Be sure that you include the timing, e.g. how many days the seedlings grew during each part of the experiment.

3. Results

Present and describe the results you obtained. You should also describe the key points of the results in words, and include statistical analysis.

You should include Tables 1A-G. Be sure that you are specific about the type and concentration of auxin that you used.

4. Discussion

Interpret and explain your results: What do they mean? Do they support or fail to support the hypotheses you proposed at the end of your Introduction? According to the statistical analysis, how confident can you be of your conclusions?

5. References Cited

Your report must include at least one in-text citation to an article from a scientific journal, and in the “References Cited” section should give the full reference for all sources of information cited in proper format, as described above.

6. Lab Notebook

Along with your lab report, you should submit pages 17-27 from your lab notebook, which you have been filling out during each lab period.

**Experiment 2: Phenotypic characterization of the enhancer:**

**Measuring root growth in response to auxin**

A major part of the project lab course for the semester will be designing and carrying out your own experiment to characterize quantitatively the phenotype of the enhancer mutant that you are studying. In Experiment 2, you will gain experience in this type of experiment by measuring one aspect of the enhancer phenotype, the root growth response to auxin.

One important plant process that is regulated by auxin is root growth. The low concentrations of auxin made by the plants themselves are necessary for the primary (main) root to grow properly. High concentration of auxin can actually inhibit the growth of the roots through effects on other hormones. The *axr4* enhancers were originally isolated for their long root growth compared to the parental *axr4* mutant on a single concentration of the synthetic auxin 2,4-D. In this experiment, you and your classmates will test how resistant an enhancer mutant is to 2,4-D by measuring its growth on a range of concentrations, and whether the enhancer mutant is also resistant to the natural auxin IAA and to another synthetic auxin with different properties, 1-NAA. Each lab group will test different concentrations of one type of auxin.

The use of three different auxins could give us important insights into the effects of the enhancer mutation, because these three auxins are known to have different properties. The natural auxin IAA and synthetic auxin 2,4-D are both taken up into cells through auxin import proteins, of which the most important is called AUX1. The synthetic auxin 1-NAA is not imported into cells via AUX1, but enters cells by diffusion. In the original *axr4* mutant, the localization of the AUX1 protein is affected (Dharmasiri et al., 2006), which means that the *axr4* mutant responds differently to 1-NAA than it does to IAA and 2,4-D. The results of these assays can therefore show whether the enhancer mutation might affect the same pathway as *axr4*.

Thus, the null hypothesis to be tested in this experiment is:

***H0*:** There will be no difference in root growth response to auxin between the enhancer and the parental *axr4-3* mutant.

The alternative hypothesis is:

***HA*:** The enhancer will have greater root growth on auxin than will the parental *axr4-3* mutant.

This experiment has the following series of steps:

Step 1, Day 0 (W Jan. 27): Sterilize seeds and spread on plates without auxin; cold-treat. Do calculations and weigh agar and sucrose for media preparation.

Step 2, Day 2 (F Jan. 29): Place plates in the growth chamber in vertical orientation.

Step 3, Day 5 (M Feb. 1): Prepare media with different concentrations of auxin. Pour plates.

Step 4, Day 7 (W Feb. 3): Transfer seedlings to plates with different concentrations of auxin and mark the root tips. Return to the growth chamber in vertical orientation.

Step 5, Day 12 (M Feb. 8): Measure the length of root growth. Enter the data in Excel.

Step 6, Day 14 (W Feb. 10): Analyze the results using SPSS; graph the results and use statistical tests to determine statistical significance.

You will need to test three genotypes of seedlings in this assay:

* wild-type Wassilewskija (abbreviated “Ws”; this is the parental strain of the *axr4-3­* mutant; genotype *AAEE*)
* *axr4-3* (the parental strain of the enhancer; genotype *aaEE*)
* the enhancer (genotype *aaee*).

Be sure to record in “Notes on today’s procedure” the identities of the strains that you use, i.e. information about the specific mutations present in each strain, the ecotype, and if applicable the generation, e.g. 1X backcrossed. Also record all the dates for everything that you do.

**Step 1: Sterilizing and plating *Arabidopsis* Seeds**

Today you will sterilize the seeds.You will spread these seeds onto hormone free (“Ats”) plates for germination. Record information specific to what you do today at the bottom of this page.

1. Sterilize the seeds following the same procedure as for the previous lab.

Genotypes to sterilize: Ws, *axr4-3*, and enhancer.

2. Spread these seeds onto labeled Ats plates (these have no hormone in them).

3. Wrap the plates with micropore tape.

4. Place the plates, wrapped in plastic film, into the refrigerator.

5. Clean up the hood, your lab bench if used, and wash your hands.

## Notes on today’s procedures

#### Genotypes of Seeds:

#### Plates Used:

Other notes

**Step 2: Plan (Weds. Jan. 27) and prepare (Mon. Feb. 1) the media**

1. Planning how to prepare the media with different auxin concentrations

The standard growth media that we will use for growing *Arabidopsis* on plates is made by adding, to a large volume of water, small amounts of six different concentrated mineral solutions (which provide essential nutrients such as potassium, calcium, nitrate, phosphate, and iron), along with agar and sucrose, then steam sterilizing (steam sterilization as done here is called “autoclaving” because a machine called an “autoclave” is used to carry it out). After the media is sterile and the agar and sucrose dissolved, the media is cooled and hormones or other additions are made, in the form of concentrated stock solutions, before pouring the plates. In order to practice the essential lab skill of making solutions, you will carry out almost all the calculations needed to make the growth media yourselves.

During the first part of this planning step, you need to do the following:

1. Calculate how much media to make.

2. Calculate how to make the liquid media.

3. Calculate how much agar and sucrose to add to this media.

4. Calculate how much of the concentrated auxin stock solutions to add.

5. Weigh out the agar and sucrose into the flasks.

6. Make the liquid media and store in the refrigerator.

1. To calculate how much media to make, here is the information you need to know:

* You will test three genotypes of plants (i.e. three types of seedlings: wild type, *axr4-3*, and *axr4-3 enhancer*).
* You will test each genotype on five different types of plates, containing hormone-free media and four concentration of auxin. Some groups will test the synthetic auxin 2,4-D, some the synthetic auxin 1-NAA, and some the natural auxin IAA.
* For each combination of genotype and auxin, you will need one plate, which will have enough room for about 12 seedlings. The combinations of genotype and plates are shown in these tables.

Tables 2.1 Auxin Concentrations and Plates Needed

|  |  |  |  |
| --- | --- | --- | --- |
| **1-NAA group**  | wild type | *axr4-3* | enhancer |
| 0 auxin | 1 plate | 1 plate | 1 plate |
| 0.5 x 10-7 M 1-NAA | 1 plate | 1 plate | 1 plate |
| 1.5 x 10-7 M 1-NAA | 1 plate | 1 plate | 1 plate |
| 3 x 10-7 M 1-NAA | 1 plate | 1 plate | 1 plate |
| 5 x 10-7 M 1-NAA | 1 plate | 1 plate | 1 plate |

|  |  |  |  |
| --- | --- | --- | --- |
| **2,4-D group**  | wild type | *axr4-3* | enhancer |
| 0 auxin | 1 plate | 1 plate | 1 plate |
| 0.5 x 10-7 M 2,4-D | 1 plate | 1 plate | 1 plate |
| 1.5 x 10-7 M 2,4-D | 1 plate | 1 plate | 1 plate |
| 3 x 10-7 M 2,4-D | 1 plate | 1 plate | 1 plate |
| 5 x 10-7 M 2,4-D | 1 plate | 1 plate | 1 plate |

|  |  |  |  |
| --- | --- | --- | --- |
| **IAA group**  | wild type | *axr4-3* | enhancer |
| 0 auxin | 1 plate | 1 plate | 1 plate |
| 5 x 10-7 M IAA | 1 plate | 1 plate | 1 plate |
| 10 x 10-7 M IAA | 1 plate | 1 plate | 1 plate |
| 25 x 10-7 M IAA | 1 plate | 1 plate | 1 plate |
| 50 x 10-7 M IAA | 1 plate | 1 plate | 1 plate |

* How many plates will you need of 0 auxin?\_\_\_\_\_\_\_
* Each individual plate requires 25 mL of media. What volume of media will you need to pour all the 0 auxin plates? \_\_\_\_\_\_\_\_\_\_\_
* How many plates will you need for each concentration of auxin?\_\_\_\_\_\_\_
* What volume of media will you need to pour the plates with each concentration of auxin? \_\_\_\_\_\_\_
* What total volume of media will you need? \_\_\_\_\_\_\_

2. To make the volume of liquid media you will need, you will add small volumes of concentrated stock solutions of various solutions to a large volume of water. You need to calculate the volumes of these stock solutions to add. This table indicates the concentrations of each stock solution and the final concentration needed in the liquid media. By carrying out the calculations as described below, you can fill in the last column with the amount of each solution to add:

Table 2.2 Making Up Liquid Media

|  |  |  |  |
| --- | --- | --- | --- |
| Solution | Stock Concentration | Final Concentration Needed | Vol. stock to add |
| KNO3 | 1 M | 5 mM |  |
| KPO4 | 1 M | 2.5 mM |  |
| MgSO4 | 1 M | 2.0 mM |  |
| Ca(NO3)2 | 1 M | 2.0 mM |  |
| Fe-EDTA | 20 mM | 50 μM |  |
| micronutrients (H3BO3, MnCl2, CuSO4, ZnSO4, Na2MoO4, NaCl, CoCl2) | 1000X (actual concentration varies for each nutrient, e.g. 70 mM H3BO3) | 1X (actual concentration varies for each nutrient, e.g. 70 μM H3BO3) |  |
| Total volume |  |  |  |

Calculating how much stock solution to add

Use this equation: civi = cfvf

c = concentration; v = volume; ci = initial concentration (i.e. of your stock solution);

cf = final concentration (i.e. needed for the media)

You know the concentration of the stock solution (ci) and the final concentration needed (cf), and the final volume you want, i.e. how much media you want to make (vf). Now you need to solve for vi which is the volume of stock solution to add (enter in last column).

Calculations

3. To calculate how much agar and sucrose to add:

For each of your five types of media, you should prepare one flask of media with the appropriate amount of agar and sucrose. In step 1 above, you calculated how much media you need for each type of media. Write that volume here\_\_\_\_\_\_\_\_\_. Based on this amount and the information that the media requires 10 g of sucrose per liter and 7 g of agar per liter (1000 mL), calculate:

* + How much agar will you need for each flask? \_\_\_\_\_\_\_\_\_
	+ How much sucrose will you need for each flask? \_\_\_\_\_\_\_

Calculations:

4. To calculate how much auxin to add:

You will prepare the media with auxin in it by adding a small volume of concentrated stock solutions of 2,4-D, 1-NAA, or IAA to the media after it is autoclaved. These stock solutions will be available at 10,000X the final concentrations needed. Calculate the concentrations and volumes of each stock solution needed and fill in these values in Table 2.3 below.

The stock solutions are dissolved in ethanol. To be sure that we are not looking at the effects of the ethanol on root growth, we will design the experiment so that the control plates have the same concentration of ethanol as the auxin-containing plates. This will require adding ethanol, without auxin, to the “0” concentration also.

Fill out the following table so you will know how to make up the media:

Table 2.3 Making up media for the root growth assay

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Vol. of media | [final auxin](fill in concentrations from Table 2.1) | Concentration of auxin stock (10,000X the final concentrations) | Vol. of ethanol or auxin to use | Pipettorsetting | Agar and sucrose to add |
|  | 0 (control) |  |  |  |  |
|  |  |  |  |  |  |
|  |  |  |  |  |  |
|  |  |  |  |  |  |
|  |  |  |  |  |  |

Check your calculations with the instructor before making up plates!

Calculations**5**. Weighing out the agar and sucrose

a. Weigh out the agar and sucrose and add to Erlenmeyer flasks. The flask should be at least 2.5 times the volume of media to avoid boiling over during autoclaving (e.g. for 400 mL of media, you should use at least a 1000 mL flask).

b. Label a piece of tape with your initials and the type of media, stick the tape to the flask, and cover the flask with small pieces of foil or inverted beakers.

 We will save these flasks until next session.

6. Making the liquid media

a. In Table 2.2, add up the total volume of all the stock solutions to be added, and subtract this from the total volume needed to get the amount of water to measure (e.g. if you are making 400 mL of media, and the stock solutions total 6 mL, you would need to measure 394 mL of water). Measure out the liquid media using a graduated cylinder and pour into the flasks.

d. Place the flasks in the autoclave (steam sterilizer), set on “liquid cycle” for 20 minutes. Ideally all the groups will autoclave their flasks at the same time.

e. While the autoclave is running, label the 100 mm plates that you will use. You should label the bottom of each plate with your initials, the concentration and type of auxin, and the genotype of seeds that you will place on that plate. Organize the plates so all the plates that will receive a single concentration of auxin are together.

f. When the autoclave cycle is done (45-60 minutes), carefully open the autoclave and remove the media as directed by the instructor.

**CAUTION**: After a run the autoclave still contains a little steam, enough to be dangerous if the autoclave is opened improperly. Be sure you stand away from the autoclave when you open it to allow this steam to escape, as demonstrated by the instructor or TA.

**CAUTION:** The flasks that have just been autoclaved are very hot. Wear protective heat-resistant gloves when carrying them.

g. Cool the flasks until the outside of the flask is warm but not hot. Then, working in the laminar flow (“sterile”) hood, and using good sterile technique as demonstrated by the instructor:

i. Add the stock solution of auxin or ethanol to each flask according to Table 2.1

ii. Swirl gently to mix the stock solution with the media, and

iii. Pour the plates, using enough media to cover the bottom of the plate.

iv. Stack and move the plates to the side when you are done, and wipe up any media that spilled. You can leave the plates in the hood overnight to harden, but should come back the next day to wrap up the plates and store them in the refrigerator.

Notes on today’s procedure

**Step 3: Put the plates with seeds into the growth chamber (Fri., Jan. 29)**

Unwrap the plates with seeds that you sterilized on Wednesday, and put them into the growth chamber in a vertical orientation (i.e. on their sides) so the seedlings will grow on the surface of the agar where they are easy to remove. You may use tape or rubber bands to hold the set of plates together to help them remain upright. Be sure that the plates are stable; if they fall over, or rock from side-to-side, the seedlings will not grow uniformly.

The instructor or TA may perform this step for you by prior arrangement.

Notes on today’s procedure

**Step 4: Transferring the seedlings to the plates (Weds. Feb. 3)**

Because this procedure demands some time in the laminar flow hood, and space is limited, we will need to schedule when different groups will work.

l. Retrieve the plates of seedlings from the growth chamber. They should have been growing for five days.

2. Clean the inside surface of the laminar flow hood by wiping it with ethanol.

3. Put on a pair of gloves, squirt your gloved hands with a small amount of ethanol, and rub them together to dry the ethanol.

4. Sterilize a pair of forceps by soaking the tips in ethanol for 30 seconds or so, then remove them from the ethanol and shake off the ethanol. Give the forceps a short time to dry before using them. Or, take a foil-wrapped packet containing forceps that have been autoclaved and carefully unwrap them without compromising the sterility.

5. Working with good sterile technique, use the forceps to remove a seedling from the plate on which it has germinated and transfer it to one of the large plates. Make sure the plates are labeled correctly with the genotype! Hold the forceps gently so you do not crush the seedling. Place 10 seedlings on the plate in a line close to one edge, all oriented

as in Fig. 6 below. To get the seedlings to lay out flat, touch the root tip to the plate and then drag them gently as you lay them down. Try to pick seedlings with about the same root length to transfer; if you pick up one that is much longer or shorter than the others, discard it. When you are done, use a marker to place a dot on the plate on the tip of each root.

6. When you have transferred seedlings to all the plates, use rubber bands to attach the



Fig. 4. Arranging seedlings for Expt. 2.

plates together and place them in the growth chamber in a vertical orientation (again, make sure the plates are stable and will not tip over). Wrap the original plates on which the seedlings germinated in plastic, label them with your initials and the date, and place them in the refrigerator as directed by the instructor.

Notes on today’s procedure

**Step 5: Measuring the seedlings**

(to be carried out the next lab period after transferring the seedlings)

1. Today you will measure the seedlings and record the amount of each seedling’s additional root growth. Remove the plates from the growth chamber. Use forceps to straighten (gently!) the roots if necessary, but do not disturb the position of the seedlings.

2. Measure the distance from the dot you place initially to the position of the root tip now, in millimeters, using the plastic ruler provided. (An alternative approach, more accurate but slower, frequently used for measurements is to take a digital picture of the samples and use computer programs to measure the length). Record this information in your lab notebook in Table 2.4 below (one seedlings per cell).

Table 2.4 Results of Root Growth Assay Auxin Used\_\_\_\_\_\_\_\_\_ Enhancer\_\_\_\_\_\_\_

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Genotype | [auxin], M | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |  |
| wt | 0 |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
| *axr4-3* | 0 |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
| enhancer | 0 |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |

3. You can discard the plates when done, as directed by the instructor.

**Step 6: Analysis of the results**

The goals of the analysis are to determine the outcome of the experiment, to present the results in an easily-understood graph, and to determine the statistical significance of the results.

1.Enter your results from Table 2.4 into an Excel worksheet, set up as follows:

|  |  |  |
| --- | --- | --- |
| Genotype | [auxin] | Root\_length |
|  |  |  |
|  |  |  |
|  |  |  |
|  |  |  |

a. For “auxin” you should substitute the name of the type of auxin that you used, e.g. IAA.

 b. Each seedling’s results are placed on a single row (line). Thus, if you had 10 wt seedlings that you tested on 0 IAA, your first 10 rows would all say “wt” in the “Genotype” column and “0” in the “[IAA]” column, and would differ only in the root length. The next 10 rows would contain your data for wt on the next concentration of IAA, and so on.

c. Save the Excel workbook as a file whose name includes your and your partner’s initials.

d. Calculate averages and standard deviations, and calculate the normalized values (i.e. how much each genotype elongated its roots on auxin compared to how much it elongated without auxin.) The instructor will describe how to use formulas in Excel to do these calculations.

2. Open the workbook in SPSS (available on Adelphi computers). The instructor will guide you in analyzing the results in SPSS.

Notes on today’s procedure

Desired outcome

1. Means and standard deviations for each genotype on each type of media;

2. Normalized data, i.e. the elongation for each genotype on the different concentrations of auxin as a percentage of the elongation on hormone-free media;

3. A graph of the results with standard deviations for each point;

4. A statistical analysis of the difference between *axr4-3* and the enhancer root growth.

After analyzing the data from this experiment, you should be able to see if the original hypothesis is supported or not. Is the enhancer significantly different from its parental line in its root auxin resistance?

**Writing the second lab report, on Experiment 2 (due Feb. 22)**

The lab report should include the same sections as the first lab report:

1. Introduction

2. Methods

Be sure that you include the timing, e.g. how many days the seedlings grew during each part of the experiment. Also include a description of how you did the statistical analysis.

3. Results

Present and describe the results you obtained, summarized in two graphs showing either root length in mm or relative root elongation in % on the y axis vs. the concentration of auxin (listed as the specific type of auxin) on the x axis, with different lines for the different genotypes. You should also describe the key points of the results in words, and show the results of the statistical analysis. Be sure that your tables and graphs include the exact concentration and type of auxin that you used—don’t just list “auxin”, as this is an incomplete description of the treatment.

4. Discussion

Interpret and explain your results: What do they mean? Do they support or fail to support the hypothesis you proposed at the end of the Introduction? According to the statistical analysis, how confident can you be of your conclusions? Are they similar to what has been previously reported in the scientific literature? You might especially want to look at the papers by Hobbie & Estelle (1995), Simmons et al. (1995), and Yamamoto & Yamamoto (1999).

5. References Cited

Be sure to include any needed references. They should be cited in the text after the information that comes from them, and then all the references should be summarized in a bibliography.

6. Lab notebook

Attach the pages on Experiment 2 from your lab notebook (or copies of them), showing all the notes that you made during the lab.

**Experiment 3: Characterizing the phenotype of your mutant**

#### Designing and carrying out an experiment;

#### Presenting your proposed experiment and your results

You and your lab partner need to begin preparing plans for characterization of the enhancer mutant. Essentially, you will test an hypothesis that should be similar in form to the hypothesis that you tested in Experiment 2, e.g. “The enhancer mutant will differ from the parental *axr4-3* mutant in [some characteristic].” (In Experiment 2, this characteristic was the root growth on auxin). On February 24, you and your lab partner will make a short presentation in which you describe your hypothesis and how you will test it. During the remainder of the semester, you will carry out your planned experiment (Experiment 3). You and your partner only need to do one experiment to characterize the enhancer, but this experiment must be repeated at least twice to verify the results. You and your partner will prepare a poster summarizing your experiments, which you will present at the Adelphi Research Conference on April 12.

**How can you decide what experiment will be useful in characterizing the mutant, and how will you be able to plan this experiment?**

In the scientific method, scientists develop an hypothesis to explain a natural phenomenon, and design experiments to test the hypothesis. Frequently the hypothesis itself arises from observations made directly by the scientist. For example, you might notice something about how your mutant grows or what it looks like that leads you to an hypothesis. In Experiment 2, the longer roots of the enhancer + *axr4* double mutant when grown on a single concentration of auxin led us to hypothesize that this double mutant would be more resistant to increasing concentrations of auxin than the parental *axr4-3* mutant. We tested this hypothesis with the root growth assay. Another way in which hypotheses are generated is by learning and thinking about what has already been observed by other scientists, as recorded in the scientific literature. This approach is likely to be very useful to you, for two reasons: 1. A lot is known about processes that auxin controls in plants. A reasonable hypothesis is that the enhancer mutant is affected in one or more of these processes; and 2. Many other mutant plants with changed responses to auxin have been previously characterized. You may find that some of these mutants are also affected in other characteristics besides auxin response, and hypothesize that the enhancer mutant is also affected in one or more of these same characteristics. The many different characteristics of a mutant all reflect the normal role of the gene that is mutated, so the more that you learn about your mutant’s phenotype, the better you will understand what the gene does. You may even learn enough about your mutant’s characteristics to be able to say that it is very similar (and possibly identical) to a previously-known mutant, or to say that your mutant does not resemble any previously-known mutant, and may therefore be a novel (new) mutant.

While you can certainly design an experiment that will test an hypothesis that is totally unrelated to the known aspects of the mutant phenotype, you would do well to have some sort of justification for testing this hypothesis. If you propose the hypothesis “my mutant is deficient in DNA polymerase”, and then do experiments to test whether the mutant is indeed deficient in this enzyme, you need to explain why you think this is a possible defect in the mutant. “I thought it might be interesting” is not a good explanation. “Smith & Jones found that an auxin-resistant mutant had a defect in DNA polymerase activity” or “Watson and Crick found that DNA polymerase regulates auxin response in roots” or “*AXR4* appears to regulate DNA polymerase” are all much stronger justifications for your hypothesis.

In addition to suggesting hypotheses, the scientific literature can also give you guidance in how to perform specific experiments. If another scientist has performed an experiment in a particular way, and it worked, then it may be a good idea for you to follow the same procedure rather than developing an experimental protocol entirely from scratch. Look at the Materials and Methods sections of the papers you read for details on how exactly an experiment was carried out.

A collection of books will be placed on reserve in the library for you to use as resources. You can also do searches of databases to see if there are already mutants known with the phenotypes you have observed. Believe it or not, you will actually NOT find the best information from doing a “Google” search. I recommend the following sources of information:

1. The Arabidopsis Information Resources (TAIR), the on-line Arabidopsis database: [www.arabidopsis.org](http://www.arabidopsis.org).

2. PubMed, the database of the biological literature: www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed

Articles that you identify in these two databases can often be obtained electronically through the “e-journals” (electronic journals) link under “Research” on the Adelphi Library Web page. For some older articles or for some journals) you will actually have to go read the paper copy. Even these articles may have useful information. Alternatively, you can check with the instructor, who is likely to have copies of many of the articles that you find in your searches.

3. Another useful source with lots of information on what is known about *Arabidopsis* physiology and development is *The Arabidopsis Book*, sponsored by the American Society of Plant Biologists and available at [www.aspb.org/publications/arabidopsis/](http://www.aspb.org/publications/arabidopsis/)

4. An excellent recent review on auxin is by Woodward and Bartel (2005) (see references at end of Introduction). A copy of this will be available on the class Web site. Links to the other sites mentioned will also be available from the class Web site.

Feel free to consult with the instructor as well for guidance.

Suggested steps for this research project

* Research in scientific literature.
* Develop hypothesis.
* Consult with instructor, and revise as necessary.
* Design experiments to test hypothesis (see below)
* Consult with instructor, and revise as necessary.
* Prepare presentation.
* Practice presentation.
* Give presentation.
* Start experiment(s).
* Collect data.
* Analyze results. Perform statistical analysis. Consult with instructor.
* Prepare poster.
* Practice poster presentation.
* Give poster presentation.

(feel free to consult with instructor as needed, even if not specifically indicated)

**Record your progress as you develop your project here:**

Articles consulted

Possible project ideas considered

## Experimental Design

1. Think about the controls that you will use:

A positive control shows that your experiment is capable of detecting the result that you expect to see. In our PCR analysis, for example, we must see amplification of the proper-sized bands using the control Ws and Ler template DNA to know that our primers work as expected. In a physiological experiment testing auxin resistance, for example, it would be a good idea to include a wild-type control to show that you remembered to put the auxin in the medium and so inhibit the plants. If you left out the auxin by mistake, you might see long roots and think that it represented auxin resistance, until you saw that your wild-type control wasn’t inhibited either.

A negative control shows that our experiment is not giving us a false result, perhaps due to contamination. In our PCR experiment, for example, we should not get any amplification if we use water as a template, nor should we see a Ws-sized band when we use Ler template DNA. If we see amplification with water as a template, or the wrong-sized band with a control DNA, then we either have contamination (of the primers, the DNA, or some other reagent) or a problem with our primers. In a physiological experiment testing auxin resistance, for example, it would be a good idea to include a mutant that is known to be auxin resistant, to show that we’re not using such a high concentration of auxin that the plants are killed!

## 2. Think about testing adequate numbers of individuals. You would not want to make a conclusion from looking at just one mutant and one wild-type seedlings—you need to know that a difference you see is reproducible and consistent. The number of individuals to test depends on the magnitude of the difference you see: if a difference in phenotype is very clear, you do not need to look at thousands of individuals to be confident that the difference is real, but a small but real difference may require large numbers of individuals to be tested to give convincing results. A good approach is to do a small-scale experiment initially, say with 10-20 individuals of each genotype, and then repeat with more individuals if it appears there may be a subtle effect.

## 3. Think about testing in a physiologically relevant way. In general, the most meaningful experiments are ones in which the conditions are close to natural conditions. knowing that *Arabidopsis* plants generally grow at fairly cool temperatures, for example, it might be problematic to do experiments at high temperatures that may kill the plants, unless you are studying specifically the plant’s response to changes in temperature.

4. Think about designing a feasible experiment. While it might be interesting to know how your mutant behaves in zero gravity, the lead time for getting experiments on the International Space Station is too long to be practical for a one semester lab course. It might also be interesting to do a time course of your mutant’s response to, for example, gravity, measuring it every 2 minutes for 48 hours, but this would also present practical difficulties unless you can automate the data-collection process as some scientists have done. The quickest experiments will be ones that examine the phenotype in seedlings rather than in mature plants.

## 5. You should perform appropriate statistical tests on your results to evaluate their significance.

Hypothesis to be tested:

Plan for experiment:

First Presentation: Your Plans for Your Experiment 3 (to be given Weds. Feb. 24)

## You will give presentations in lab on your plans for mutant characterization. Complete your plans for characterizing your mutant, and check them with the instructor. Your Powerpoint presentations should involve both members of the lab team, and should take no more than 10 minutes. If you are not familiar with Powerpoint, the instructor will help you. Allow 2 minutes for questions. You must schedule an appointment with the instructor to practice your presentation at least two days before you present to the class. It is a good idea to practice your presentation with your partner before you present it to the instructor! If you have questions, consult the instructor.

This presentation would generally include the following (make each part brief!):

1. An introduction to auxin and to the evidence that it’s involved in the process you will study (include a reference to the literature, and possibly a figure from the literature);

2. A description of the *axr4* mutant and relevant aspects of its phenotype, also with reference to the literature;

3. A description of the enhancer;

4. A description of the hypothesis about the enhancer phenotype that you plan to test;

5. An outline of your planned experiment (mention the reference whose procedure you are following, if applicable);

6. Possible results and the interpretations each would yield, e.g. hypothesis is supported?;

7. References

To give the best presentation possible, try to do the following:

1. Keep the text on each slide short and to the point—use bullet points, not complete paragraphs. Too much text makes the slides hard to read.

2. Include relevant and interesting illustrations.

3. Be clear and organized. Connect each slide to the one before and the one after.

4. Use the slides to guide you through the presentation, as the basis for your verbal explanations, and to make it easy for your audience to follow, but **do NOT read each slide word-for-word**. If you don’t enjoy it when a professor reads every word of his Powerpoints to the class, then your classmates probably won’t enjoy it if you read your presentation off the slides either!

Presentations will be evaluated using this rubric:

|  |  |  |  |
| --- | --- | --- | --- |
| Feature | Pointspossible | Pointsreceived | Comments  |
| Giving practice presentation on time to instructor | 10 |  |  |
| Choice and design of experiments and controls: good rationale for choice? use of scientific literature? clear statement of hypothesis? well-designed controls?  | 30 |  |  |
| Understanding and explaining background and related knowledge  | 30 |  |  |
| Use of Powerpoint and manner of presentation | 20 |  |  |
| Written summary of plans handed in day of presentation | 10 |  |  |
| TOTAL | 100 |  |  |

##### At the end of your presentation you should give the instructor a written summary of your plans for your project. This should include the hypothesis that you are testing and a detailed outline of what you plan to do and your timetable for doing it.

Insert a copy of this written summary of your project plans here!

##### Planning ahead for your experiments

If your experiments will require any special materials or supplies, you must inform the instructor as soon as possible so that these can be obtained or ordered. Be aware that ordering special items can take a while, so the earlier you let the instructor know the more likely it is that you will actually have the items in time for your experiments!

##### **Keep careful records of your Experiment 3 experiments! You can use pages 49-61 for these records.**Final Presentation (poster presentation)

**The conclusion of this project** will be a poster presentation session. Each pair should summarize their results in a poster which will be presented to the instructor and to other faculty and students at the Adelphi Research Conference on April 12. An abstract of the work must be submitted by March 13 through the Adelphi Research Conference Web site. The poster should present the results of the enhancer characterization experiments.

You and your partner must prepare, by April 5, a rough draft of your poster (this can be printed out on regular 8-1/2 x 11 paper). The instructor will review this rough draft with you during lab and give suggestions. On April 7 you and your partner need to give a practice presentation of your poster to the instructor. You should be prepared to explain the project without reading off the poster. For the final poster, the text should be printed in 18 point type (titles may be larger) and should include the following sections:

Title and authors’ names and affiliations

Abstract (200 words maximum)

Introduction

Results: Include figures, graphs, tables, and/or photographs that display your results. Include any needed experimental detail in the figure legend.

Discussion

References & Acknowledgments

The instructor will have examples of scientific posters for you to see. During the poster session each pair will make a 5-10 minute presentation of their poster to the instructor.

The poster will be evaluated using this rubric:

|  |  |  |  |
| --- | --- | --- | --- |
| Feature | Pointspossible | Pointsearned | Comments  |
| Rough drafte of poster on April 5 | 5 |  |  |
| Practice poster presentation on April 7 | 5 |  |  |
| Abstract | 5 |  |  |
| Introduction & Background: Clear? Good explanation of why the experiment was done? Hypothesis? | 20 |  |  |
| Results: Clearly presented?Statistical analysis?Correct controls? | 20 |  |  |
| Discussion: Clear conclusion on hypothesis?Good understanding of meaning of *p* value?Correct interpretations of results? | 20 |  |  |
| Verbal presentation | 15 |  |  |
| Appearance of poster | 10 |  |  |
| Both partners contributed |  |  |  |
| If problems: sought help? |  |  |  |
| TOTAL | 100 |  |  |

##### Planning for the remainder of the semester

For the next month or more you will carry out your plan for your own project (Experiment 3). Note that you are still required to come to the scheduled lab period at the beginning to get any announcements or discussion, but may if you choose (and you can arrange it with the instructor and/or TA) do most of your work at other times during the week.

This does NOT mean that you are done with lab work for the semester, only that you are now knowledgeable enough to plan your own work schedule. The instructor will expect to see you put progress, time, and effort into lab at least equivalent to that required in the first part of the semester, as demonstrated by the characterization experiments you and your partner carry out.

**Be sure to keep good notes on what you do each time you are in lab. You can do so on the following pages.**

* When you make solutions, it’s best to plan out in advance exactly how you will make them (write down your plans and check your calculations!), and then follow your plans exactly.
* Write down what seeds you use on what days.
* Record the conditions used to grow plants: temperature, hours of light and darkness, and intensity of light.
* When you record results, again, write down the dates and the genotypes. Be sure to include the units for any measurements (are you measuring the roots in µm, mm, or cm?).
* Include a summary of your conclusions and a table of your results in the notebook too.

**Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

Notes on today’s procedures

**Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

Notes on today’s procedures

**Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

Notes on today’s procedures

**Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

Notes on today’s procedures

**Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

Notes on today’s procedures

**Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

Notes on today’s procedures

**Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

Notes on today’s procedures

**Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

Notes on today’s procedures

**Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

Notes on today’s procedures

**Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

Notes on today’s procedures

**Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

Notes on today’s procedures

**Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

Notes on today’s procedures

**Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

Notes on today’s procedures

### Introduction to Experiment 4, Molecular Mapping of the Enhancer Mutation

#### Summary

In this project you will determine the chromosomal location of a gene through the technique of molecular mapping. You will map the same gene that you are studying in the genetics project described above. Although you will be mapping a gene found in plants, the techniques you will use are the same as those used in identifying the genes responsible for human genetic diseases such as muscular dystrophy or Huntington’s disease. As part of the mapping, you will isolate DNA from plants and then characterize the DNA with the techniques of polymerase chain reaction and gel electrophoresis.

The plant gene that you will map is known at the moment only because we have identified a mutant plant with a defect in this gene, i.e. the enhancer mutation. All students in the class will work on the same mutant, which will ensure replication of results and enable the class to share results and discuss conclusions. This mutant could represent a gene that is already known or a gene that is unknown. The results that you obtain in this lab could be important in the progress of this research project. A first step towards a better understanding of these genes and their roles is to determine their chromosomal locations.

**Mapping a Mutationally-Identified Gene in *Arabidopsis***

Mapping of a mutationally-identified gene in *Arabidopsis*, such as the one that you are studying, relies on comparing the inheritance of the mutation of interest to that of “markers” from different chromosomal locations. A marker is essentially a DNA polymorphism that allows the inheritance of a piece of a chromosome to be followed through crosses; markers will be explained in more detail later. Markers that are close to the mutation on the chromosome will tend to be inherited along with the mutation. Markers that are not close to the mutation will be inherited independently of the mutation.

In order to follow the inheritance of both the mutation and of markers, we must use plants produced from a “mapping cross”. The mapping cross must be between plants of two different strains or varieties (in *Arabidopsis*, strains are called “ecotypes”): one strain contains the mutation to be mapped (i.e. the mutant allele) and the other does not (instead, it contains the wild-type allele of the same gene). An example of ecotypes or strains with which you should be familiar is the different types of apples: Red Delicious, Granny Smith, and Golden Delicious are all apples and can be crossed, yet they contain important DNA sequence differences that give them different phenotypes. With some effort almost any pair of *Arabidopsis* strains can be used for mapping. Commonly used strains in mapping include Landsberg *erecta* (abbreviated Ler, and so called because of the *erecta* mutation it contains, which makes the plants compact and easy to work with in the lab), Columbia (abbreviated Col), and Wassilewskija (abbreviated Ws). DNA from essentially any two strains differs by many small sequence changes, which can be single base pair changes, deletions, or insertions. An extensive list of these differences between Ler and Col (called “polymorphisms”) is available, making it possible to identify a useful polymorphism at any location in the *Arabidopsis* genome for this cross. Using the Arabidopsis database it is possible to identify likely polymorphisms between any pair of strains. A cross between the enhancer mutant in the Ws ecotype and a wild-type Ler plant gives F1 progeny that are heterozygous both for the mutation and its corresponding wild-type allele, and for all the DNA polymorphisms between Ws and Ler. When the F1 plants self-fertilize, they produce the actual F2 mapping population.

In order to visualize these DNA polymorphisms easily, we will use Polymerase Chain Reaction (PCR) amplification, in which cycles of DNA synthesis create large amounts of a specific DNA sequence. In our PCR we will begin using just one type of primers, those that amplify polymorphisms called “simple sequence length polymorphisms” (SSLPS). These amplify a region that differs slightly in size between Landsberg and Wassilewskija. Short sequences of nucleotides (usually just one or two bases) are repeated many times at certain locations along the chromosome, e.g. AGAGAGAGAGAG. Such sequences commonly vary in number between ecotypes; for example, there might be 16 copies of the AG repeat in Wassilewskija and 20 copies in Landsberg. Thus, PCR primers that bind just outside these repeated regions, to sequences that are identical in Wassilewskija and Landsberg, amplify this sequence will give different-sized bands from the two ecotypes. The primers that amplify the SSLP enable us to detect easily the polymorphism. Essentially these primers convert the polymorphism into a molecular marker, one that can easily be used to determine the identity of a particular region of DNA.

**Mapping The Enhancer Mutation**

The following description assumes that the enhancer mutation is recessive; if it is dominant, the principle is the same, but some of the details are different. The enhancer mutation we are studying, originally identified in Wassilewskija ecotype plants that already contained the *axr4-3* mutation, was crossed to wild-type Landsberg *erecta* plants (so the cross is *aaee* Ws *x AAEE* Ler). The F1 plants (genotype *AaEe*)were allowed to self-fertilize. From among the F2 progeny, the plants that are homozygous for both the *axr4* and the enhancer mutation are identified (i.e. the *aaee* plants) by their long roots (longer than those of *axr4*). If the enhancer is recessive and auxin-sensitive, then the only plants showing the enhanced (longer root phenotype) will be those that are homozygous both for the *axr4* mutation and the enhancer mutation. These F2 plants with long roots, homozygous for both the *axr4* and the enhancer mutations, can be identified and form a population that can be used to map the enhancer mutation. All of the F2 plants have a mixture of Ws and Ler DNA at most points along their five chromosomes because of independent assortment and crossing-over. However, at the locations of the mutations that make them auxin-resistant (both the *axr4-3* mutation and the enhancer mutation), the F2 plant with enhanced auxin resistance should contain the same type of DNA that is present around the original mutations, i.e. only Ws DNA. These mutations were originally identified in Wassilewskija plants and will therefore be associated with Wassilewskija DNA; the wild-type alleles of the *AXR4* and enhancer genes (*A* and *E*) are, in this mapping population, associated with the Landsberg *erecta* DNA. We will identify the location of the mutations by testing a number of molecular markers distributed around the Arabidopsis genome, and looking for one that always is inherited along with the mutation, i.e. shows a high percentage of Ws DNA in the enhanced resistant *aaee* F2 seedlings. We already know where the *AXR4* gene is; we should see linkage to the *axr4* mutation. The other location to which we see linkage should be where the enhancer mutation is. The figure on the next page shows the mapping cross and its analysis in diagrammatic form, for a gene called *ENHANCER* (the mutant allele is *e* and the wild-type allele is *E*).

This semester we will use the F2 plants directly for mapping. This approach has a slight risk if the phenotype is one that could be misscored (e.g. it’s possible that a seedling that we classify as “long” really only has “medium” *axr4* roots). Therefore we will check our results by keeping track of which F2 plant each sample comes from, and retesting any plants that give significant or confusing results. The best way to retest the genotype of the F2 plants is to let them self-fertilize and then test the growth of the resulting F3 seedlings on auxin-containing plates. If the F2 plant was homozygous for both the *axr4* and enhancer mutations, then the F3 family should also be homozygous and all the seedlings will have long roots.

We will begin this project by analyzing the F2 seedlings from the cross *aaee* Ws x *AAEE* Ler and separating out those with enhanced resistance to create a mapping population (see Fig. 5). We will plant these seedlings so that they can grow up. Once the plants are grown, we will number the plants, collect a small bit of tissue from each one, extract DNA from this tissue, and use the DNA as the template for PCR. The PCR will use different molecular markers from across the *Arabidopsis* genome to determine where Ler and Ws DNA is located in the plants of our mapping population (see Fig. 6). If we identify markers that appear to be close (linked) to the mutant gene, we will verify this by further tests. If we have time we will attempt to define a map position even more precisely by identifying and testing additional markers from a small region.

**Fig. 5. Mapping the *enhancer* mutation in *Arabidopsis*, part 1 (**explanation is on next page)**.**

****

**Fig. 5 (previous page). Mapping the *enhancer* mutation in *Arabidopsis*, part 1.**

A plant containing a recessive *enhancer* mutation (*e*) together with the original recessive *axr4-3* mutation (*a*), in a Wassilewskija ecotype plant, was crossed to a wild-type Landsberg ecotype plant. Wassilewskija DNA is indicated by straight lines, Landsberg DNA by wiggly lines. The heterozygous F1 plant undergoes meiosis, which produce a variety of gametes resulting from crossovers. For simplicity, only the enhancer gene (*E* or *e*) is shown and only four of the many possible gametes are shown. These gametes then combine with other gametes to produce zygotes of various genotypes. 1/4 of the F2 are homozygous for the enhancer mutation (*ee*) (these are in the lower right portion of the figure). Only 1/4 of these are also homozygous for the *axr4* mutation (*aa*; not shown), giving only 1/16 of the F2 total that will show the enhanced mutant phenotype and can be selected to create a mapping population. All of them must be homozygous for Ws DNA at the location of the two mutations selected for.

**Fig. 6. Mapping the *enhancer* mutation in *Arabidopsis*, part 2.**

In the diagram below, the four *aaee* F2 enhancer mutants from Fig. 4 (1-4) are characterized with three molecular markers (A-C); the location of the markers on the chromosomes are indicated. These markers enable the type of DNA (Ws or Ler) at each location to be determined in each DNA sample. From the results we can know that the enhancer mutation must be close to marker C, as this marker detects the highest frequency of Ws DNA.

|  |  |
| --- | --- |
| Marker | Total |
| A | 0 W, 8 L |
| B | 4 W, 4 L |
| C | 8 W, 0 L |

In fact, the diagram shown is a bit misleading as the marker farthest away, A, would be expected to have ½ Ws, ½ Ler, not all Ler as shown.



Marker: A LL LL LL LL

 B WW LW WL LL

 C WW WW WW WW

**Experiment 4**

## **Mapping the Enhancer Mutation**

In this experiment, you will determine the chromosomal location of the enhancer mutation. This experiment will take the remainder of the semester. You will be guided through the basic procedures once and will then carry out further mapping independently, in consultation with the instructor and TA.

The steps in mapping are the following:

1. Cross the mutant to be mapped to a wild-type plant from another ecotype.

2. From the resulting F­2, identify a mapping population (usually the least frequent class).

3. Collect tissue from controls and F2 plants in the mapping population.

4. Isolate DNA from the tissue collected in step 3.

5. Set up Polymerase Chain Reactions (PCRs) using control and mapping populationtemplate DNAs and mapping primers.

6. Analyze the PCRs by gel electrophoresis.

7. Repeat steps 5 and 6 with additional primers as needed to identify 1 or 2 markers close to the gene.

8. Verify linkage for the linked markers identified in steps 5-7 by carrying out PCRs with more individual F2 DNA samples.

9. Refine the map position of the gene using additional markers (as time permits).

10. Re-test the F3 progeny from individual F2 plants that gave significant results to verify the genotype & phenotype.

The goals are to identify one or two linked markers using a small set of 21 F2 DNA samples, and then to verify this linkage using a larger set of individual F2 DNAs.

#### Step 1 was carried out prior to the start of the semester. You carried out step 2 as part of Experiment 1. We plan to carry out steps 3-7 during these labs.

#### Steps 1 & 2: Generate and identify a mapping population

The mapping population is the collection of plants that is useful for mapping a gene. In our case, we want to map the gene in which the enhancer mutation occurred. As discussed in the Introduction, the mapping population is derived from the cross between the enhancer (which came from a Wassilewskija ecotype plant) and a wild-type Landsberg erecta plant. In Experiment 1, you identified in this F2 population the plants that had either an *axr4*-like phenotype orenhanced auxin resistant phenotype in root growth.

**An updated version of the protocols for Experiment 4 will be distributed prior to the start of this experiment.**

**Step 3: Collect tissue samples from the mapping population and control plants**

The trays containing the F2 plants from the mapping population that you put into dirt earlier will be retrieved from the plant room where they have been growing.

1. Observe your plants. They should be clearly bigger than before. Count how many healthy plants you have from the F2 population and record it here:\_\_\_\_\_\_\_

Inform the instructor of this number.

2. You will be assigned a series of numbers for your F2 plants. Write these numbers on labels and put one numbered label by each individual F2 plant.

Record the range of numbers here:­\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Also label 0.5 mL microcentrifuge tubes (small tubes) with these numbers and “F2”. Label the tubes on both the lid and the side.

NOTE: Be sure that the tubes are properly and completely labeled and will not be confused with tubes from another group!

3. Using scissors, cut off a small piece from a healthy leaf of each plant and place it in the corresponding numbered tube. The piece of leaf should be about the size of one of the holes in a piece of looseleaf notebook paper. Do NOT pull the plant out of the dirt while doing this! Wipe the scissors carefully with an ethanol-soaked Kimwipe between each sample—you do not want to carry over any tissue from one sample to the next.

4. One group will collect two tissue samples from each of the three types of control plants (wt Wassilewskija, wt Ler, and *axr4-3*) into labeled microcentrifuge tubes (samples 22, 23, & 24).

5. Put the tissue samples numbered above 24 and the extra control samples in the designated box. These will be stored in the -80 degree freezer for future use.

**Step 4: DNA extraction**

In previous years we used a traditional DNA isolation procedure which required grinding a relatively large amount of frozen tissue in liquid nitrogen, extracting the DNA with a strong solvent, removing proteins using chloroform, and precipitating the DNA with isopropanol. This procedure gave lots of high quality DNA but was long and somewhat hazardous. This semester we will use a recently-developed very short and simple procedure that requires only incubating a tiny amount of tissue in a patented extraction solution at high temperature. Thanks to the miracle of PCR, the small amount of DNA that results is still enough for our purposes.

CAUTION: The extraction solution should not be gotten in your eyes or on your skin. Follow the instructor’s and TA’s directions, wear safety goggles and gloves, and observe proper safety precautions.

1. Add 100 μL of extraction solution to each of the F2 tissue samples (numbers 1-24). Be sure that the pieces of plant tissue are immersed in the extraction solution.

2. Be sure that the lids of the tubes are tightly closed. Place the tubes at 65 degrees for 6 minutes and then at 98 degrees for 2 minutes using the PCR machines.

3. While these tubes are incubating, begin labeling five strips of eight 0.2 mL tubes. Put a letter on the end of the set, and numbers on the individual tubes. Also number strip caps just with the letters. Follow the chart below to know what labels to use:

|  |  |  |
| --- | --- | --- |
| Group | Labels | And adds proper DNA to tubes  |
| 1 | 5 strip tubes “MA”, tubes 1-8 | 1-4 in all MA strips |
| 2 | 5 strip tubes “MA”, tubes 1-8 | 5-8 in all MA strips  |
| 3 | 5 strip tubes “MB”, tubes 9-16 | 9-12 in all MB strips |
| 4 | 5 strip tubes “MB”, tubes 9-16 | 13-16 in all MB strips |
| 5 | 5 strip tubes “MC”, tubes 17-24 | 17-20 in all MC strips |
| 6 | 5 strip tubes “MC”, tubes 17-24 | 21-24 in all MC strips |

4. When the incubations are done, spin the tubes containing the tissue and extracted DNA briefly and then place them on ice.

5. Once you have finished labeling the strip tubes in step 3, aliquot the DNA as follows: transfer 1 μL of DNA from your first 0.5 mL tube (with tissue and DNA extraction buffer) into the corresponding numbered tube in each of your strips. You can use the same pipet tip for these transfers. Note: Because 1 μL is a very small volume, it is important to be sure that the tiny drop actually ends up in the tube. Watch carefully to make sure, and try to put the drop in the bottom of the tube.

Change tips, and transfer 1 µL of DNA from your second 0.5 mL tube into each of the corresponding labeled strip tubes. Continue for all of your 4 labeled samples.

6. Pass the strip tubes on to the other group that is preparing the same strip tubes, and receive from them their strip tubes. Repeat step 5 so that all the tubes in all the strips now have DNA. Put the lids on the strip tubes when you are done.

7. Exchange strip tubes with other groups so that each group has at least one complete set of strips A, B, and C. 4 groups will have two complete sets. Give the strip tubes a quick spin.

8. Store your original DNA samples (in 0.5 mL tubes) and the sets of strip tubes (with lids on) in your group’s designated box in the -20 freezer.

A note on storage of samples

The DNA you prepared today will be needed for the rest of the course. Constant freezing and thawing of DNA causes it to degrade, so be careful when removing samples from the freezer or refrigerator boxes not to allow your samples to thaw unnecessarily. Follow the instructor’s instructions on how to store your samples.

Notes on today’s procedure

**Step 5: Set up Polymerase Chain Reactions (PCRs) using control and F2 DNAs and mapping primers**

##### Prelab preparation

Read through the instructions for this lab and complete Tables 3 B, C, D, and E.

## Overview

Today you will use the control DNA (from Ws, Ler, *axr4-3*, and enhancer plants) and the F2 mapping population DNA that you prepared to set up a total of 25 polymerase chain reactions (PCRs) with a single pair of primers. Each pair of students will test a different marker (primer pair), such that together the entire class will cover, in no more than two sets of PCRs, the entire *Arabidopsis* genome at a resolution that should be adequate to identify an approximate location for the enhancer gene.

CAUTION: It is very important during this work:

* to change pipet tips when necessary (i.e. almost every time) to avoid any possibility of cross-contamination.
* to keep all reagents and tubes on ice as much as possible;
* however, you should remove the tubes from ice when pipetting out of them.

Each polymerase chain reaction (PCR) requires a number of components:

* template DNA
* DNA polymerase (in our case, a thermostable version called *Taq* polymerase)
* DNA precursors (deoxynucleoside triphosphates, or dNTPs)
* primers (oligonucleotides=short single-stranded pieces of DNA): two per reaction, binding close together on the chromosome and on opposite strands
* buffer (to ensure optimal conditions of pH and ionic strength for the polymerase)
* magnesium (required for the polymerase to work)

We will use the approach and the set of primers (with additions and modifications) described in the following paper: Lukowitz W, Gillmor CS, and Scheible W-R. 2000. Positional cloning in Arabidopsis. Why it feels good to have a genome initiative working for you. Plant Physiology **123**, 795-805.

Planning Polymerase Chain Reactions

First, you need to plan what will go into each PCR. You should complete this section **before** coming to lab.

1. The instructor will assign you and your lab partner one molecular marker. For each marker, find in Table 4A below and note down the name (letter code), chromosomal location, and predicted sizes of the Columbia and Landsberg alleles of the marker.

Marker:\_\_\_\_\_\_\_\_ Chromosomal location:\_\_\_\_\_\_\_\_\_\_

Predicted size of Col allele:\_\_\_\_\_\_\_ Predicted size of Ler allele:\_\_\_\_\_\_\_\_\_

An updated version of Table 4A will be distributed before the lab.

Table 4A.

*Arabidopsis* Mapping Set of Markers/Primers, spring 2010

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Name | Chromosome/Position (cM) | Position (bp) | Size of Col allele | Size of Ler allele | Forward primerReverse primer |
| AB | 1.15 |  | 154 | 134 | AAATGGGAGATGTTGATGTTCTTCCCAAAGGGTCACGAGTGAAGTCTAG |
| AE | 1.49 |  | 241 | 191 | TTGGATTATCTCTTGAATGGGTTTGGCCGTCTTTAGCATTAGGAACTCTGGTG |
| CB | 1.83 |  | 180 | 190 | ATCTTGAAACCTTTAGGGAGGGGGTCTGTTGATGTCGTAAGTCG |
| CL | 1.110 |  | 138 | 118 | TTGGTGAAAGAGTGTCCTTGGGAAACGTACAGTTCAAGAAGAGAGG |
| AJ | 2.10 |  | 254 |  | Tcctttcttagcagccttcacagattcatgaagaagatgttcgtgtaggagc |
| CT | 2.30 |  | 273 | 244 | GAACGAAGCCGATCGTTAGACGATCTTTGATAATTCTAGGTTTCG |
| AN | 2.65 |  | 199 | ? | GTCTGGATCTTGTATCGCAAACCAAATGCTTCAGAGCTGATGAACAAAAGAC |
| DB | 2.75 |  | 173 | 131 | CATGCTGATCAAGGGTGTGATGACCAGTTCAGAAGCAGGA |
| AQ | 3.07 |  | 165 | ? | CATCCGAATGCCATTGTTCAGCTGCTTCCTTATAGCGTCC |
| CO | 3.30 |  | 201 | 182 | AAGTAGCCCAAAGCCGTACAGCAGGGACAATCCGTAAAGA |
| AS | 3.53 |  | 151 | 129 | TCAATGGCAGCAAAGTCCATTAAATCAACAGATACCAACTCCACAGGGACAA |
| CQ | 3.88 |  | 224 | 205 | GAAATGCAACGAGACAAACTCAGGTTTGGTGGGAGAGAATGA |
| AU | 4.05 |  | 183 | 291 | Aataacagagcaaacggtggcaataaatgagcctgatccaaaaccaggtaa |
| AW | 4.30 |  | 122 | 102 | Tgctgtgaagtgttcaatggtaagacagcatagacaatcctcaacctccagaa |
| AZ | 4.64 |  | 271 | 213 | GCGACGTTATCTTTCCCTTCTTTTGTTGACTACCGTCACAATCCTGACTCAA |
| BD | 4.100 |  | 162 | 144 | Tatcgtcggttaatacaactttccctctatgtaaaaccttcgaaatactgatacaacaaa |
| CG | 5.13 |  | 177 | 120 | TCCAAAGCTAAATCGCTATCTCCGTCTATTCAAGATGC |
| BH | 5.30 |  | 249 | 233 | CCCAGTCTAACCACGACCACAATCCCAGTAACCAAACACACA |
| BJ | 5.60 |  | 177 | 135 | Aattgtgggaaggacaacaaccaaagagagagcacgtgagatgtcacaga |
| VC | 5.88 |  | 184 | 162 | TCGGAAAAAGTATGTTGGGAGTTGTTCAACAATAGCTGCCAAA |
| VH | 5.113 |  | 175 | 155 | TCACCTTACTTAATTCAACTGCAAACCAGATTCGATGTACTTCACTTTC |
| BO | 5.119 |  | 149 | 128 | Tgaaaactaaaaggcgactactagcataagatcttgccatttatttggtcaacaca |

2. Read this explanation, and then fill out the last column of Table 4B below.

As already mentioned above, each polymerase chain reaction (PCR) requires a number of components. These components are supplied as concentrated stock solutions, and are therefore added in small amounts to the reaction to give the correct final concentration:

* template DNA: while it is possible to use too much or too little template DNA, in general this amount is flexible within a range of 10 to 100 ng, or even wider. We will simply use 1 µL of our DNA preparation.
* *Taq* DNA polymerase: concentration of this enzyme is measured in activity units; we will use 0.5 activity unit (at 5 units/µL, this is 0.1 µL of the *Taq* polymerase).
* DNA precursors (dNTPs): generally a final concentration of 200 µM is adequate; our stock solution is 2 mM.
* primers (oligonucleotides): two per reaction; we will use primers here at a concentration of 250 nM. Our primer stocks are at 12.5 μM.
* buffer: here we will use a stock that is 10X more concentrated than our desired final concentration, which is 50 mM KCl, 10 mM Tris, pH 8.3, 1.5 mM MgCl2
* magnesium (provided as MgCl2): the optimum concentration varies for different primers. The primers in our mapping set have all been chosen to have an optimum Mg concentration of 2 mM. As there is already 1.5 mM in the 10X buffer, we need to add only enough MgCl2 to bring the concentration up to 2 mM, from a stock solution that is 10 mM.
* water is added to bring the reaction up to the desired final volume. In our case, we will set up reactions of 20 µL.

An example of how you will calculate the amount of a reagent to add to a reaction:

How much magnesium should I add to a 50 µL PCR to give a final concentration of 2 mM, if the 10X buffer will already contribute 1.5 mM? So, I need to add a final concentration of 2-1.5 = 0.5 mM. The stock magnesium solution that I will be using is 10 mM.

Use the formula **civi = cfvf,** where ci = the initial concentration (i.e. the concentration of the stock solution), vi = the initial volume (the volume of the stock solution), cf = the final concentration (i.e. in the reaction tube), and vf = the final volume (of the reaction). We need to find vi, the volume of the stock solution to be added to the reaction.

Solving for vi: vi = cfvf/ci

Substituting in the known values: vi = (0.5 mM)(50 µL)/10 mM = 2.5 µL

So 2.5 µL of 10 mM magnesium stock must be added to a 50 µL total reaction volume to give a final magnesium concentration of 2 mM.

NOTE: Be careful that your units are consistent! You cannot mix millimolar and micromolar in the same calculation and expect to get the correct answer—convert all values to the same units, e.g. 2 mM = 2000 µM.

In the table below, you will calculate how much of each concentrated stock reagent to add to make a reaction with a total volume of 20 μL, using the approach shown in the example above.

Table 4B. Components of polymerase chain reactions.

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **[Stock solution]** | **[Desired final]** | **Vol. of stock to add** **for one 20 μL reaction** |
| dNTPs | 2 mM | 200 µM |  |
| MgCl2 | 10 mM | 2 mM(but have 1.5 mM already) |  |
| buffer | 10X | 1X |  |
| DNA template | -- | -- | 1 µL |
| Primers | 12.5 µM | 250 nM |  |
| Taq polymerase | -- | -- | 0.1 µL |
| water |  |  | To give a final volume of 20 µL: \_\_\_\_\_\_\_\_\_\_ |

Saving time with a master mix

If you are setting up many reactions that require the same reagents (as you are here), a time-saving approach is to make a “master mix” or “cocktail” containing all these reagents in the proper proportions, and then distribute the proper volume of the master mix into each reaction tube. For example, instead of adding water to each of 25 tubes, then adding 10X buffer, then adding dNTPs, then adding MgCl2, then adding primers, and then adding Taq polymerase (150 separate pipettings), you could combine water, 10X buffer, MgCl2, dNTPs, primers, and Taq in 1 tube (6 pipettings), and then pipet the mix into each of the 25 tubes (25 pipettings, for a total of 31 pipettings). This approach saves on pipet tips, time, and effort, and reduces errors.

Two useful tips when making a master mix:

* Calculate the volumes to add based on the number of tubes you need + a few additional: that way you have a little extra so you don’t run out for the last tube. So, if you need to set up 25 reactions, combine enough reagents for 27 reactions. If you find you still run out of reagents, ask the instructor to check your pipetting technique and your pipettors.
* Add the water first to the master mix. Sometimes adding together the concentrated reagents by themselves without water may lead to precipitation.

You can calculate the ingredients of a master mix in the Table 4C below. The “vol. of stock for 1 reaction” (column 2) comes from your calculations in Table 4B. The “vol. of stock to add to master mix” (column 4) is calculated by multiplying the values in column 2 by the values in column 3.

Note that you can only include in the master mix the ingredients that will be the same in each tube. Therefore, the template DNA is not usually part of the master mix. In some cases you might be testing many pairs of primers in one set of reactions, in which case you could not include the primers either. The Taq polymerase is sometimes added separately after all the other ingredients have been placed in the tubes, to prevent the reactions from starting prematurely. Here, be sure to add the Taq polymerase last to the master mix, and only after the reactions are otherwise all ready to go and the PCR machine is heated up.

Table 4C. Master mix for polymerase chain reactions.

|  |  |  |  |
| --- | --- | --- | --- |
| **Column 1** | **Column 2** | **Column 3** | **Column 4** |
| **Reagent** **(stock concentration)** | **Vol. of stock for 1 reaction** | **# of reactions + 2** | **Vol. of stock to add to master mix** |
| water |  |  |  |
| 2 mM dNTPs |  |  |  |
| 10 mM MgCl2 |  |  |  |
| 10X buffer |  |  |  |
| 12.5 μM primers |  |  |  |
| *Taq* polymerase |  |  |  |
| Sum: Vol. to add to each tube |  | xxxxxx | xxxxxx |

5. You can now determine what to put into each PCR tube. You and your partner will together carry out 25 reactions: the first 21 using F2 DNA and the last 4 using control DNA. You will fill in the identities of the individual DNA templates later.

To fill out Table 4D below:

Fill in the “primer pair” column with the name of the marker you will use. Fill in the “Vol. master mix” column with the volume you calculated at the bottom of Column 2 in Table 3C. All volumes are in microliters.

Table 4D. Details of 1st Set of Polymerase Chain Reactions Tubes for Primer Pair\_\_\_\_\_\_

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Tube | DNA template | vol. template DNA | Vol. master mix | Tube | DNA template | vol. template DNA | Vol. master mix |
| 1 |  | 1 |  | 14 |  | 1 |  |
| 2 |  | 1 |  | 15 |  | 1 |  |
| 3 |  | 1 |  | 16 |  | 1 |  |
| 4 |  | 1 |  | 17 |  | 1 |  |
| 5 |  | 1 |  | 18 |  | 1 |  |
| 6 |  | 1 |  | 19 |  | 1 |  |
| 7 |  | 1 |  | 20 |  | 1 |  |
| 8 |  | 1 |  | 21 |  | 1 |  |
| 9 |  | 1 |  | 22 | wtCol | 1 |  |
| 10 |  | 1 |  | 23 | wtLer | 1 |  |
| 11 |  | 1 |  | 24 | *axr4-2* | 1 |  |
| 12 |  | 1 |  | 25 | enhancer | 1 |  |
| 13 |  | 1 |  |  |  |  |  |

Setting Up Polymerase Chain Reactions

1. Fill a styrofoam container (one per pair) with crushed ice. You must keep the reagents on ice as you prepare the PCRs.

2. Collect the tubes of the reagents, such as water, 10X buffer, and dNTPs, that you will need. The proper way to do this is to take your ice container to the reagent container and transfer the tubes directly into your ice container so that they remain cold.

3. Collect from your classmates and from your own storage boxes the 21 tubes of F2 DNA that the instructor has designated as the initial set to be tested. Also collect from your storage boxes one each of the four control DNA tubes that you previously prepared. Add to the labels on the tubes the name of the marker that you are testing in the PCR. For example, if you had tubes 1-21 and were testing primer pair BH, your tubes would now be labeled BH1, BH2, BH3,…BH21, BH-Col, BH-Ler, BH-axr4, and BH-enh. Keep these tubes on ice.

4. Prepare the master mix as follows:

Label one 1.5 mL tube with the primer pair name and “Mix” (e.g. “BH Mix”). Into this tube, on ice, pipet the proper volume of each of the ingredients of the master mix as listed in Table 3C, starting with the water. Do NOT add the *Taq* yet.

5. Before continuing, prepare the PCR machine: turn it on, and start program “SSLP” so that the lid heats up. The machine should pause when the lid is hot.

6. a. Add the Taq polymerase to the master mix.

b. Close the lid of the master mix tube and invert it gently several times to mix together the ingredients.

c. Add the required volume of master mix to each PCR tube.

7. Close the caps, mix by flicking with your finger if necessary, and spin the tubes briefly to bring all the reagents to the bottom. Note that when you spin the small tubes, you need to have holders in the rotor, as the rotors are designed for larger tubes. Return the tubes to ice.

8. Continue the SSLP program on the PCR machine so that the block begins to heat up. You can now put the tubes into the machine. The standard program “SSLP” that we will be using for these primers is as follows:

Table 4E. PCR Program “SSLP”

|  |  |  |  |
| --- | --- | --- | --- |
| **Step** | **Temp.** | **Time** | **Purpose** |
| 1 | 94 °C | 5 min. | initial denaturation |
| 2 | 94 °C | 30 sec. | denaturation |
| 3 | 55 °C | 30 sec. | annealing |
| 4 | 72 °C | 30 sec. | extension |
| Repeat **steps 2, 3, & 4** 39 more times (40 times total) |
| 4 | 72 °C | 10 min. | final elongation |

This program will take about two hours to run. At the end of these steps we have programmed the machine to go to 10 °C and hold until we stop it. This temperature is cool enough to keep our samples safe. We can therefore retrieve the samples later tonight or tomorrow; we do not have to retrieve the samples as soon as the program is done.

9. Clean up: store the buffer, magnesium solution, water, and dNTPs in your group’s box in the freezer. Discard other tubes as instructed and put away equipment.

10. Check the PCR machine to be sure that it is running properly. If you have any questions, ask the instructor or TA. **NOTE: YOU AND YOUR PARTNER are responsible for collecting the PCR tubes from the machine when the reactions are done and storing them in your rack in the freezer.** If there are no other tubes in the PCR machine when you remove yours, STOP the PCR program and shut off the machine. Do NOT leave the machine running after it is done and do NOT forget your tubes! Forgotten tubes will be placed in the “Rack of Shame” or discarded!

## Notes on this lab’s procedures

**Step 6: Analyze the PCRs by gel electrophoresis**

## Overview

You will prepare and run agarose gels to analyze the PCRs that you set up in the previous lab.

## Agarose gel electrophoresis is the standard approach used to analyze most types of experiments involving nucleic acids. Agarose, a branched polysaccharide purified from sea algae, is melted in a buffer solution, poured into a mold with a “comb’ to create holes, and then submerged in an electrophoresis chamber containing the same electrophoresis buffer. Samples, mixed with a loading dye, are pipetted into the holes (wells”) in the gel, and then an electric field is applied across the gel. The negatively-charged nucleic acid fragments in the gel are attracted towards the positive electrode and are separated by size as they move through the gel. Smaller fragments can move more quickly through the gel and so migrate farther down the gel. At the end of the electrophoresis, the DNA fragments are visualized by staining with a dye such as ethidium bromide that fluoresces under UV light.

For the first lab, the agarose solution will be provided already melted in running buffer at the proper concentration. For future labs, we will either re-use old gels, or prepare new gels. The buffer we will use is called “TBE”, for Tris, boric acid, and EDTA (the three ingredients). We will use a special agarose blend called “3:1” at a relatively high concentration of 3-4%. This combination of agarose type and concentration will enable us to separate clearly the small fragments we expect to see for our collection of molecular markers.

##### Procedure

1. Pour an agarose gel.

2. Thaw and prepare your samples (PCR tubes) by adding loading dye.

3. When the gel is ready, place it in the electrophoresis chamber with buffer.

4. Load the samples onto the gel; include a molecular weight marker.

5. Run the gel.

6. Stop the gel, and stain the gel with ethidium bromide.

7. Destain the gel in water or running buffer.

8. Photograph the gel.

9. Analyze the results.

#### Protocol for Agarose Gel Electrophoresis

You will need one gel tray, two combs (white), one electrophoresis chamber, and a chamber lid with black and red cords. There are several sizes of gel apparatuses, and combs with different numbers of teeth. We will use a type of apparatus called the “Scooter 100”, together with a comb that has 26 teeth (i.e. makes 26 wells).

1. Place a gel tray on a clear and level area of your bench.

2. a. Take the prepared agarose solution and pour the agarose carefully into the gel tray. Do not pour too fast or the agarose may spill out the sides. Use the volume of agarose directed by the instructor. For the standard large “Scooter 100” gel, this volume is 50 mL.

b. Put a comb or combs into the appropriate positions of the gel tray. Allow the agarose to harden (about 10-15 minutes).

c. If you needed to make your own agarose solution, you would measure out the proper volume of 1X TBE buffer into an Erlenmeyer flask at least 5 times greater than the volume of buffer, weigh out the proper amount of agarose (for example, if you wanted to make 50 mL of a 4% gel, you would weigh out 0.04 x 50 = 2 g of agarose), add it to the buffer, and then microwave gently, stopping occasionally to swirl the solution so it doesn’t boil over.

3. While the gel is hardening, prepare your samples. Add 4 µL of 6X orange loading dye to each PCR tube (Note: 20 µL of PCR solution + 4 µL of 6X dye = 24 µL at 1X dye). The loading dye enables you to see the sample as you load it, helps you monitor how the samples are running (the orange dye runs faster than our DNA samples), and contains a high concentration of sucrose to cause the sample to sink into the well.

4. Prepare or obtain a molecular weight size marker to use, as directed by the instructor. The molecular weight marker is usually labeled “MW” and is provided already mixed with loading dye. The standard DNA marker that we use is provided by the company New England Biolabs, is called the “Low Molecular Weight Marker” mix, and contains bands of the following sizes, in basepairs of DNA: 25, 50, 75, 100, 150, **200**, 250, 300, 350, 500, and 766. The 200 bp band contains more DNA than the others and so can be easily identified on the gel. The smallest bands may not be visible or may be fuzzy.

4. When the gel has hardened (it will look white), gently remove the comb by pulling straight up and transfer the gel, still in its tray, to the electrophoresis chamber, as follows:



a. Tilt the gel tray so the end without the notches along the top goes in first;

b. Slide this end in to the end of the electrophoresis chamber with the rubber strip;

c. Push the gel tray firmly against the strip;

d. Lower the other end of the gel tray until it locks into place.

e. Have the instructor or TA check that the gel tray is properly positioned. Be sure that you are all ready to load your samples before proceeding to the next step.

5. Pour chilled 1X TBE buffer (electrophoresis buffer) into the two sides of the electrophoresis chamber.

6. Load the samples: Pipet 10 µL of the first PCR reaction into the 2nd well of the gel: put the pipet tip at an angle in the top of the well and gently depress the plunger so that the sample drops down into the well. Do NOT put the pipet tip all the way down into the well, nor should you squirt the sample forcefully down into the well.

7. Load the remaining samples in the same manner. Put the MW size marker into the first well—just load the entire contents of the tube; you should not need to add extra loading dye.

8. Put the lid on the electrophoresis chamber, attach it to a power supply (put the red lead in the red socket, and the black lead in the black socket!), and start it at the voltage indicated by the instructor (usually 120 V).

\*CAUTION: Have the instructor or TA check that your gel is properly connected before starting it. Be sure that the DNA will run towards the red electrode. Electrophoresis uses high voltages; be careful that the chamber is properly closed while the power is on, and be sure to turn off the power before touching the inside of the chamber. After starting the gel, check that bubbles begin to come off the electrodes and that the dye is moving in the proper direction. You should be able to see this within a few minutes!

Note the times of starting and stopping:

Voltage\_\_\_\_\_\_\_\_\_\_ Time started\_\_\_\_\_\_\_\_\_\_\_ Time stopped\_\_\_\_\_\_\_\_\_\_\_

9. At the end of the electrophoresis, turn off the power supply (see CAUTION above), disconnect the leads and remove the lid. Carefully lift out the gel tray and pour the used TBE buffer into the designated container.

10. Use the large white plastic “gel scoop” to help remove the gel from the gel tray by sliding it out the end that was against the rubber strip in the electrophoresis chamber (it won’t fit out the end where the comb was) and into a plastic container (see diagram).



11. Cover the gel with ethidium bromide solution and let it stain for about 15 minutes on the rocking platform. CAUTION: Ethidium bromide is a known mutagen. Wear gloves at all times when handling solutions or gels containing it; be careful not to spill it.

12. At the end of this time, carefully return the staining solution to the appropriate bottle by pipetting it out with a large plastic pipet and an electronic “Pipet-Aid”. Be careful not to get liquid up into the Pipet-Aid itself. Rinse the gel once with water, cover it with water, and let it soak (“destain”) for about 10 minutes.

13. Under the instructor’s or TA’s supervision, examine the gel on the UV light box, and photograph (if there’s anything to see). CAUTION: UV light is dangerous. Be sure that the gel viewing chamber is properly closed so that your eyes and skin are not exposed to the UV light.

Notes on this lab’s procedures

14. Record and analyze your results here.

a. Tape in the gel photo. Above or below the gel photo you should label each lane.

b. In Table 4F, record the bands that you saw in your gel. Determine the approximate sizes of each band by comparison with the MW size marker. Note that small (<50 bp) fuzzy bands seen in every lane are likely to be the primers themselves, not a PCR product.

c. By comparison with the control samples, you should be able to identify which bands represent the Col allele and which represent the Ler allele of the particular marker that you tested. Record the alleles present in each DNA sample in the last column.

##### Gel Photo

Table 4F. Results of 1st PCR and Gel Electrophoresis:

Sizes of DNA fragments for marker \_\_\_\_\_\_\_ (Predicted: Col\_\_\_\_\_\_, Ler\_\_\_\_\_\_\_).

|  |  |  |  |
| --- | --- | --- | --- |
| Tube | DNA template | Size of bands | Alleles detected(CC, CL, or LL) |
| 1 |  |  |  |
| 2 |  |  |  |
| 3 |  |  |  |
| 4 |  |  |  |
| 5 |  |  |  |
| 6 |  |  |  |
| 7 |  |  |  |
| 8 |  |  |  |
| 9 |  |  |  |
| 10 |  |  |  |
| 11 |  |  |  |
| 12 |  |  |  |
| 13 |  |  |  |
| 14 |  |  |  |
| 15 |  |  |  |
| 16 |  |  |  |
| 17 |  |  |  |
| 18 |  |  |  |
| 19 |  |  |  |
| 20 |  |  |  |
| 21 |  |  |  |
| 22 | wtCol |  |  |
| 23 | wtLer |  |  |
| 24 | *axr4-2* |  |  |
| 25 | enhancer |  |  |

Low Molecular Weight Marker” mix contains bands of the following sizes, in basepairs of DNA: 25, 50, 75, 100, 150, **200**, 250, 300, 350, 500, and 766. The 200 bp band contains more DNA than the others and so can be easily identified on the gel. The smallest bands may not be visible or may be fuzzy.

Answer these questions:

i. Do these sizes match what you expected to see for this pair of primers?

ii. Can you easily distinguish between the Col and Ler bands?

iii. What type of DNA is present in *axr4-2* and in the enhancer mutant at the location of the marker?

iv. In the 21 F2 DNA samples, how many Col alleles and how many Ler alleles did you detect?

v. Does this result support the idea that the enhancer mutation is linked or unlinked to the marker that you tested? Explain.

The instructor will collect each group’s results and share the composite results with the class.

## **Step 7: Identifying a linked marker**

You have now performed essentially all the techniques that you will use in the molecular mapping project: preparing plant DNA, setting up PCRs, and analyzing the PCRs by agarose gel electrophoresis. Therefore you are capable of proceeding with the molecular mapping project on your own. Your first goal is to test, using the set of 25 DNA templates that you already used, the remaining molecular markers (primer pairs) in the mapping set. These markers are spaced close enough together that you should be able to detect linkage to at least one of them no matter where in the genome the enhancer mutation is located. All the lab groups will work together to test all the markers. Try to complete the testing of all markers using the initial set of 21 F2 DNAs, and the next step (step 8, below) by the end of October when the first lab report on molecular mapping is due. Tables to use in preparing the 2nd set of PCRs are given on the next page.

If everything went well in the first set of PCRs, you would have seen DNA products (bands) in all lanes of the gel and you may already have seen a marker that might be linked to the enhancer mutation. If everything did not work the first time and not all of your samples gave DNA products (a very common occurrence), you and your partner need to troubleshoot and try again until you obtain results. Remember to keep good notes of what you do, and to check with the instructor whenever you have questions or need guidance. At the same time, you will need to continue with the other project.

What you do next depends on the results you obtained in the previous steps.

a. If your and your partner’s PCRs did not work (i.e. gave no visible product), then you should troubleshoot and try again with the same markers. Think about and discuss with your lab partner and with the instructor what might have caused your PCRs not to work, and try modifying or changing whatever possibilities you identify. Sometimes it is as simple as being more careful in pipetting (do your PCR tubes have the same volumes as other groups? If not your pipetting may be in error), or being sure to keep tubes on ice until the PCR machine is warmed up. Be sure to include as many controls as possible. For example, if you are not certain if your DNA template was good, be sure to include a control DNA template that is known to be good (how would you know that a DNA template was good?). In some cases you might need to repeat the DNA isolation procedure to get good quality DNA template. Did your gel work? If you could see the molecular weight marker clearly separated on the gel, then the gel is not the problem.

b. If your PCRs did work, then you do not need to test more samples with the same markers. Instead, you should test an additional marker from the initial marker set using the same approach as before. Even if someone in the class saw linkage with the first set of markers tested, the class should continue testing until all markers in the set have been tested.

**PCR #2**

**Marker tested:\_\_\_\_\_\_ Date tested:\_\_\_\_\_\_\_\_\_**

Table 4G. Details of 2nd Set of Polymerase Chain Reactions Tubes

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Tube | DNA template | vol. template DNA | Vol. master mix | primer pair tested |
| 1 |  | 1 |  |  |
| 2 |  | 1 |  |  |
| 3 |  | 1 |  |  |
| 4 |  | 1 |  |  |
| 5 |  | 1 |  |  |
| 6 |  | 1 |  |  |
| 7 |  | 1 |  |  |
| 8 |  | 1 |  |  |
| 9 |  | 1 |  |  |
| 10 |  | 1 |  |  |
| 11 |  | 1 |  |  |
| 12 |  | 1 |  |  |
| 13 |  | 1 |  |  |
| 14 |  | 1 |  |  |
| 15 |  | 1 |  |  |
| 16 |  | 1 |  |  |
| 17 |  | 1 |  |  |
| 18 |  | 1 |  |  |
| 19 |  | 1 |  |  |
| 20 |  | 1 |  |  |
| 21 |  | 1 |  |  |
| 22 | wtCol | 1 |  |  |
| 23 | wtLer | 1 |  |  |
| 24 | *axr4-2* | 1 |  |  |
| 25 | enhancer | 1 |  |  |

Table 4H. Master mix for 2nd PCRs.

|  |  |  |  |
| --- | --- | --- | --- |
| **Column 1** | **Column 2** | **Column 3** | **Column 4** |
| **Reagent** | **Vol. of stock for 1 reaction** | **# of reactions + 2** | **Vol. of stock to add to master mix** |
| water |  |  |  |
| 2 mM dNTPs |  |  |  |
| 10 mM MgCl2 |  |  |  |
| 10X buffer |  |  |  |
| 12.5 μM primers |  |  |  |
| *Taq* polymerase |  |  |  |
| Sum: Vol. to add to each tube |  | xxxxxx | xxxxxx |

Order of loading of samples:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Date of electrophoresis:\_\_\_\_\_\_\_\_\_\_ Type of gel:\_\_\_\_\_\_\_\_\_\_ Buffer:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Voltage\_\_\_\_\_\_\_\_\_\_ Time started\_\_\_\_\_\_\_\_\_\_\_ Time stopped\_\_\_\_\_\_\_\_\_\_\_

Gel photo:

Table 4I. Results of 2nd PCR and Gel Electrophoresis for Marker \_\_\_\_\_\_\_

Predicted sizes: Col\_\_\_\_\_\_, Ler\_\_\_\_\_\_\_

|  |  |  |  |
| --- | --- | --- | --- |
| Tube | DNA template | Size of bands | Alleles detected (CC, CL, or LL) |
| 1 |  |  |  |
| 2 |  |  |  |
| 3 |  |  |  |
| 4 |  |  |  |
| 5 |  |  |  |
| 6 |  |  |  |
| 7 |  |  |  |
| 8 |  |  |  |
| 9 |  |  |  |
| 10 |  |  |  |
| 11 |  |  |  |
| 12 |  |  |  |
| 13 |  |  |  |
| 14 |  |  |  |
| 15 |  |  |  |
| 16 |  |  |  |
| 17 |  |  |  |
| 18 |  |  |  |
| 19 |  |  |  |
| 20 |  |  |  |
| 21 |  |  |  |
| 22 | wtCol |  |  |
| 23 | wtLer |  |  |
| 24 | *axr4-2* |  |  |
| 25 | enhancer |  |  |

**Conclusion: Step 8: Confirming linkage with all available individual F2 samples**

When the class has tested all markers in the starting marker set with the 21 F2 DNA samples, there should be one or more markers that show linkage to your gene, i.e. for which most of the 21 samples tested show that they carry the same allele as the parental enhancer mutant. To confirm that you have identified linkage to this region of the genome, the class should now carry out another set of PCRs using the one or two linked markers and as many individual F2 DNA templates as possible (remember that you saved many of your original DNA preparations in the -20 freezer and did not test them in the initial set of PCRs). The instructor will help the class plan this set of PCRs. Of course you will need to include control templates in the set of PCRs as well, although you may not need to include all 4 controls that you used previously; discuss your plans with the instructor. Note that, because of the number of wells on a gel, you should plan to do no more than 25 PCRs at once.

Again, you should make a “master mix” as described previously.

##### Prelab Preparation

You need to write out before lab, and be ready to show to the instructor:

1. A table showing each tube that you will prepare and its contents (use template Table 3J below).

2. A table showing the contents of the master mix, i.e. each ingredient and its volume (use template Table 3K below). The master mix will not include the template, but will include all the other components. The instructor will help you to decide which primers to use.

##### Lab Procedure: Setting Up and Analyzing PCRs

After the class and instructor have discussed how to proceed, you should set up the appropriate PCRs. Be sure that the instructor has approved your plans, and that you take good notes on what you do. Here is a brief summary of the procedure, with which you should already be familiar:

1. Setting up the PCRs:

a. Label the PCR tubes.

b. Add the template DNA to each PCR tube, following your table. Keep the tubes on ice.

c. Prepare the master mix, following your table. Use 1.5 mL microcentrifuge tubes on ice.

d. Start the PCR machine. When the lid of the PCR machine is hot, put the tubes into the machine and close the lid tightly.

e. Add Taq polymerase to the master mix. Mix the master mix tube gently by inverting it several times.

f. Add the proper volume of master mix to each tube, following your table.

g. Close the lids of the PCR tubes and spin them briefly in the microcentrifuge. Keep the tubes on ice.

h. Remember to come back when the PCRs are done (this will be in about 2 hours, or the next morning if you start the reactions at night) to retrieve your samples. Stop the PCR program on the machine, turn off the machine, and put your samples into your freezer rack if you will not analyze them immediately by gel electrophoresis.

2. Analyze the PCR samples by gel electrophoresis, and record the results.

Be sure to record the key features of the electrophoresis in the indicated spaces: what kind and percentage of agarose, how you ran the gel, and what order the samples were in. Your gel photos should be stored in your lab notebook. The best way to store them is on the same page as the one where you wrote out what you put into the PCR reaction. Tape the gel photo onto the page and then label the lanes, clearly indicating what sample is in each lane. After you analyze the results of the gel, it’s a good idea to write your conclusion on the same page so you remember what the result meant; also write any additional information that might explain why you got the results you did.

3. If the reactions do not work, trouble-shoot and try again.

See the instructor with any questions.

**PCR #3**

**Marker tested:\_\_\_\_\_\_ Date tested:\_\_\_\_\_\_\_\_\_**

Table 3J. Details of 3rd Set of Polymerase Chain Reactions Tubes

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Tube | DNA template | vol. template DNA | Vol. master mix | primer pair tested |
| 1 |  | 1 |  |  |
| 2 |  | 1 |  |  |
| 3 |  | 1 |  |  |
| 4 |  | 1 |  |  |
| 5 |  | 1 |  |  |
| 6 |  | 1 |  |  |
| 7 |  | 1 |  |  |
| 8 |  | 1 |  |  |
| 9 |  | 1 |  |  |
| 10 |  | 1 |  |  |
| 11 |  | 1 |  |  |
| 12 |  | 1 |  |  |
| 13 |  | 1 |  |  |
| 14 |  | 1 |  |  |
| 15 |  | 1 |  |  |
| 16 |  | 1 |  |  |
| 17 |  | 1 |  |  |
| 18 |  | 1 |  |  |
| 19 |  | 1 |  |  |
| 20 |  | 1 |  |  |
| 21 |  | 1 |  |  |
| 22 | wtCol | 1 |  |  |
| 23 | wtLer | 1 |  |  |
| 24 | *axr4-2* | 1 |  |  |
| 25 | enhancer | 1 |  |  |

Table 3K. Master mix for 3rd PCRs.

|  |  |  |  |
| --- | --- | --- | --- |
| **Column 1** | **Column 2** | **Column 3** | **Column 4** |
| **Reagent** | **Vol. of stock for 1 reaction** | **# of reactions + 2** | **Vol. of stock to add to master mix** |
| water |  |  |  |
| 2 mM dNTPs |  |  |  |
| 10 mM MgCl2 |  |  |  |
| 10X buffer |  |  |  |
| 12.5 μM primers |  |  |  |
| *Taq* polymerase |  |  |  |
| Sum: Vol. to add to each tube |  | xxxxxx | xxxxxx |

Order of loading of samples:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Date of electrophoresis:\_\_\_\_\_\_\_\_\_\_ Type of gel:\_\_\_\_\_\_\_\_\_\_ Buffer:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Voltage\_\_\_\_\_\_\_\_\_\_ Time started\_\_\_\_\_\_\_\_\_\_\_ Time stopped\_\_\_\_\_\_\_\_\_\_\_

Gel photo:

Table 3L. Results of 3rd PCR and Gel Electrophoresis for Marker \_\_\_\_\_\_\_

Predicted sizes: Col\_\_\_\_\_\_, Ler\_\_\_\_\_\_\_

|  |  |  |  |
| --- | --- | --- | --- |
| Tube | DNA template | Size of bands | Alleles detected (CC, CL, or LL) |
| 1 |  |  |  |
| 2 |  |  |  |
| 3 |  |  |  |
| 4 |  |  |  |
| 5 |  |  |  |
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| 25 |  |  |  |

**Conclusion:**

Writing the lab report (due May 10) on the molecular mapping project

This report should summarize your results to date for the molecular mapping project. You should also include relevant results from other groups working on the same mutant. You should include the same sections as in the first lab report:

1. Introduction

Clearly describe the background and goal of the lab, i.e. how does molecular mapping work and why are we doing it?

2. Methods

Give an outline of the procedures followed. Describe the main steps in a procedure, e.g. “Prepared DNA from F3 seedlings” or “Prepared PCR with primers x & y”. However, for the detail of experimental procedures you can state “according to the procedures in the lab handout”. Do NOT copy the procedures word-for-word from the laboratory manual.

3. Results

Present and describe the results you obtained, even if negative. They can be given in the form of a table or figure. You may also wish to describe them in words.

DO include your gel photos. Analyze the gel photo: label the molecular weight markers and determine an approximate size for any bands visible on the gel.

Do NOT make this a narrative (“we tried this and got that, then we tried that and got this”). Condense the results if possible: there is no need to show every gel if all the results were similar.

4. Discussion

Interpret and explain your results: what do they mean? How do they represent progress towards the goals that you outlined in the introduction? What would the next step or steps be if you were to continue this research?

Be sure to include any needed references. They should be cited in the text after the information that comes from them, and then all the references should be summarized in a bibliography.

**Step 9: Refining the map position of the gene by testing additional markers**

After completing the previous steps, the class should now have identified the one or two markers from our original mapping set that are the most closely linked to the enhancer mutation. As the initial set of markers are spaced from 10 to 50 map units apart, it is unlikely that one of them is very closely linked to the enhancer gene, i.e. within a few map units. Our next goal is therefore to identify additional markers that are even more closely linked to this mutation. You will accomplish this by: 1. finding a number of markers in the region where your gene is located; and 2. testing them. Step 1, finding markers, is a new procedure and is described below. Step 2, testing the markers, is the same as you have already followed, i.e. performing PCRs with the markers using the template DNA from individual mutant F2 plants. The new markers will need to be tested with as many individual F3 DNA samples as possible.

##### Locating a marker’s exact position

An important first step is to identify the exact location of the marker or markers that you already found that appear to be linked to your gene. From Table 3A, you know the names and approximate locations of these markers. However, as you will discover if you use the *Arabidopsi*s database, there are different genetic maps of *Arabidopsis*, based on different techniques, and it is not always easy to figure out how the different maps correspond. The map positions in genetic map units given for the markers in Table 3A are based on the “Lister & Dean RI map”, which was developed based on classical genetic mapping techniques. Positions on this map are given in genetic map units (centiMorgans), and define a marker’s position relative to other markers. The position of each marker is also given by DNA sequence. DNA sequence positions are given in basepairs (bp) from the top of each chromosome, and therefore define an absolute position. To find this position, go to the Arabidopsis database (www.arabidopsis.org); under “Tools”, select “Seqviewer”. Copy and paste the sequence of one of the primers from the marker into the box under “Whole View Options”, select “Search by sequence”, and then select “Submit”. In the Web page that appears with the results, under the red type at the top, click on “SELECT”, and select “1” to see the view of the sequence. The sequence corresponding to the primer will be highlighted. The nucleotide numbers are given at the left side of the display. This should give you the absolute position for the marker.

##### Identifying useful markers

There are two ways to identify new molecular markers in the region of interest:

1. Search the database of existing markers.

2. Create a new marker based on known polymorphisms.

##### Searching the database of existing markers

1. Go to TAIR ([www.arabidopsis.org](http://www.arabidopsis.org)).

2. Click on “Markers” under “Advanced Search.”

3. Set the search parameters to restrict your search. Suggested restrictions are:

* “SSLP” marker
* on the chromosome where your gene maps
* “Polymorphic Between Ecotype Columbia and Ecotype Landsberg”.

4. Examine the resulting output and find markers that are in the region of interest. Choose four based on ease of use and even distribution across the region (the instructor will discuss choosing markers). Submit electronically to the instructor a list of these markers. The list must include:

your name & the date

the marker name

the marker location

the sequence of the primers for the marker

Creating a new marker based on known polymorphisms

Techniques are now available whereby essentially any polymorphism can be converted into an easy-to-use molecular marker. While it is likely that already-created markers will be adequate for our purposes in this course, a brief overview of different molecular markers is given here so you can understand the range of resources available.

**SSLP: simple sequence length polymorphism**  (for definition, see lab introduction)

To convert to a marker: design primers on either side of the simple sequence repeats. These primers should amplify products of different sizes in the two different ecotypes. Primers can be designed using a number of freely-available programs, for example “Primer3”.

**SNP: single nucleotide polymorphism** (a single basepair change)

To convert to a marker:

a. If the SNP changes a restriction enzyme recognition site, simply design primers that amplify the sequence including the SNP. One allele will have the enzyme recognition site, and so will be cut by the restriction enzyme, and the other allele will lack the recognition site and so will not be cut. To detect which allele is present, one amplifies the product, digests the product with the enzyme, and then separates on a gel. This type of marker is called “**CAPS**” (cleaved amplified polymorphic sequence).

b. If the SNP does not change a restriction enzyme recognition site, there are two options:

i. **dCAPS** (“derived CAPS”): uses primers that incorporate small sequence changes so that, together with the SNP, a restriction enzyme recognition site is created in one allele but not in the other. To detect which allele is present, the procedure is the same as for a CAPS marker (see above). A program is available on the Internet to help design dCAPS primers.

ii. **allele-specific primers**: uses primers that incorporate small sequence changes so that, under the right conditions and together with the SNP, one pair of primers will only amplify one allele and a similar but slightly different pair of primers will only amplify the other allele. To detect which allele is present, two PCRs are run for each template DNA, one with the pair of primers specific for one allele (e.g. Columbia) and one with the pair of primers specific for the other allele (e.g. Landsberg). Only gel electrophoresis is needed, not restriction digestion, to identify the outcome of the analysis. A program is available on the Internet to help design allele-specific primers for *Arabidopsis*.

The instructor will discuss how to design new SSLP primers for a given region if we are able to get to this step.**PCR #4**

**Marker tested:\_\_\_\_\_\_ Date tested:\_\_\_\_\_\_\_\_\_**

Table 3M. Details of 4th Set of Polymerase Chain Reactions Tubes

|  |  |  |  |
| --- | --- | --- | --- |
| Tube | DNA template | vol. template DNA | Vol. master mix |
| 1 |  | 1 |  |
| 2 |  | 1 |  |
| 3 |  | 1 |  |
| 4 |  | 1 |  |
| 5 |  | 1 |  |
| 6 |  | 1 |  |
| 7 |  | 1 |  |
| 8 |  | 1 |  |
| 9 |  | 1 |  |
| 10 |  | 1 |  |
| 11 |  | 1 |  |
| 12 |  | 1 |  |
| 13 |  | 1 |  |
| 14 |  | 1 |  |
| 15 |  | 1 |  |
| 16 |  | 1 |  |
| 17 |  | 1 |  |
| 18 |  | 1 |  |
| 19 |  | 1 |  |
| 20 |  | 1 |  |
| 21 |  | 1 |  |
| 22 | wtCol | 1 |  |
| 23 | wtLer | 1 |  |
| 24 | *axr4-2* | 1 |  |
| 25 | enhancer | 1 |  |

Table 3N. Master mix for 3rd PCRs.

|  |  |  |  |
| --- | --- | --- | --- |
| **Column 1** | **Column 2** | **Column 3** | **Column 4** |
| **Reagent** | **Vol. of stock for 1 reaction** | **# of reactions + 2** | **Vol. of stock to add to master mix** |
| water |  |  |  |
| 2 mM dNTPs |  |  |  |
| 10 mM MgCl2 |  |  |  |
| 10X buffer |  |  |  |
| 12.5 μM primers |  |  |  |
| *Taq* polymerase |  |  |  |
| Sum: Vol. to add to each tube |  | xxxxxx | xxxxxx |

Order of loading of samples:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Date of electrophoresis:\_\_\_\_\_\_\_\_\_\_ Type of gel:\_\_\_\_\_\_\_\_\_\_ Buffer:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Voltage\_\_\_\_\_\_\_\_\_\_ Time started\_\_\_\_\_\_\_\_\_\_\_ Time stopped\_\_\_\_\_\_\_\_\_\_\_

Gel photo:

Table 3O. Results of 4th PCR and Gel Electrophoresis for Marker \_\_\_\_\_\_\_

Predicted sizes: Col\_\_\_\_\_\_, Ler\_\_\_\_\_\_\_

|  |  |  |  |
| --- | --- | --- | --- |
| Tube | DNA template | Size of bands | Alleles detected (CC, CL, or LL) |
| 1 |  |  |  |
| 2 |  |  |  |
| 3 |  |  |  |
| 4 |  |  |  |
| 5 |  |  |  |
| 6 |  |  |  |
| 7 |  |  |  |
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**Conclusion:**

**Step 10: Re-test the phenotype of F3 progeny from individual F2 plants to verify the genotype and phenotype.**

In order for our mapping to be accurate, we must be completely sure that all the plants in our mapping population have the desired genotype and phenotype (i.e. that of the least frequent class). Classifying a phenotype based on just a single F2 seedling, as we did initially, is liable to error, so we would like to verify that the F2 phenotypes were correctly scored. Remember that the F2 plants were allowed to grow up and self-fertilize to produce F3 seeds. The F3 seeds produced by a single F2 plant make up an “F3 family”.

By testing the F3 families derived from our F2 individuals, we will be able to examine multiple plants that should be identical and thereby score the phenotype with much more certainty. If the F2 seedlings were properly selected and were homozygous, all the F3 seedlings from the same F2 plant, i.e. one F3 family, should also have this phenotype. Ideally we would re-test all of our F3 seedlings, but it is most important to re-test any that are especially significant, e.g. that appear to have a crossover close to the location of the enhancer gene.

To re-test the F3 seedlings, you will harvest the seeds, sterilize them, and plate them on high auxin (4 x 10­-7 M 2,4-D) as you did for Experiment 2. After 10 days you will determine if all the seedlings in a given family have enhanced auxin resistant root growth (are all the seedlings long?).

Outline of procedure

**1. Seed harvesting**

a. Remove a dried plant from the pot or tray, getting rid of as much of the dirt as possible.

b. Hold the plant over 2 thicknesses of nylon mesh on top of 2 pieces of clean paper. Rub the plant between your hands to break open all the seed pods (siliques), letting the seed and chaff (the dried stems, seed pod walls etc.) fall onto the nylon mesh.

c. Shake the nylon mesh gently to let the seed fall through to the paper. Some of the chaff will fall through also.

d. Discard the chaff that has accumulated on top of the nylon mesh.

e. Pour the seed through the mesh again, with the second sheet of paper underneath. Again shake the mesh.

f. Pour the seed on the paper into a labeled seed envelope. Discard the chaff.

**2. Seed sterilization and plating**

a. If you are not given tubes of seed ready to sterilize, then you will need to carefully pour the seed from the packets into labeled tubes. You should sterilize the following genotypes of seed: wild-type Landsberg *erecta, axr4-2 gl1,* the enhancer mutant seed (backcrossed), and the F3 seed to be re-tested from a cross between the enhancer mutant and Ler.

b. Then, follow the protocol given for seed sterilization on the first day of lab. You and your partner should each sterilize some of the tubes of seeds.

## During the sterilization procedure, label the plates with your initials, the cross (e.g. “FE61.1.1, 1X x Ler”),, and the proper number of the F3 family that you spread on it. For example, F3 family #20 must go on the plate labeled “F3 #20”.

## c. Spread the seeds on plates that contain 4 x 10-7 M 2,4-D (“4D” plates) in a nutrient agar solution. The technique for spreading seeds is described at the end of the seed sterilization protocol.

## d. Wrap your & your partner’s plates with plastic wrap and place them in the refrigerator (NOT the freezer!), labeled for easy identification.

## e. Arrange with your partner, the instructor, or the TA so that the plates get transferred to the growth chamber one day before the next lab class. Consult with the TA or instructor if this schedule poses a problem.

Notes on today’s procedure

**3. Analysis of the F3 seedlings**

Materials and equipment needed

## Plates of F3 and control seedlings

Forceps

“Blank” plates

Ruler

## **Analyzing the F3 seedlings**

1. First you must determine the root length of the control wild-type and mutant seedlings.

Growth medium used:\_\_\_\_\_\_\_\_\_\_\_\_ Age of seedlings:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Table 10A. Root lengths of Landsberg seedlings, in mm

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  |  |  |  |  |

Average =

Range =

Table 10B. Root lengths of *axr4-2 gl1* seedlings, in mm

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  |  |  |  |  |

Average =

Range =

2. Repeat the above analysis for the mutant seedlings. Mutant\_\_\_\_\_\_\_\_ Generation\_\_\_\_\_

Table 10C. Root lengths of mutant seedlings, in mm

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  |  |  |  |  |

Average =

Range =

3. Analyze the F3 seedlings. Your goal is to determine which F2 individuals’ results can be included in our analysis. To be worth using, the F3 seedlings must meet two criteria: 1. All the seedlings in a plate should have the same phenotype; and 2. The seedlings’ phenotype must match the desired phenotype, either enhanced auxin resistance, i.e significantly longer roots than *axr4* (for a recessive enhancer) or *axr4-*like (for a dominant enhancer).

a. Pull out 12 seedlings from each plate and line them up on a blank plate.

b. If they are not similar in length—if you see two or more distinct size classes—then the family is not homozygous. Make a note of your results in the table below.

If the seedlings in a family are all similar in length, then measure and record the lengths as shown in the example below.

Table 10D. Results of F3 Analysis

| F3 family # | Root lengths of individual seedlings (mm) | Ave. | Conclusion |
| --- | --- | --- | --- |
| example | 12, 11, 13, 11, 13, 14, 12, 13, 11, 12, 12, 11 | 12 | homozygousmutant—use  |
|  |  |  |  |
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4. Before proceeding, show your plates of F3 seedlings to the instructor so that he can verify you scored the seedlings correctly. Instructor’s initials:\_\_\_\_\_\_\_\_\_

5. You also collect additional tissue for more DNA preparation if necessary. Collect seedlings as follows:

a. Label one tube for each family, with the mutant and the family number. Be sure to use the tubes designated “For tissue collection.” Label on both the side and lid. Also label one tube for Ler and one tube for the FE backcrossed line.

b. Use forceps to remove F3 seedlings from the plate. If there are any seedlings that look abnormal (there might, for example, be a rare wild-type contaminant), don’t collect them. Blot the seedlings gently on a Kimwipe to remove any agar that is stuck to them. Then place the seedlings into the tube. Collect about 3 seedlings, if possible, from each family.

c. Place the tubes in the designated rack. They will be stored in the -80º Celsius freezer for storage until you prepare DNA from them.

Notes on today’s procedure

How easy was it to distinguish the seedlings with different root lengths? Was there any ambiguity in scoring the F3 families?

Final Molecular Mapping Report Due May 10

You will present the final results of your mapping in a final lab report, essentially an update and revision of your first lab report on the molecular mapping. Include the same sections as before.

In addition to the usual sections, in this lab report you should include the following:

* a Summary Table giving all the results obtained by the class for all the markers tested with the F2 DNA. This table should include the names and locations of the markers tested and the numbers of Col and Ler alleles detected in the F2 samples.
* If you tested any markers not in our original set, include the name, type, primer sequence, and other information in addition to the results
* a clear conclusion in which you should explain what these results indicate about the location of your gene.
* a one page summary of your Genetics Project. This summary should include the following:
* the mode of inheritance, and a brief summary of the evidence for this (Experiment 2)
* a brief summary (a graph + a one or two sentence statement) of the lateral root growth assay (Experiment 1)
* a brief summary of the results of your phenotypic characterization (i.e. the conclusion from your poster; Experiment 4)

# Summary Table for Molecular Mapping Results

Names:

Mutant:

|  |  |  |  |
| --- | --- | --- | --- |
| Marker Name | Marker Location | # of Col/# of Ler alleles | Conclusion: linkage or no linkage? |
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