Arabidopsis Gene Expression Experiment
(Labs 1, 2, 3) – GROUPS OF 2

Goals: 1. Use RT-PCR to characterize regulation of gene expression in plants in response to differing levels of light.
2. Use a mutant strain in an experiment to identify what happens with the loss of a gene's function.

Background:

Plants are dependent for life on their ability to turn light energy into ATP by fixing carbon. Since plants are unable to move to new locations in response to changing light conditions, plant regulation of light is tightly linked to plant morphology. Normally, genes involved in a response to a signal are only expressed when a signal is either present or absent (but not both). Genetic screens are used to select for mutants that cannot respond correctly to a signal (e.g. light). In our experiment, regardless of the presence of light, these mutants generally will result in one of two phenotypes: 1) the response gene is always expressed
2) the response gene is never expressed
Genetic analyses are then applied to determine whether the mutation has resulted in a "gain-of-function allele" or a "loss-of-function allele", and whether the mutation acts in *cis* or *trans* to the regulated gene.

In this experiment you will see if a light-response mutant has altered expression levels of two light regulated genes that are chlorophyll a/b-binding proteins: CLAB (accession number NM_102733.2), and CHAB (accession number NM_101450.4). The expression levels of polyubiquitin 10 (accession number NM_116771.5), which is not light-regulated, will be used as our control.

Reference material on gene expression: Modern Genetic Analysis 2nd Edition**,** pages 419-440

The following articles will help you understand the background behind this experiment.

A review of signaling in plants. Chory J. and Wu D. (2001) Weaving the complex web of signal transduction. Plant Physiol. 1125:77-80.

Review paper Fankhauser C. and Chory J. (1997) Light control of plant development. Annu Rev Cell Dev Biol. 13:203-229.

The full paper Ausubel et al. (1989) Arabidopsis thaliana mutant that develops as a light-grown plant in the absence of light. Cell. 58:991-999.

Schedule:

Lab 1: - RNA preparation
- Pictures of plants
- Spec of RNA for quality/quantity

Lab 2: - agarose gel of RNA
- RT-PCR

Lab 3: - Polyacrylamide gel electrophoresis; gel documentation

MATERIALS/ LAB PREPARATION

Table of primer pairs

Gene primer name	Primer sequence	Product Size (bp)
CLAB Forward	TACCCCGGTGGCAGCTTCGACC	260
CLAB Reverse	GAAGCAAAATACAGATAAAACTTGG	
CHAB Forward	GATCCGTTGGGTCTCGCCGGG	274
CHAB Reverse	TGATGATCTTTTCCCAAATGTCAC	
Ubiquitin Forward	TCTCATCTTCGCTGGAAAGC	182
Ubiquitin Reverse	CCCCAAAACACAAACCACCA	

Arabidopsis growth plates

1 litre of media makes ~30-35 plates. The Petri dishes are taller (100X20) than normal to allow for plant growth on the medium.

Media (1L)

4.3 g Murashige&Skoog salt mixture (1 container/packet)
10g Sucrose
800 ml H20

When sucrose and salts have dissolved, adjust pH to 5.7 using 1N KOH. Make up the volume to 1L.

Add 8g Agar and autoclave.

When temperature of media cools to approximately 50 degrees C, add 10ml 100X B5 vitamin mix (stored in aliquots in freezer).

Pour plates and let plates dry for several hours or overnight. It is preferred to use plates once they are made as opposed to storing since it can result in growth of contaminants over time.

100X B5 vitamin mix (500ml)

5.0g myoinositol
0.05g nicotinic acid
0.05g pyridoxine HCl
0.5g thiamine HCl

Filter-sterilize and dispense into aliquots in sterile tubes and freeze at –20C.

Surface sterilization of seeds

200mg of seed = approx. 10 000 seeds

-Imbibe seeds in a 1.5 mL tube with sterile water 0.1% Tween 20. First vortex a couple of times and then place on a shaker for 20 min. laying down
-Decant water, replace with 95% ethanol 0.1% Tween 20. Place 5 min. on shaker.
-Decant ethanol, replace with 1.6% w/v sodium hypochlorite (bleach) 0.1% Tween 20. Place on shaker for 10 min. (Remember to check what the starting concentration of sodium hypochlorite is in your stock bleach prior to diluting).
-Decant hypochlorite solution, wash 5 times with 1 mL of sterile water
-Decant water. Plate seeds with a p1000 micropipettor (may need to add small amounts of sterile water to make it easier to pipette. Plate 50-100 seeds per plate (or whatever you find ideal)
-Spread seeds around plate using a small sterile pipette tip.
-Wrap plates in parafilm and poke a couple of holes in parafilm to allow for air circulation.

Growth conditions of plants

There will be two growth conditions for two genotypes.

1: wild-type grown in light
2: *det1* mutants grown in light
3: wild-type grown in the dark
4: *det1* mutants grown in the dark

To grow the plants in light conditions grow under a cool florescence light (a florescence desk lamp should be fine). Grow under light 24 hrs/day.

For dark-adapted plates: First place under light until germination occurs (2-3 days). Then, to grow the plants in dark conditions wrap groups of plates in aluminum foil (loosely to allow some air flow), such that no light can get into the plates. Place wrapped plates into a drawer. Grow plants for 2-3 weeks before needed in lab.

PROTOCOLS FOR GENE EXPRESSION EXPERIMENT
GROUPS OF 2

Lab 1: RNA isolation, Pictures of plants, spec of RNA

You will be extracting RNA from 4 samples:

1. wild-type grown in the light
2. wild-type grown in the dark
3. mutant grown in the light
4. mutant grown in the dark

Pictures of plants:

1.) Pick seedlings from the four plates.
2.) Arrange seedlings in a petri dish (separated into four quadrants drawn on with a marker).
3.) Using the digital camera provided, take a digital image of the seedlings, making sure your group # is visible in the photo.
4.) Record a description of the plant's appearance in your lab notebook.

RNA isolation:

1.) Using tweezers pick your seedlings (approximately 50 mg of tissue).
2.) Place seedlings in a 1.5 ml homogenization tube that has been immersed in liquid nitrogen. Add some liquid nitrogen.
3.) Let sit for 30 seconds and then briefly crush the tissue with the homogenizer pestle that you have dipped in liquid nitrogen.
4.) Add 500 ul of Trizol reagent. Trizol is a very harmful substance: **BE CAREFUL**.
5.) Homogenize the tissue for 1 minute.
6.) Let stand for 5 minutes at room temperature.
7.) Add 100 ul of chloroform, close lid tightly, and shake the tube for 15 seconds.
8.) Let stand for 2-3 minutes at room temperature.
9.) Centrifuge at top speed in a microcentrifuge for 10 minutes.
10.) Transfer the colourless upper phase (contains RNA) to a new labeled 1.5 ml microcentrifuge tube. Take care not to get any of the interphase (contains DNA).
11.) Add 250 ul of isopropanol to the transferred upper phase.
12.) Let stand at room temperature for 10 minutes.
13.) Place on ice for 3 minutes.
14.) Centrifuge for 10 minutes at top speed in a microcentrifuge. **BALANCE THE TUBES.**
15.) Remove and discard the supernatant and retain the pellet.
16.) To wash the pellet: Add 500□ ul of 75% Ethanol and vortex to resuspend the pellet.
17.) Place on ice for 3 minutes.
18.) Centrifuge for 5 minutes at top speed in a microcentrifuge.
19.) Remove and discard the ethanol.
20.) Let air dry for 10 minutes.
21.) Add 25 ul of DEPC treated water.
22.) Incubate at 45°C for 10 minutes.

Testing RNA concentration:

1.) **Student A** will process RNA samples 1 & 2, while **Student B** will process RNA samples 3 & 4.
2.) Label a 1.5uL eppendorf tube for each RNA sample.
3.) Add 99uL of DEPC water to each tube.
4.) Add 1uL of RNA to the appropriately labeled tube (i.e. RNA sample 1 to tube 1, etc.). Place the original tube of RNA (the remainder of your RNA) in the freezer.
5.) Mix the DEPC/RNA solution (diluted RNA) by gently pipetting the solution up & down.
6.) Label disposable cuvettes with your sample number.
7.) Transfer your diluted RNA into the appropriately labeled cuvette (i.e. diluted RNA sample 1 in cuvette 1, etc.).
8.) With the TAs assistance, quantify your RNA with the Biophotometer.

TABLE 1

RNA Sample	**Biophotomoeter Reading (ug/ul)**	**Volume needed for 1ug RNA** Needed for Lab 2 cDNA synthesis. Note maximum of 9ul.
1		
2		
3		
4		

Lab 2: RT-PCR

Agarose gel of RNA

1) **Student A** will Transfer a 5 uL aliquot of each RNA prep to a clean, labeled 1.5mL tube. Add 5 uL of 5X sample buffer to the aliquot. **DO NOT ADD BUFFER TO YOUR ENTIRE SAMPLE!**
2.) **Student B** will make an agarose gel and load the samples (in order: ladder, samples 1, 2, 3, 4) and **Student A** will take a picture of the gel. **Save the file with your group # and "RNA"**

Reverse transcription of mRNA (done as a group):

1) Set up the following 4 reactions (TALBE 2) in 1.5 ml microcentrifuge tubes. Remember to fill in the table with the correct volumes of RNA (from TABLE 1) and water – all of the boxes marked with "?".

TABLE 2

Component	1	2	3	4
1 ug total RNA from wild-type grown in light	?			
1 ug total RNA from wild-type grown in dark		?		
1 ug total RNA from mutant grown in light			?	
1 ug total RNA from mutant grown in dark				?
Water (9 ul minus volume of RNA added)	?	?	?	?
dNTPs	2 ul	2 ul	2 ul	2 ul
oligo(dT)12-18 primer	2 ul	2 ul	2 ul	2 ul
Total volume	**13 ul**	**13 ul**	**13 ul**	**13 ul**

2) To melt secondary structure: incubate the tubes at 70°C for 5 minutes, and chill on ice.
3) Spin the tubes in a microcentrifuge for 20 seconds.
4) Make a Master Mix (fill in the table with the correct volumes) and add the correct volume of Mix to each of the four reaction tubes.

TABLE 3

cDNA Synthesis Components	Volume	Master Mix (x_____)
5X 1st strand buffer	4 ul	
0.1 DTT	2 ul	
M-MLV reverse transtriptase	1 ul	
Total volume	**7 ul**	

Note that the final volume in each tube is now 20ul (13ul from Table 2 plus 7 ul from Table 3).

5) Mix gently by pipetting up and down.
6) Incubate in a 37 °C waterbath for 50 minutes.
7) To stop the reaction: incubate the reactions at 70°C for 15 minutes, and place tubes on ice.

At this point you will have made the first stand of a cDNA copy of the mRNA population. The next step is to PCR amplify a specific template from this cDNA copy of an mRNA population.

Multiplex PCR amplification (done individually).
TABLE 4 lists the reagents needed to perform RT-PCR:

TABLE 4

Component	Tube 1 (ul)	Tube 2 (ul)	Tube 3 (ul)	Tube 4 (ul)	Tube 5 (ul)	Tube 6 (ul)	Tube 7 (ul)	Tube 8 (ul)
cDNA from wildtype grown in light	2	-	-	-	2	-	-	-
cDNA from wildtype grown in dark	-	2	-	-	-	2	-	-
cDNA from mutant grown in dark	-	-	2	-	-	-	2	-
cDNA from mutant grown in dark	-	-	-	2	-	-	-	2
Primer Ubiquitin Forward	1	1	1	1	1	1	1	1
Primer Ubiquitin Reverse	1	1	1	1	1	1	1	1
Primer CLAB Forward	1	1	1	1	-	-	-	-
Primer CLAB Reverse	1	1	1	1	-	-	-	-
Primer CHAB Forward	-	-	-	-	1	1	1	1
Primer CHAB Reverse	-	-	-	-	1	1	1	1
10X Genscript PCR buffer (contains $MgCl_2$)	5	5	5	5	5	5	5	5
5mM dNTP	1	1	1	1	1	1	1	1
Water	37.8	37.8	37.8	37.8	37.8	37.8	37.8	37.8
Taq DNA Polymerase	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Total Volume	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**

8) **Student A** amplifies CHAB; **Student B** amplifies CLAB. **Draw a table** (**TABLE 5**) for your Master Mix calculations.

9) Set up the reactions in 200 ul PCR tubes. Add the appropriate volume of Master Mix to each tube, then your cDNA. Check that the final volume is 50ul.

10) Give to the TA to start the PCR reaction. 28 cycles: 94°C for 30sec
60°C for 1 minute
73°C for 45 sec

11) Store PCR reactions in the freezer.

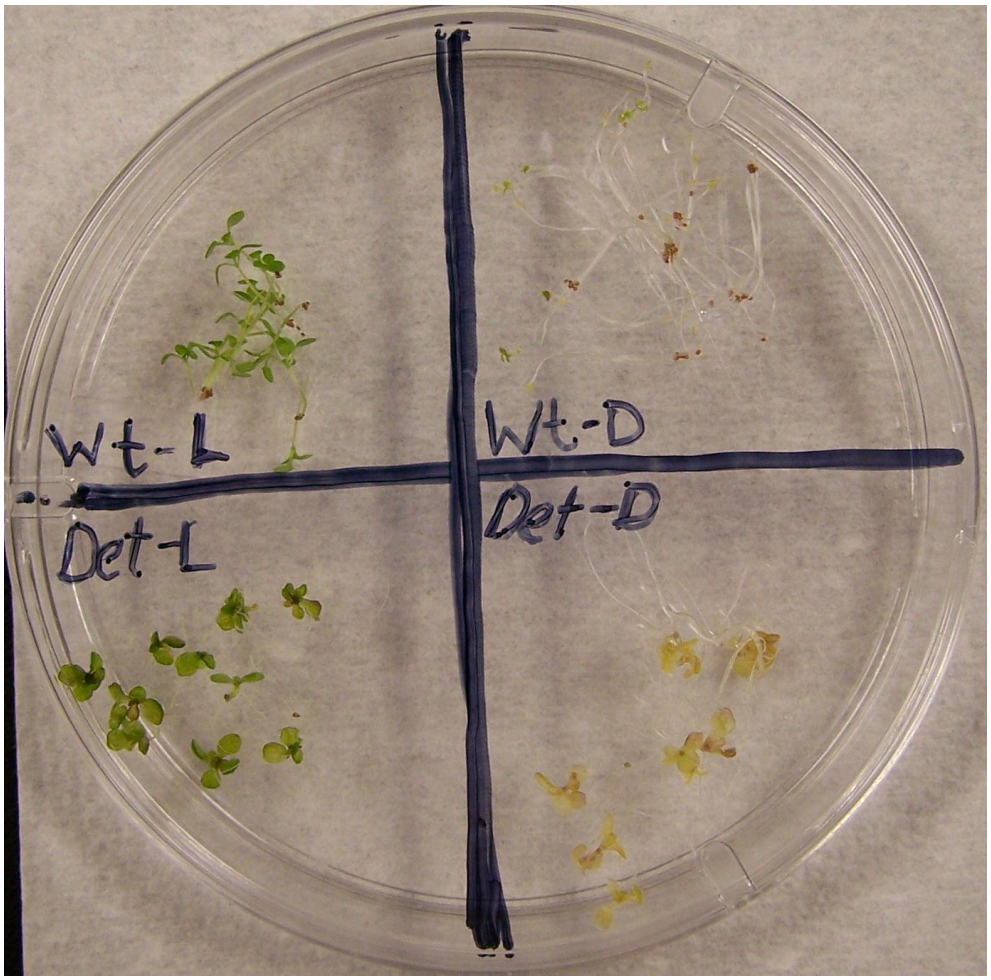
Lab 3: Polyacrylamide gel electrophoresis of RT-PCR samples
1) **Student B** will Set up an 6% polyacrylamide gel with 15 wells.

2) Each student will load their own samples from **Lab 2**. Add 13 ul of 5X loading dye buffer to each RT-PCR reaction and load 20 ul on gel. **Student A** will load CHAB/Ubiquitin in lanes 1-4. **Student B** will load the ladder in lane 5 and then load CLAB/Ubiquitin in lanes 6-9.

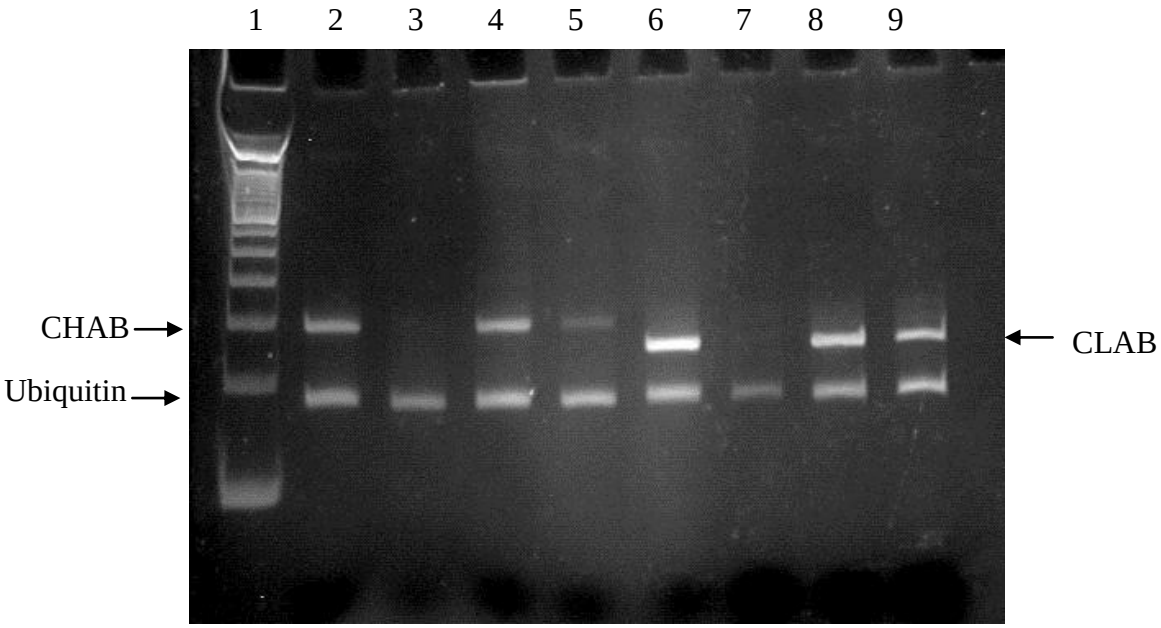
3) **Student B** will run the gel at 100 volts, stain with SYBR Safe for 10 minutes, and then image the gel. **Save the file as a .tif with your group # and "RT"**

Sample picture of wild-type and det1 mutant plants grown in the presence or absence of light.

Wt-L = wild-type grown in light
Wt-D = wild-type grown in dark
Det-L = det1 mutant grown in light
Det-D = det1 mutant grown in dark



Sample gel depicting results of RT-PCR.



Legend:

1 – 100 bp ladder
2 – wild-type light (CHAB)
3 – wild-type dark (CHAB)
4 – det1 light (CHAB)
5 – det1 dark (CHAB)
6 – wild-type light (CLAB)
7 – wild-type dark (CLAB)
8 – det1 light (CLAB)
9 – det1 dark (CLAB)