EXPERIMENT 3 - IDENTIFYING FEATURES OF MUTANT SEEDS USING NOMARSKI MICROSCOPY (GENE ONE)

STRATEGY

I. OBSERVATION OF SEEDS USING LIGHT MICROSCOPY AND FIXING SEEDS FOR OBSERVATION WITH NOMARSKI OPTICS

II. OBSERVATION OF SEEDS AND EMBRYOS USING NOMARSKI OPTICS

III. OBSERVATION OF THE MATURE PLANT PHENOTYPE

I. Observation of Seeds Using Light Microscopy and Fixing Seeds for Observation with Nomarski Optics

Purpose: To introduce the **D**ifferential **I**nterference **C**ontrast (**DIC**) or **N**omarski **I**nterference **C**ontrast (**NIC**) microscopy technique as a tool to identify features of defective embryos in knockout mutants.

Reference: The protocol was written by Dr. Miguel Aguilar in Professor Robert L. Fischer's laboratory at University of California, Berkeley.

Materials Needed:

- Siliques containing seeds with a wide range of embryo stages (globular to mature green) from *Arabidopsis*
    a. wild type
    b. homozygote or heterozygote mutant
- 100% ethanol
- Acetic acid
- Sterile water
- Chloral Hydrate (Cat. #C8383, Sigma-Aldrich; should be fresh)
- Glycerol (Invitrogen)
- Double-distilled water

Materials Needed:

- Pipettes
- Pipette tips (regular, non-filter)
- 1.5 mL microcentrifuge tubes
- Microcentrifuge tube rack
- Black ultra-fine sharpie
- Rulers with METRIC scale (mm)
- Plant layout chart
- Phenotype observation record
- Fine point forceps
- 30-gauge hypodermic needles
- Fine-point scissors or razor blades
- Coverslips
- Microscope Slides
- Double-sided tape
- Dissecting microscopes (borrowed from Dr. Pei Yun Lee)
- A microscope equipped with Nomarski optical parameter (Leica CTR5000)
- Microscope camera

PROCEDURE

Each student collects the following from wild type and his/her homozygous or heterozygous mutant:

a) 5 siliques containing seeds with embryo stages of globular to torpedo.

b) 2 siliques containing seeds with mature green embryos.

Note: *Be sure to collect a wide* ***range*** *of stages. Do not collect yellow or brown siliques; these contain dry seeds.*

1. Prepare 5 mL of a fixative solution of ethanol: acetic acid (9:1, v/v) in a 14 mL centrifuge tube using disposable 5 mL pipets.

FIXATIVE SOLUTION	
100% ethanol	4.5 mL
Acetic acid	0.5 mL
Total volume	5.0 mL

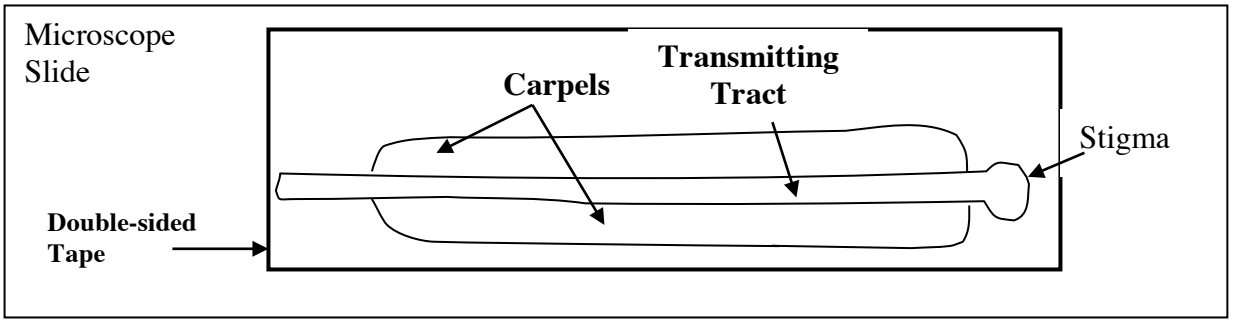
Tightly snap the cap on the tube. Make sure the cap clicks. **Invert** the tube to mix the contents.

2. Pipet **1 mL** of the **fixative solution** into **FOUR** 1.5 mL microcentrifuge tubes sitting on a microcentrifuge tube rack. Label each tube in step 2 with your **initials**, the **plant #** and the **plant genotype**. *These tubes will be used in step 5i.*
3. Bring the following materials to the Plant Growth Center (PGC).
   - Bucket of ice
   - FOURTEEN 1.5 mL microcentrifuge tubes
   - Microcentrifuge tube rack
   - Black ultra-fine sharpie
   - Ruler with METRIC scale (mm)
   - A pair of fine point forceps
   - Plant layout chart with information about plant number and the genotype of those plants
   - This protocol
   - Bruincard with access to the PGC
   - Key to growth chambers in the PGC

4. Measure and collect siliques according to the chart below. Place each silique in a 1.5 mL microcentrifuge tube. Write your **initials**, the **plant #**, the **plant genotype** and the **length** on the tube. Keep the tube on **ice**. *Note: Collect the **same length** of siliques for wild type and homozygous/heterozygous mutant so that you can compare them.*

Plant Genotype	**Seed Stages Collected**	**Length of Siliques Collected**	**Number of Siliques Collected**
Wild type	globular to torpedo	*0.5 - 1.0 cm*	5
Wild type	mature green	*1.0 - 1.9 cm*	2
Heterozygous or homozygous mutant	globular to torpedo	*0.5 - 1.0 cm*	5
Heterozygous or homozygous mutant	mature green	*1.0 - 1.9 cm*	2

5. Go back to the lab. **Dissect** the siliques and **observe** the seed phenotype using a dissecting microscope. *Note: Work quickly so the seeds don't dry out. You may also place a drop of water on the silique.*
    a. **Place** a **piece of double-sided tape** on a **microscope slide**. Label the microscope slide with a small piece of white tape with your **initials**, the **plant #**, the **plant genotype** and the **length**.
    b. Carefully, use **fine-point forceps** to place a silique on the tape.
    c. Under a dissecting microscope, use **fine-point forceps** to carefully **arrange** the silique such that the **transmitting tract** is facing you (see diagram below, NOT drawn to scale).



d. With your left hand, use forceps to hold the silique on the side closest to the stem.

e. With your right hand, use a **28G** or **30G hypodermic needle attached to a 1 cc syringe** to **slice** the **carpels along each side of the transmitting tract.**

f. **Gently** peel back the carpels and stick them to the tape to reveal the seeds.

g. Observe the phenotype. Note any phenotypes that you observe on your **Screening Seeds Using Light Microscopy** chart.

In what stage of development are the seeds?

How many seeds are in the silique?

How many are green?

How many are white?

How many are brown?

What is the expected ratio of wild type seeds to mutant seeds if the mutation is seed lethal?

What is the observed ratio of wild type seeds to mutant seeds?

Are the observed results significantly different from the expected results?

Use a Chi-Square test.

$$\chi^2 = \Sigma \frac{(observed - expected)^2}{expected}$$

Probability that the deviation is due to chance alone

Degrees of Freedom	0.5	0.1	0.05	0.02	0.01	0.001
1	0.455	2.706	3.841	5.412	6.635	10.827
2	1.386	4.605	5.991	7.824	9.210	13.815
3	2.366	6.251	7.815	9.837	11.345	16.268
4	3.357	7.779	9.488	11.668	13.277	18.465
5	4.351	9.235	11.070	13.388	15.086	20.517

What is your null hypothesis?

How many degrees of freedom are there?

(The degrees of freedom is one less than the number of different phenotypes possible.)

What is your chi-square value?

(The chi-square statistic is a probability that indicates the chance that, in repeated experiments, deviations from the expected would be as large or larger than the ones observed in this experiment)

What is the probability that the deviation of the observed values from the expected values was a chance occurrence?

(Look up your degrees of freedom in the table. Find where your chi-square value falls in that row.)

Can you reject the null hypothesis?

If the probability is less than 0.05 (5%), reject your null hypothesis. If the probability is 0.05 (5%) or greater, then you cannot reject your null hypothesis.

h. Ask your TA to take pictures of the seeds within the siliques.

i. **Before the seeds dry out**, use the fine-point forceps to transfer the cut silique into the tube with fixative solution from **step 2**.

j. Repeat steps a-i for the other siliques. *Note: You collected an excess of siliques so that you would have some to practice dissecting and to have a*

range of developmental stages for each genotype. However, you only need to fix FOUR siliques.

i. *Wild type, early development*
ii. *Heterozygous (or homozygous), early development*
iii. *Wild type, late development (mature green stage)*
iv. *Heterozygous (or homozygous), late development (mature green stage)*

6. Fix seeds and siliques in the fixative solution for at least 2 hours. *Note: It is recommended to fix the siliques* ***overnight*** *to ensure that the fixative solution penetrates the seeds and their embryos. It is okay to leave siliques in the fixative solution for up to 3 days.*
7. **Carefully**, pipet off **900 µL** of the fixative solution using a **P-1000** pipette and discard into a beaker labeled "acetic acid waste." Then remove the **remaining volume** with a **P-200** pipette. *Note: Do not let the seeds and siliques dry out, and do not pipet up your seeds.*
8. **Immediately**, pipet **1 mL** of **90% ethanol** solution into the tube using a P-1000 pipette. *Note: The 90% ethanol solution will remove chlorophyll from the embryos.*

90% ETHANOL SOLUTION

Absolute ethanol	4.5 mL
Double-distilled water	0.5 mL
Total volume	5.0 mL

9. Incubate seeds and siliques in the 90% ethanol solution for **0.5 - 1 hour**. *Note: It is safe to store the materials in the ethanol indefinitely.*
10. Replace the 90% ethanol solution with **70% ethanol** as in steps 7 & 8.

70% ETHANOL SOLUTION

Absolute ethanol	3.5 mL
Double-distilled water	1.5 mL
Total volume	5.0 mL

11. Incubate seeds and siliques in the ethanol solution for **0.5 - 1 hour**. *Note: It is safe to store the materials in the ethanol indefinitely.*

II. Observation of Seeds and Embryos Using Nomarski Optics

Note:

- ***Before observation of the seeds and their embryos, seeds must be submerged in the clearing solution. For young seeds, clearing is usually fast (~5 minutes). The older the silique, the longer it takes to clear (~ 1 hour). Seeds are ready for observation after they sink in the clearing solution.***
- ***Tissues CANNOT be stored in the CLEARING solution for more than TWO days because they will lose their structures quickly.***

1. Prepare a ***fresh*** clearing solution of chloral hydrate/glycerol/water (8:1:2, w/v/v) in a 14 mL centrifuge tube. *Note: The TA will prepare this solution before the lab class begins.*

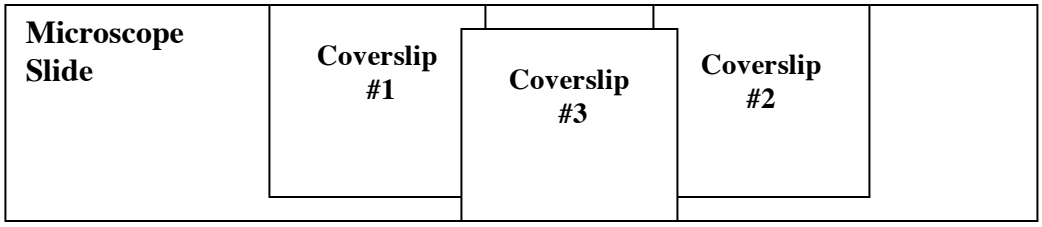
CLEARING SOLUTION

Chloral hydrate	8 g
Glycerol	1 mL
Water	2 mL
Total volume	~7 mL

2. **Carefully**, pipet off **900 µL** of the 70% ethanol solution using a **P-1000** pipette and discard into a beaker labeled "ethanol waste." Then remove the **remaining volume** with a **P-200** pipette. *Note: Do not let the seeds and siliques dry out, and do not pipet up your seeds.*
3. Replace the 70% ethanol solution with **100 µL of clearing solution**.
4. Incubate seeds and siliques in the clearing solution for **5 min - 1 hour**. Wait until the seeds **sink** to the bottom of the tubes. You may lay the tube on its side so that the

silique is immersed in the clearing solution. *Note: Tissues CANNOT be stored in the CLEARING solution*.

5. Set a new glass microscope slide on the bench. Label it with your **initials**, the **plant #**, the **plant genotype** and **silique length**.
6. Use forceps to remove a silique from the clearing solution and place it on the labeled glass slide.
7. Pipet the remaining clearing solution and seeds onto the slide with the silique.
8. Carefully, place two square coverslips, one on each side of the solution. Then, place a third coverslip over the clearing solution. Avoid trapping bubbles in the solution (see diagram below).



9. Observe the seeds under Nomarski optics using the Leica CTR5000 microscope.
10. Take pictures of the embryos.

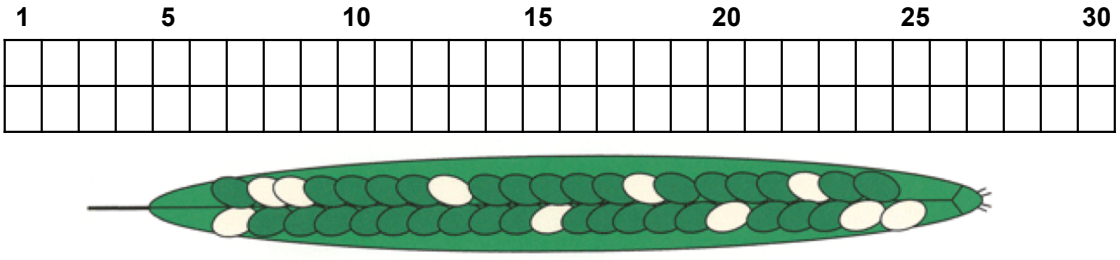
    In what stage of development are the seeds?
11. Repeat steps 2-10 for the remaining 3 fixed siliques.

Screening Seeds Using Light Microscopy

AGI# _____ SALK # _____ Plant #_____Genotype _____

Silique # _____ Length of Silique (cm) _____ Total Seeds _____ Total Mutant Seeds _____

<u>Instructions:</u> The grid represents the layout of the silique. Put a number in each square that corresponds to a mutant seed. Describe the seed phenotypes in the chart below. The base of the silique is defined as the region closest to the pedicel and main stem, which is at the left of the grid.



Seed	Seed Coat Color	Embryo Color	Notes
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			

III. Observation of the Mature Plant Phenotype

1. Observe T-DNA tagged plants for abnormal phenotypes. Write your observations on the **Phenotype Observation Record**. Take pictures of the plants to document the phenotype. Take pictures of the tags to identify the plants in the pictures. You may take flowers back to the lab to observe the phenotype under a microscope.

PHENOTYPE OBSERVATION RECORD

Gene ID: **At__ g _____ Salk line#: _____ Date: _____**

LEAF	**Mutant**	**Wild Type**
What do the leaves look like, green or yellow, elongated or round?		
What is the range of their length in cm?		
How many leaves does each plant have?		
Is the range of leaf sizes of the mutant plant smaller or larger or similar to wild type leaves?		

STEM	**Mutant**	**Wild Type**
What is the height of the main (or longest) stem?		
What is the thickness of the stem?		
How many stems (or branches including the main and side ones) does the plant have?		

FLOWERS	**Mutant**	**Wild Type**
Do the flowers have all FOUR floral organs (green sepals, white petals, yellow anthers, green pistils)?		
How many sepals are on each flower?		
How many petals are on each flower?		
How many anthers are on each flower?		
How many pistils are on each flower?		

SILIQUES, SEEDS AND EMBRYOS	**Mutant**	**Wild Type**
How many siliques are on each plant?		
Do you see a difference in the lengths of siliques?		
How many seeds are in EACH silique?		
What is the average number of seeds in FIVE siliques?		
Do you see different COLORED seeds within a single silique?		
If yes, what colors are the seeds? How many seeds of each color?		
What stage of embryos (globular, heart, torpedo, cotyledon, mature green, or post mature green) do you see?		