

## EXPERIMENT 8 - OBSERVATION AND CHARACTERIZATION OF KNOWN AND UNKNOWN

**Purpose:** To introduce Differential Interference Contrast (DIC) or Nomarski Interference Contrast (NIC) microscopy technique as a tool to identify features of defective embryos as illustrated in a T-DNA knockout mutant *dicer-like*, *lec1*, *raspberry3*, and *titan*.

**Reference:** The protocol was written by Dr. Miguel Aguilar in Professor Robert L. Fischer 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. T-DNA knockout mutant *raspberry3* or *titan* or *dicer-like* or *lec1*
  - c. homozygote or heterozygote mutant
- Absolute ethanol
- Acetic acid
- Chloral Hydrate (C-8383, Sigma-Aldrich)
- Glycerol (Invitrogen)
- Fine point forceps
- 30-gauge hypodermic needles
- Microcentrifuge tubes (1.5 mL)
- Microcentrifuge tube rack
- Disposable transfer pipets or Pasteur pipets
- Double-distilled water
- Pipetman P-200 and P-1000
- Pipette tips
- Rulers with METRIC scale (cm)
- Fine-point scissors or razor blades
- Coverslips
- Microscope Slides

### Equipment Needed:

- Dissecting microscopes (we got 10 extra dissecting microscopes from our Biology department storage)
- A microscope equipped with Normarski optical parameter (Leica CTR5000)

## PROCEDURE

*Each student collects the followings from Wild type, one of the known mutants, and his/her homozygous/heterozygous mutant:*

*(a) 5 siliques containing white seeds with embryo stages of globular to heart or torpedo. Note: silique length is in a range of 0.5 cm to 1.5 cm.*

*(b) all seeds from One silique containing mature green embryos.*

### A. Fixing Plant Materials

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

Absolute ethanol	4.5 mL
<u>Acetic acid</u>	<u>0.5 mL</u>
Total volume	5.0 mL

- Close the tube with its cap.
- Invert the tube to mix the content.

15. Pipet **0.75 mL** of the **fixative solution** into **SIX** 1.5 mL microcentrifuge tubes sitting on the microcentrifuge rack at room temperature.

16. Label on the lid and side of each tube in step 2 with your initial, number (1-6), and information (mutant/wild type, siliques/seeds).

17. Label on the lids and sides of **THREE** microcentrifuge tubes with information (Wild type, known mutant, or your homozygote/heterozygote mutant). *Note: siliques with different lengths from wild type, known mutant, and homozygote/heterozygote mutant plants will be collected into each of these three tubes.*

18. Bring the **tubes in step 4** and the **Plant Chart** with information about plant number and the genotype of those plants to the Plant Growth Center (or greenhouse).

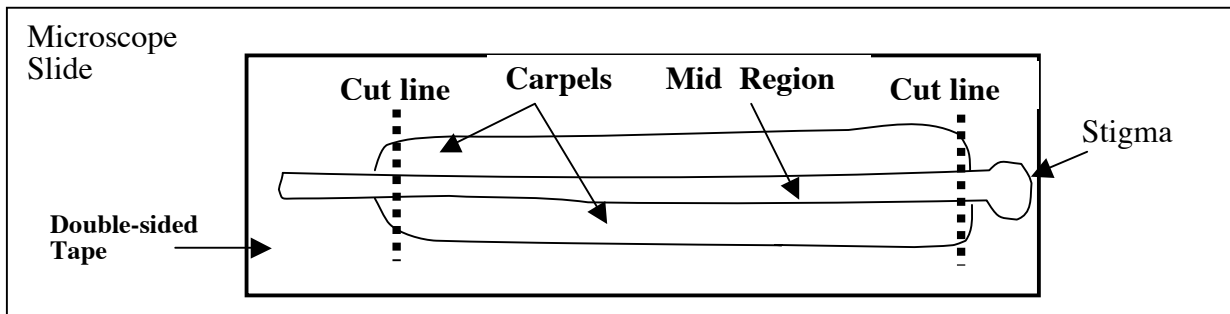
19. Measure and collect **3 siliques** for **each** of **two different lengths** (one short and one long) in a **range of 0.5-1.5 cm** from the wild type, a known T-DNA knockout (*raspberry 3*, *dicer-like*, *lec1*, or *titan*), and homozygous/heterozygous mutant.

*Note:* collect the **same length** of siliques for wild type, known mutant, and homozygous/heterozygous mutant.

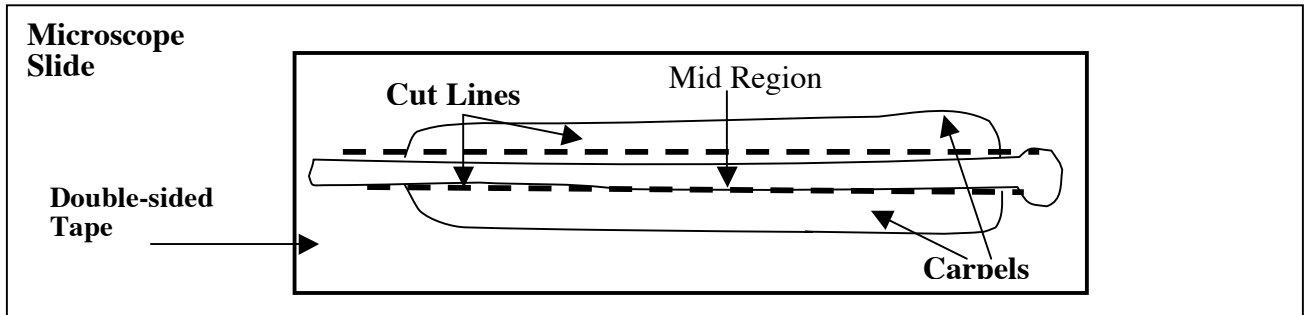
What are the genotype and phenotype of the mutant plants?

20. Collect **siliques** and **seeds** and put them in the fixative solution as follows:

- a. For **siliques with length < 0.7 cm**, Place a **piece of double-sided tape** on a **microscope slide** --> Carefully, use a **fine-point forceps** to place a silique on the tape --> **Excise the top and bottom of the silique** at its **ends of carpels** to allow solutions to penetrate the seeds and embryos during fixation, washing, and clearing steps (see a diagram below) --> **Immediately**, use the fine-point forceps to transfer the cut silique into the Fixative solution. Repeat the cutting of siliques for other siliques.



- b. For **siliques with length > 0.7 cm**, including those containing seeds with mature green embryos, **dissect** the seeds out of the siliques as illustrated below (NOT drawn to scale): Place a **piece of double-sided tape** on a **microscope slide** --> Carefully, use a **fine-point forceps** to place a **silique** on the tape and **rearrange** the silique such that the **mid region** is facing you --> Use a **28G or 30G hypodermic needle attached to a 1cc syringe** to **slice the carpels** along the **mid region** as shown in the diagram, then Use the fine-point forceps to **tear off the carpels** to expose the seeds --> Use either the forceps or the needle to **collect and transfer seeds** into the **Fixative solution**.



21. Fix seeds and siliques in the fixative solution for 2 hours to overnight.

*Note: It is recommended to fix the seeds from siliques with length > 0.7 cm **overnight** to ensure that fixative solution penetrates the seeds and their embryos.*

22. Next day, CAREFULLY pipet off **650  $\mu$ L** of the fixative solution using a **P-1000** pipetman and then the **remaining volume** with a **P-200** pipetman. *Note: Do not let the seeds and siliques dried out.*

23. **Immediately**, pipet 0.5 mL of 90% ethanol solution into the tube using a P-1000 pipetman. *The 90% ethanol solution will remove chlorophyll from the embryos.*

**90% ETHANOL SOLUTION**

Absolute ethanol	4.5 mL
<u>Double-distilled water</u>	<u>0.5 mL</u>
Total volume	5.0 mL

24. Incubate seeds and siliques in the ethanol solution for 0.5 - 1 hour.

*Note: It is safe to leave the materials in the ethanol solution longer than 1 hour (or indefinite).*

25. Replace the 90% ethanol solution with 70% ethanol similar to steps 9 & 10.

**70% ETHANOL SOLUTION**

Absolute ethanol	3.5 mL
<u>Double-distilled water</u>	<u>1.5 mL</u>
Total volume	5.0 mL

26. Incubate seeds and siliques in the ethanol solution for 0.5 - 1 hour.

*Note: it is safe to leave the materials in the ethanol solution longer than 1 hour (or indefinite).*

## B. Observation of Seeds and Embryos

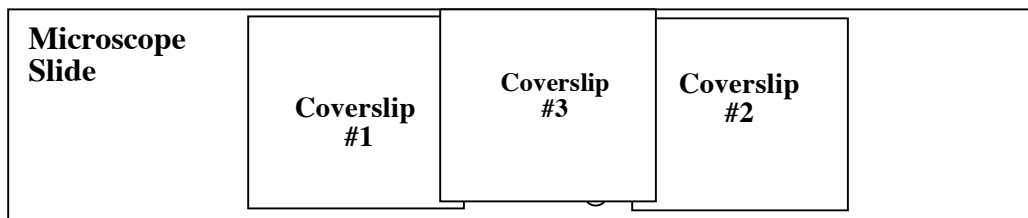
***Note:*** At least **ONE** hour before observation of the seeds and their embryos. Seeds must be submerged in the clearing solution. (a) The older the silique, the longer it takes to clear and sink. For young seeds, clearing is usually fast, i.e., seeds can be observed under a microscope within 30 minutes. (b) Tissues **CANNOT** be stored in the **CLEARING** solution for more than **TWO** days because they will lose their structures quickly.

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

### **CLEARING SOLUTION**

Chloral hydrate	8 g
Glycerol	1 mL
Water	<u>2 mL</u>
Total volume	3 mL

10. Replace the 70% ethanol solution with the CLEARING solution. Wait for 30-60 minutes or until the seeds and siliques **SINK** to the bottom of the tubes.
11. Set a new glass microscope slide on the bench.
12. Gently, pipet the clearing solution up and down for 5 times to mix up the seeds using a P-200 pipetman.
13. Dispense **100-150  $\mu\text{L}$**  of the clearing solution with seeds in the center of the microscope slide.
14. Carefully, place two squared coverslips, one on each side of the solution. Then, place a third coverslip over the clearing solution (see below)



15. Observe the seeds under Nomarski optic using Leica CTR5000 microscope.

16. Take pictures of the embryos.

***Note: For siliques, you need to dissect seeds out of the silique on a microscope slide under a dissecting microscope. Then, proceed to steps 5-8.***