Methods and Approaches Used to Study Knockout Arabidopsis Plants

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Methods of Gene Discovery

Conceptually there are two methods:

Forward Genetics: Hunt for observed mutations in the phenotype in the genome.

Reverse Genetics: Mutate a specific part of the genome and observe the effect on the phenotype.

Reverse Genetics

- Most genomes of model and commercially important organisms have been sequenced.
- Many genes, especially more complex regulatory sequences, produce no obvious phenotype or produce variable phenotypes.
- Advantages of forward genetics negligible in model organisms

Mechanisms of Reverse Genetics

- "Knockout" (KO) a gene and observe the resulting phenotype from its absence
- "Knockdown" by limiting its expression and observe the resulting phenotype
- Over-express a gene my modifying its regulatory sequences and observe the resulting phenotype

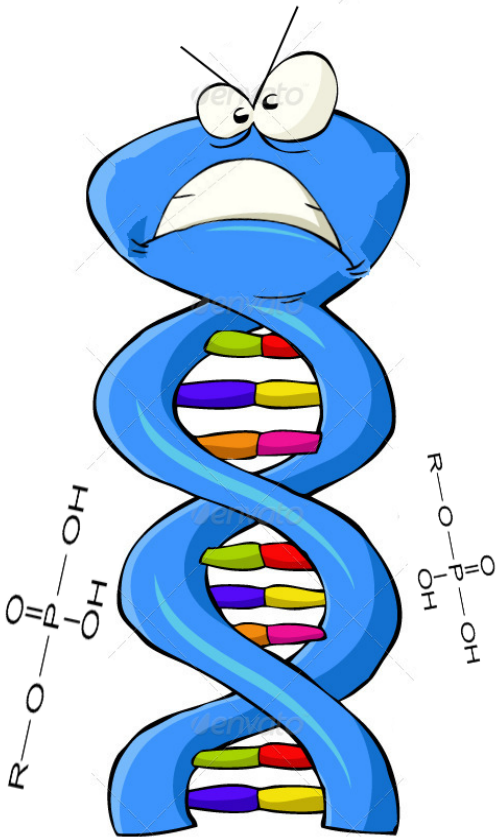
Overview of our Experiment

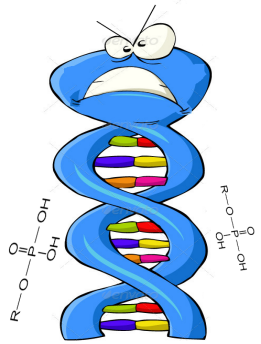
- *Arabidopsis Thaliana* are modified via a T-DNA from *Agrobactium.* T-DNA "knockout" a gene.
- Plants are screened for mutants using PCR primers specific to the T-DNA and the inhibited gene.
- Phenotype of mutant plants are compared to known wild-type plants.

The differences illuminate the gene's function!


Tris-EDTA Buffer
10 mL for Luciferin Substra
Expiry date Jan-2013

Gel electrophoresis





Materials

- Agarose powder
  - Many pores; more powder=more accurate
- Tris Acetate EDTA buffer
  - pH 8.0, cations
- SYBR Safe
  - interchelates with DNA, fluoresces under UV light
- Loading dye
  - Weighs down DNA solution
- Ladder

Spectophotometry

- DNA exposed to UV light---->DNA absorbs UV light---->Less light is measured by photo-detector

Why does DNA absorb UV light?

- Structure of purines and pyrimidines makes them absorb photons

Beer-Lambert Law

- How do we find the concentration of DNA from light absorbance?
- Beer-Lambert Law establishes a linear relationship between absorbance and concentration of material that absorbs
- Absorbance=epsilon*path length*concentration
  - Path length=1.1 mm
- That's why we always start NanoDrop with TE buffer blank with known concentration

Bringing it all together

gDNA isolation is process of isolating genomic DNA from rest of plant

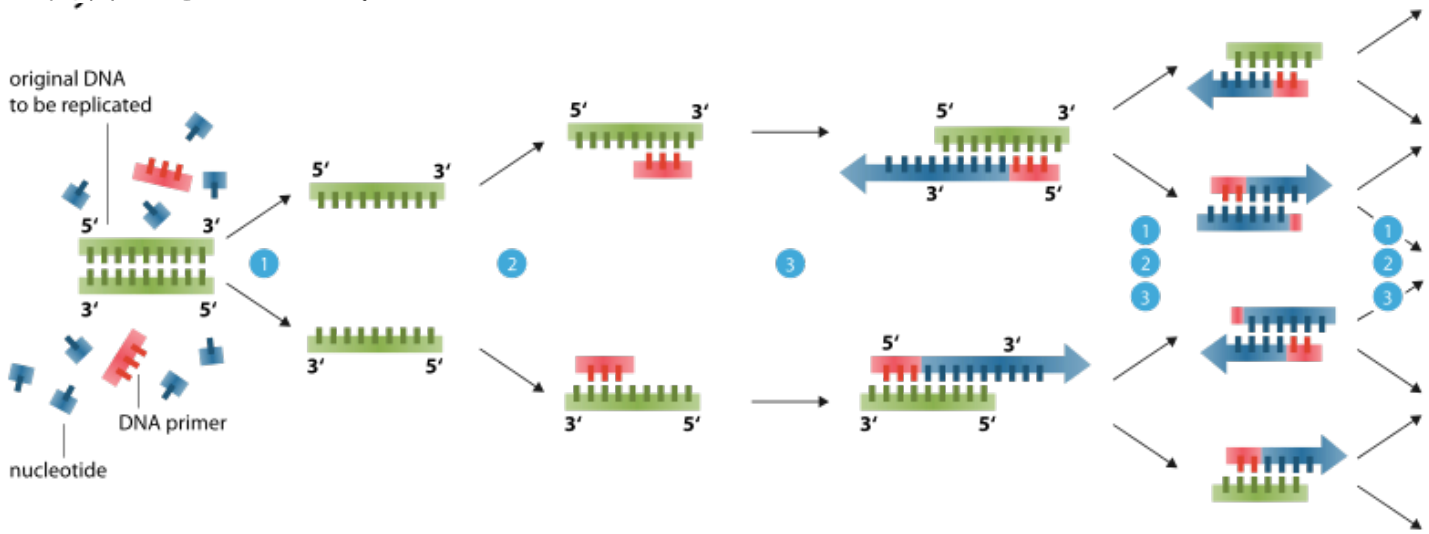
Gel electrophoresis and spectophometry are used to verify this process.

What's next?

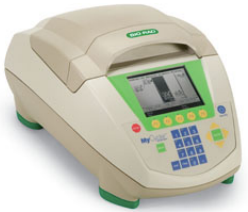
- How do we identify plants with the T-DNA?
- How do we determine the genotype of the plants?
- How do we determine where the T-DNA is inserted?
- How do we know where the gene is expressed?

PCR

- What is PCR?

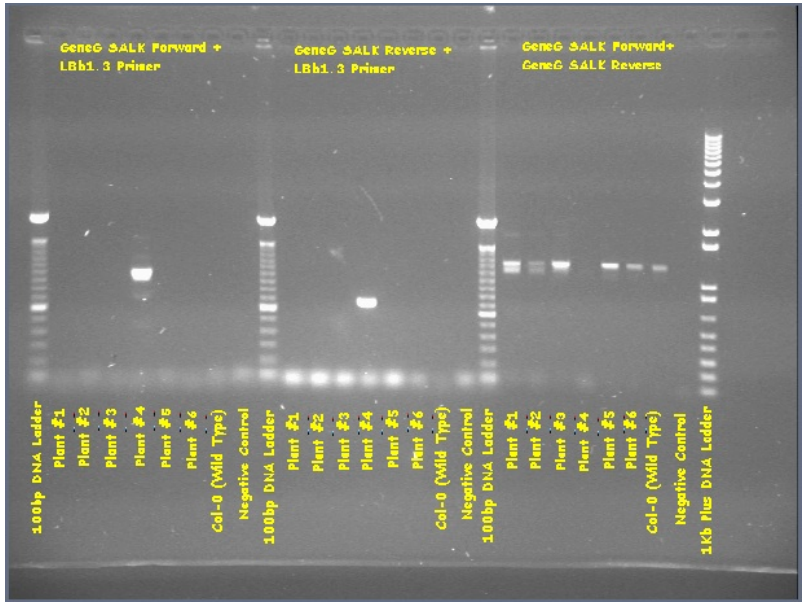


- What do we need in a PCR?
  - DNA template, 10x Ex Taq Buffer, dNTP mix, Primers, Ex Taq DNA Polymerase
  - Thermocycler

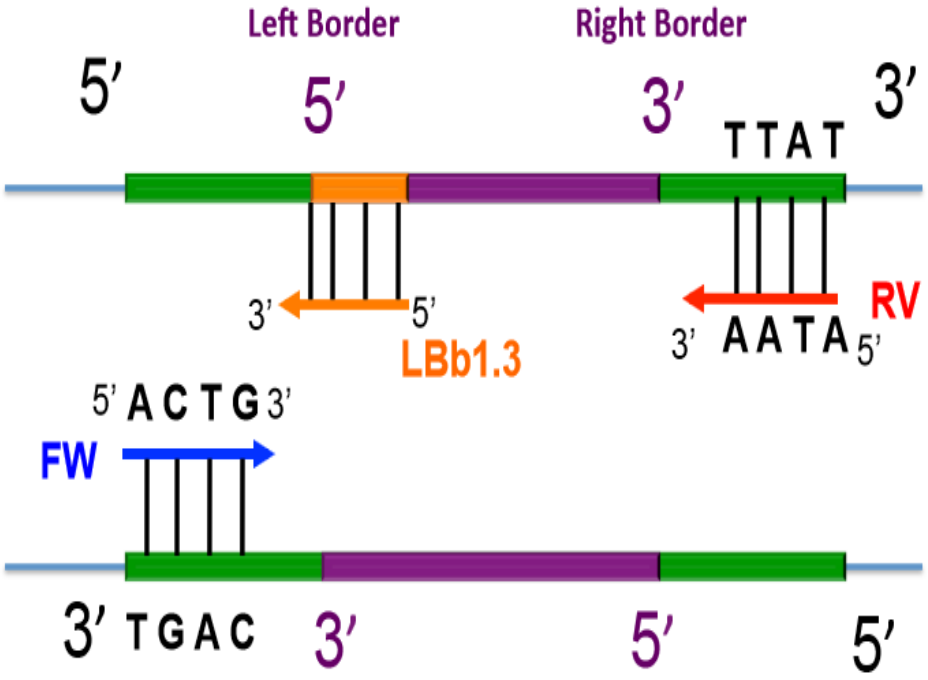


Genotyping

- Primers
  - Binds specifically to gene of interest and T-DNA
- Gel electrophoresis
  - Visualize the PCR products in different reactions

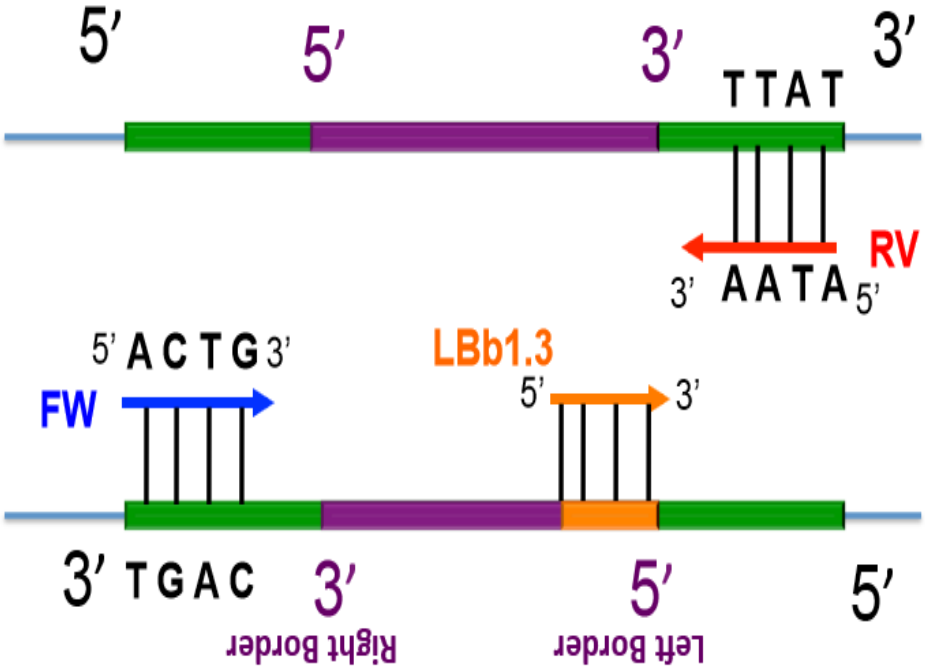


What happens if T-DNA is present in the forward orientation?



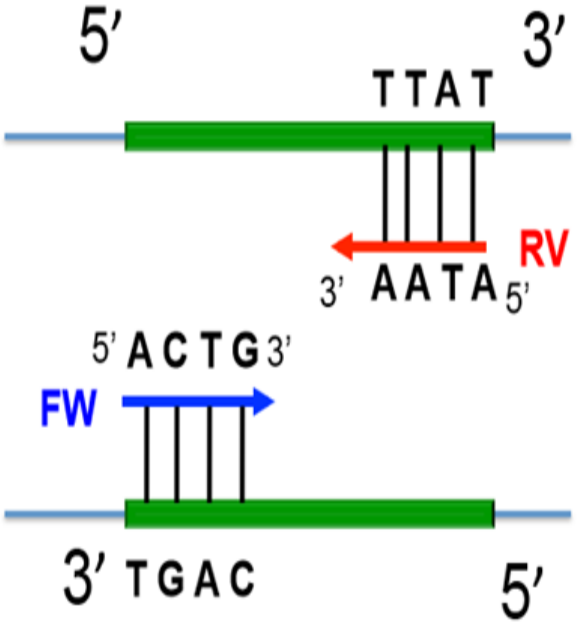
A PCR product is formed between the gene-specific forward and left border primers.

What happens if T-DNA is present in the reverse orientation?



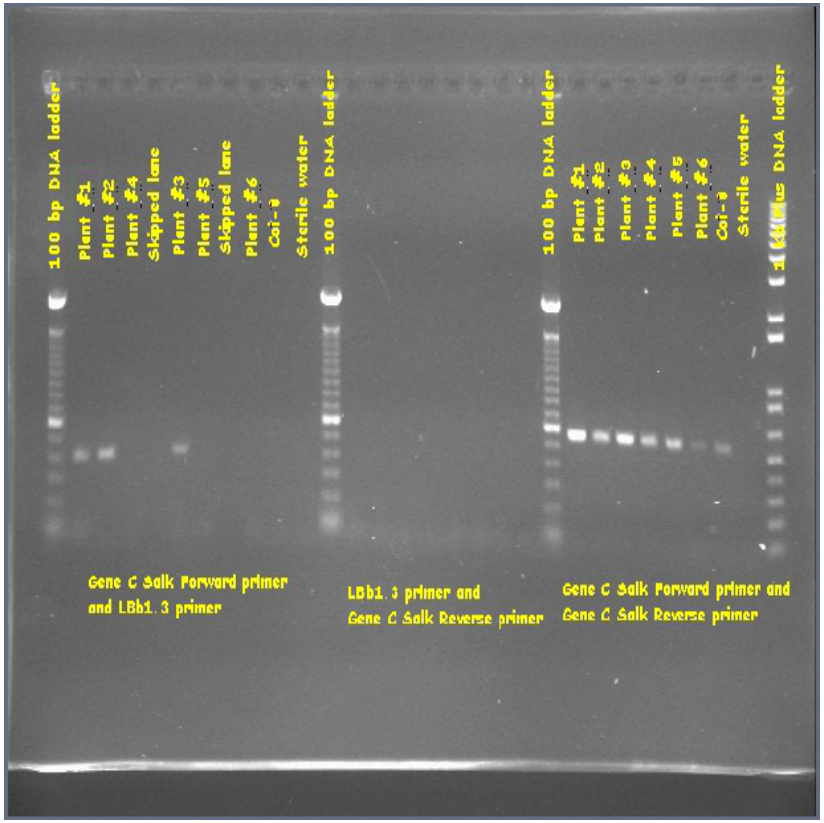
A PCR product is formed between the gene-specific reverse and left border primers.

What happens if no T-DNA is present?



A PCR product is formed between the gene-specific forward and gene-specific reverse primers.

How do we interpret gel electrophoresis results?



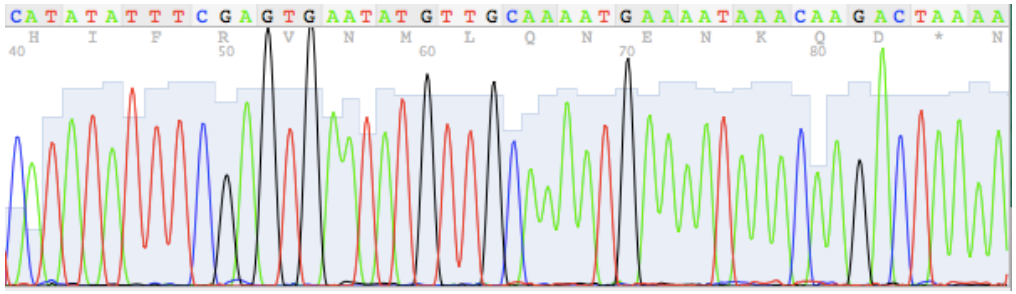
- Product in FW+RV only: WT
- Product in FW/RV +LBb1.3: homozygous for T-DNA
- Product in FW+RV and FW/RV+LBb1.3: heterozygous for T-DNA

PCR Purification/Gel Extraction

- Purify DNA fragments from primers, nucleotides, polymerases, salts
- DNA binds to QIAquick column membrane while impurities are washed away with buffer solutions
- Purified DNA eluted from column with addition of elution buffer

Sanger Sequencing

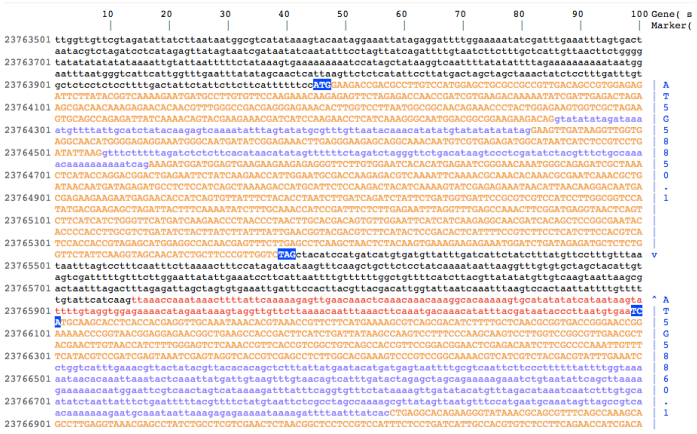
- Fluorescently labeled terminator ddNTPs used



- Helps determine:
  - Sequence of DNA
  - Location and structure of gene
  - Location of T-DNA insert
  - But how?

Bioinformatics

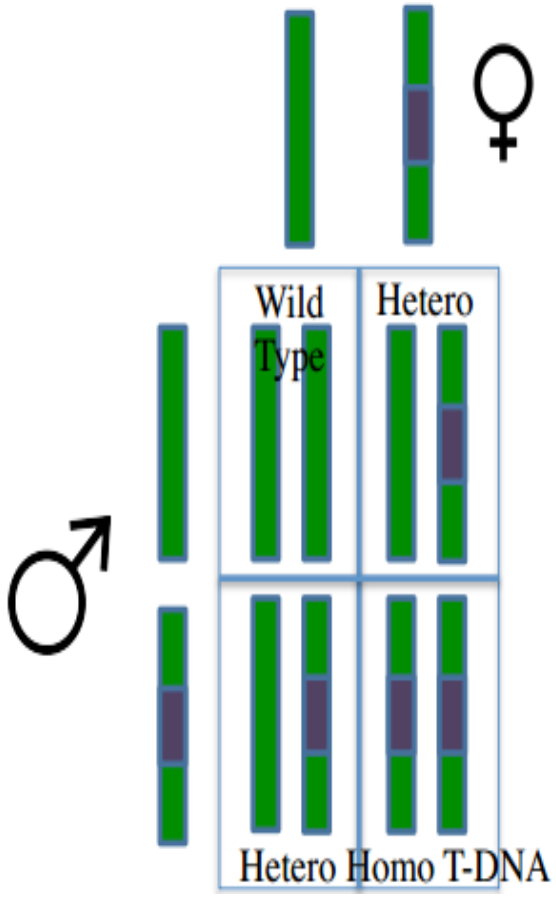
- TAIR BLAST
  - Aligns query sequence with *Arabidopsis* genome database
  - Look up additional information about the gene
- SeedGeneNetwork
  - Look up information about when and where the gene of Interest is expressed



How do we observe and analyze phenotypes?

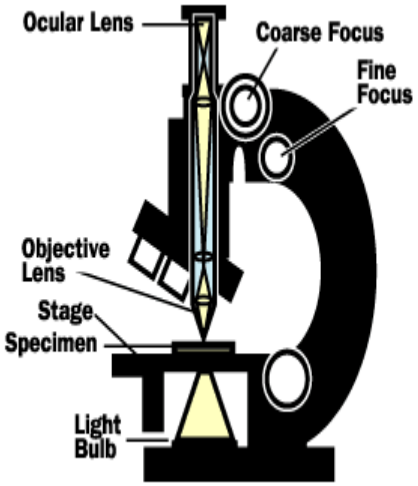
- Use light microscopy to get a cursory view of seed phenotype, that is its colour and shape, and to prepare seeds for Nomarski microscopy.
- Use Nomarski microscopy to observe seed phenotype at a greater detail, looking at tissues including the embryo and the suspensor.
- If any potential mutants were found, analyze the results with a chi-squared test.
- Observe and take measurements of mature plant stems, leaves, flowers and roots.

What do we expect to observe?

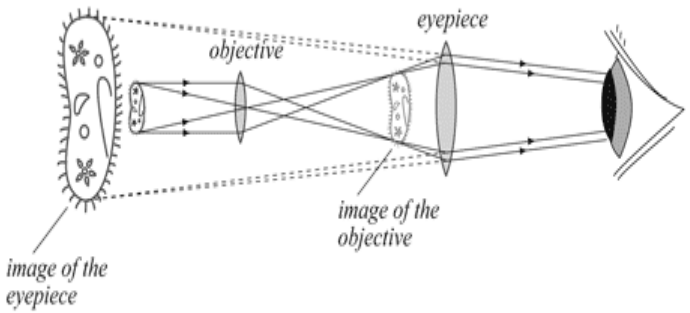


- Given a heterozygous parental line, one would expect on average $\frac{1}{4}$ seeds to be wild type, $\frac{1}{2}$ heterozygous and $\frac{1}{4}$ homozygous.
- If the mutation is embryo lethal, we would expect a 3:1 ratio of green to white seeds.

What is light microscopy?

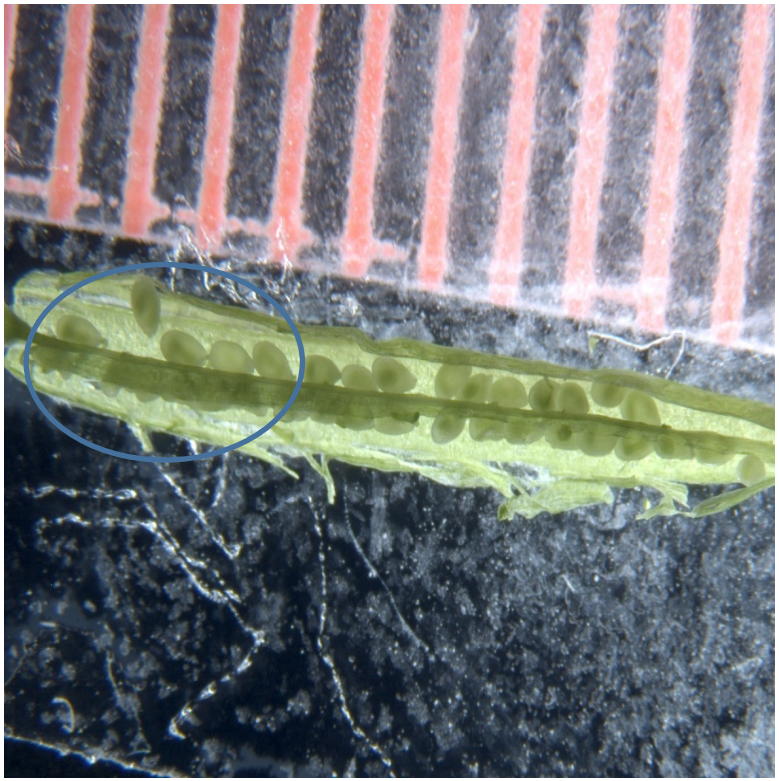


- Light, emitted by the light bulb, illuminates the specimen.
- The image is brought into focus within the microscope tube, which is then magnified by the ocular lens.
- We can adjust magnification, focus and brightness of the light source as desired to obtain better images.

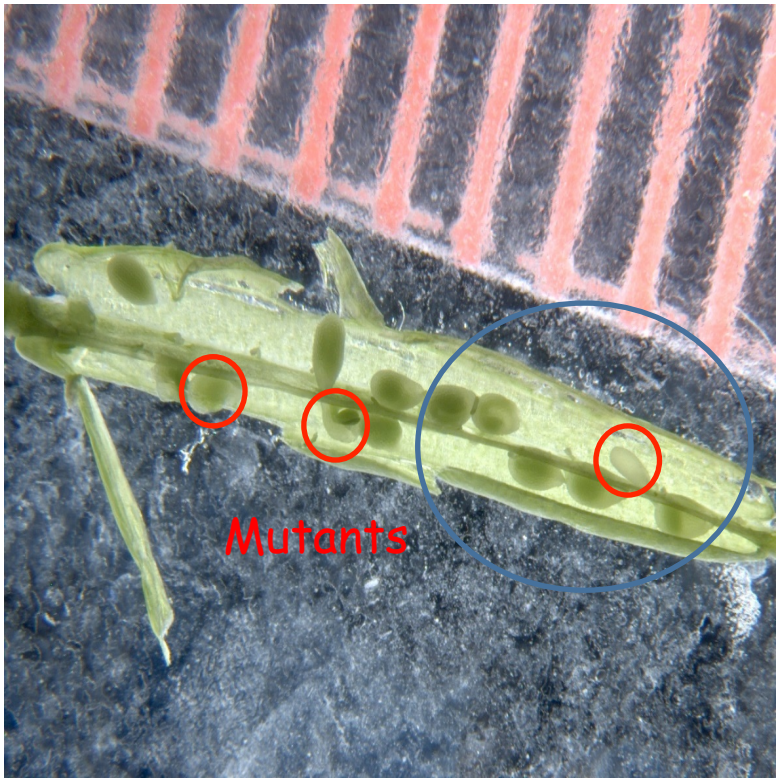


What can we observe with a light microscope?

Observing a wild type silique with a light microscope

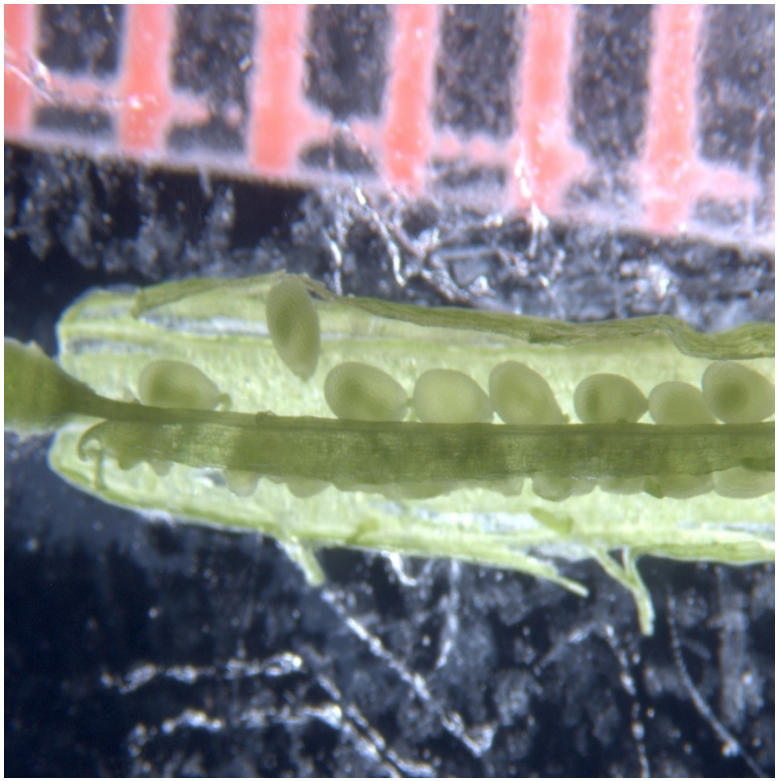


Observing a mutant silique with a light microscope

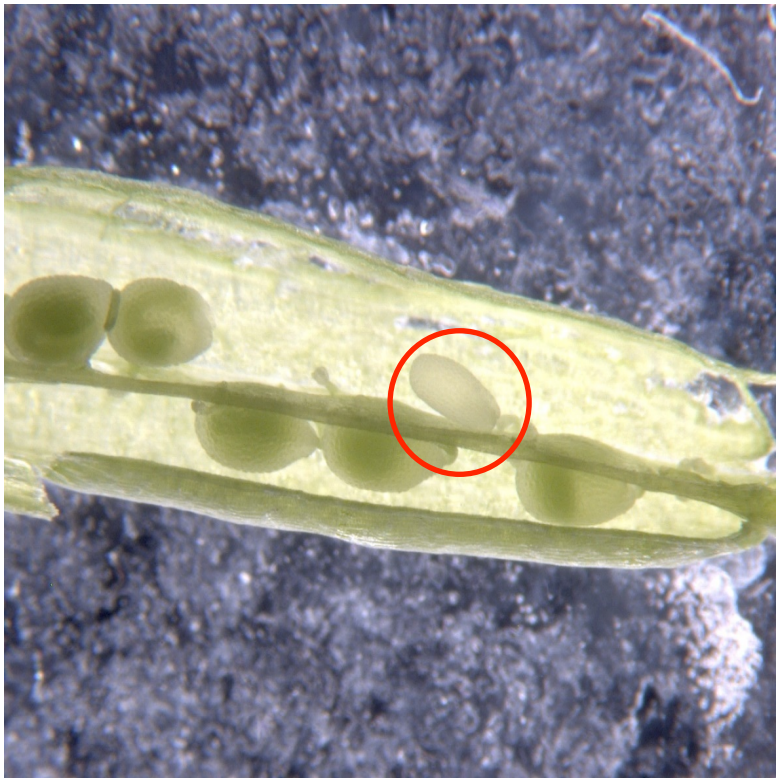


What can we observe with a light microscope?

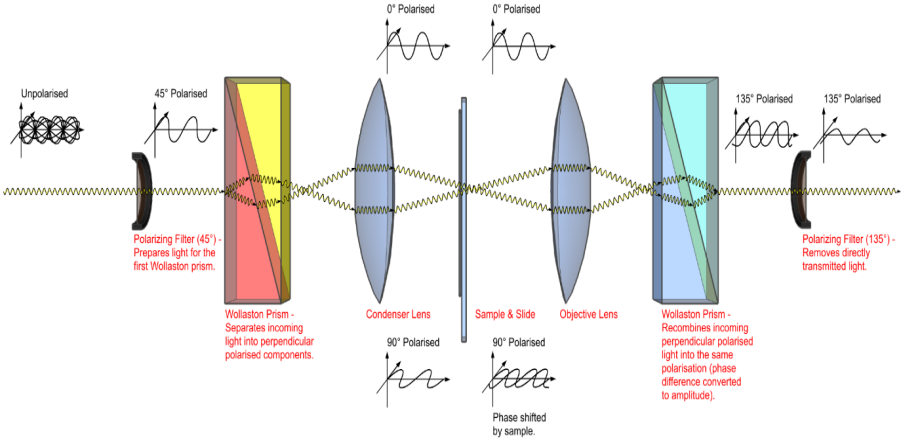
Taking a closer look at a wild type silique



Taking a closer look at a mutant silique



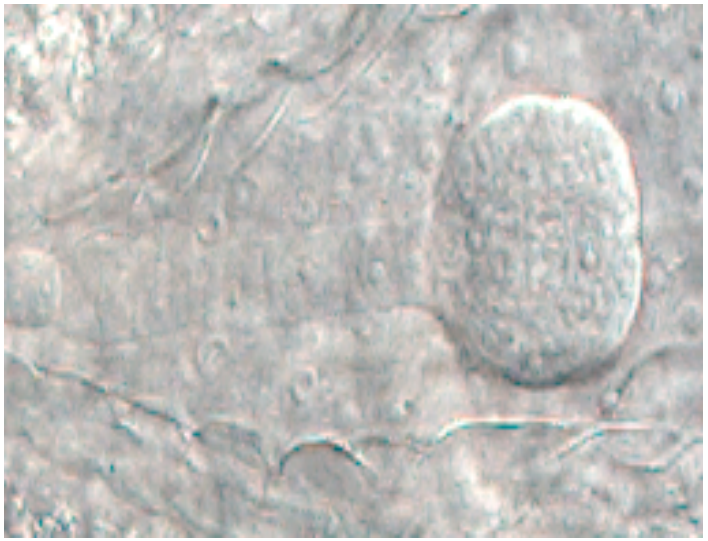
What is Nomarski microscopy?



- Also known as differential interference microscopy.
- Light is polarized at 45°, then separated into two coherent light waves polarized at 0° and 90°, then focused onto the sample 0.2 microns apart.
- The rays experience different phase shifts as they travel through the sample, and are recombined to produce wave interference, making the ray brighter or darker. A second polarizing filter removes any light which did not experience phase shifts.

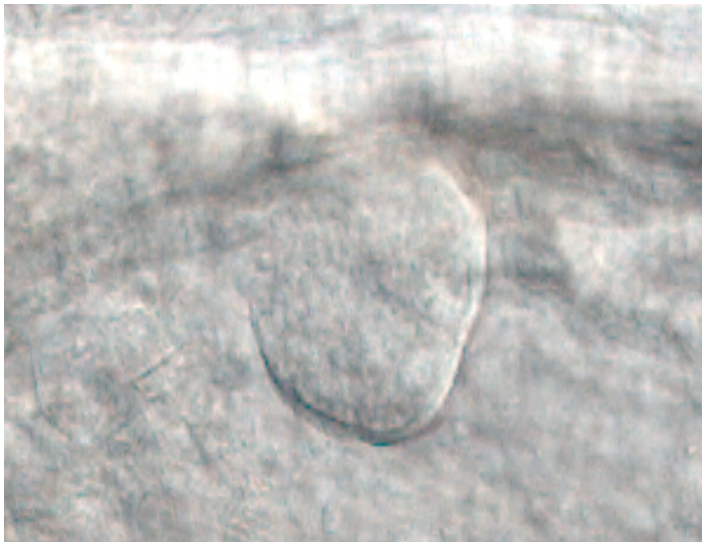
What can we observe with a Nomarski microscope?

Observing a wild type seed with a Nomarski microscope



A globular stage embryo with normal phenotype.

Observing a mutant seed with a Nomarski microscope



A globular stage embryo with abnormal phenotype.

How do we perform a chi-squared test?

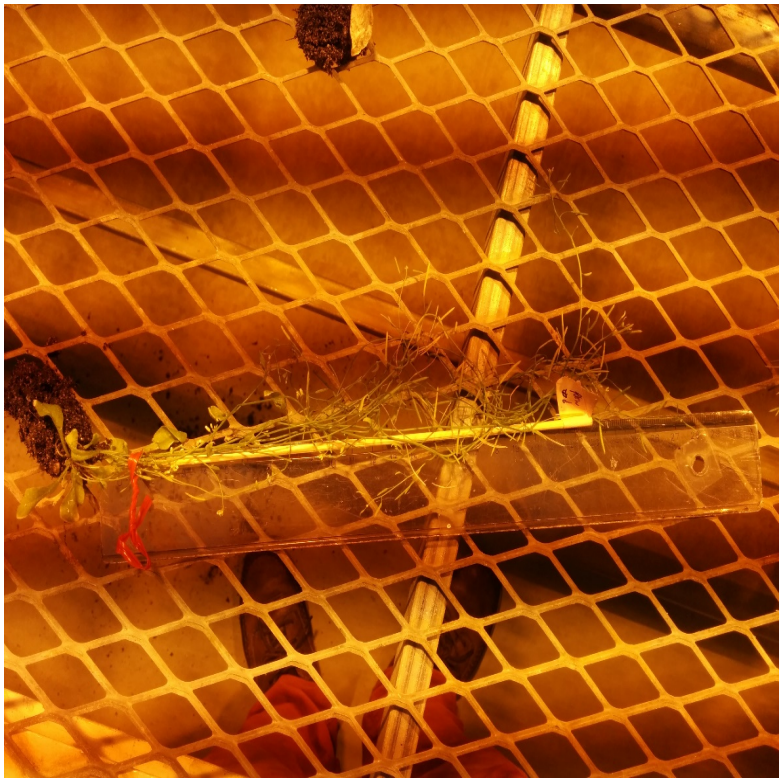
- $X^2=\Sigma(O-E)^2/E$, where X^2 is the chi-squared test statistic, O is the observed frequency, and E is the expected frequency.
- Let our null hypothesis be that the expected ratio is 3:1 for green to white seeds, and calculate appropriate expected values.
- Compare the test statistic to a chi-squared distribution table and get a p-value, with 1 degree of freedom, as there are only two possible phenotypes.
- Reject the null hypothesis if the associated p-value is less than 0.05.
- Otherwise, fail to reject the null hypothesis.

What can we observe on mature plants?

- Stems: Height of main stem, thickness, number of branches.
- Leaves: Shape, colour, length, size.
- Flowers: Sepals, petals, anthers, pistils.
- Roots: Length, number of branches.

Measuring mature plants

Measuring main stem height



Measuring root length