Methods and Approaches used to Study Knockout Arabidopsis Thaliana Plants

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Reverse Genetics

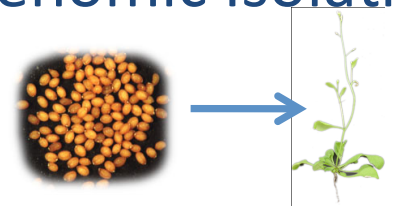
- Question
  - What is the function of the gene of interest?
- Method
  - Observe the phenotypic effects of the knockout mutant

What is a Knockout Mutant?

- Method
  - Knockout a gene by placing a T-DNA insert in the middle of an exon to disrupt sequence
- Homozygous T-DNA mutant plant
  - Both alleles contain T-DNA inserts and cause the gene to not be expressed
    - Does not always cause lethality or a physical phenotype
      - Other genes may compensate
      - Gene may not have a crucial role in seeds

Genomic Isolation

- Sow Seeds

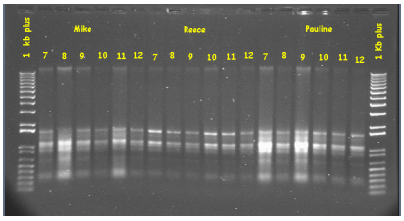


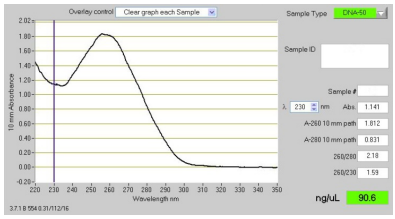
- Extract Nucleotides from a Leaf
  - Extraction Buffer, Isoproponal, Ethanol, SpeedVac, TE Buffer

- Determine quality of Nucleotides
  - Gel Electrophoresis and Nanodrop

What does Nanodrop and Electrophoresis Measure?

- Nanodrop
  - Measures the concentrations of nucleotides in a substance

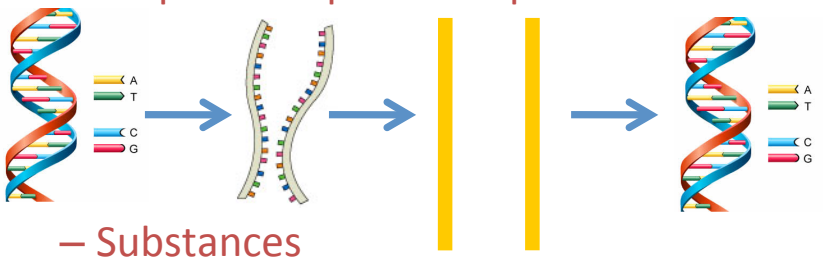




- Electrophoresis
  - Measures the quality of genomic DNA which corresponds with the top band
    - Syber Safe, Agarose, TAE Buffer, Loading Dye

Polymerase Chain Reaction (PCR)

- PCR
  - Amplifies a specific sequence of DNA



  - Substances
    - 10x Taq Buffer, dNTP Mix(deoxyribonucleotide triphosphate), Primers, Ex Taq DNA Polymerase

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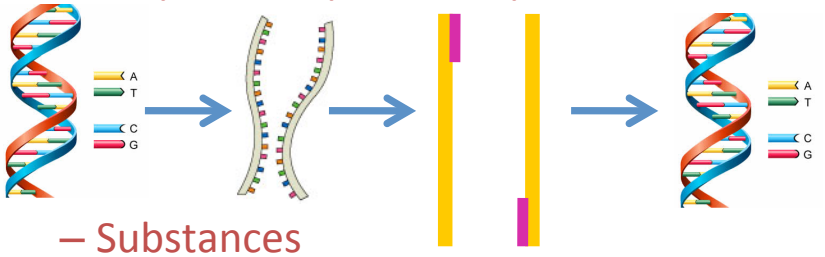

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Genotyping

- PCR
  - Primers specify what allele is isolated and amplified
- Biorad
  - Denaturing, Annealing, Replication and Cycles
- Gel Electrophoresis
  - The expected sizes for each type of allele should indicate what genotype the plant has for that specific gene

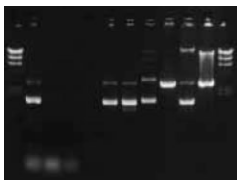
PCR Purification

- PCR purification purifies the products produced by PCR in order to sequence

- Methods
  - Gel Extraction
  - PCR QuickSpin purification

Gel Purification

- Cut out desired DNA fragment from agarose gel
  - Use a razor under UV light to cut out gel
- Add QG Buffer
  - Dissolves gel during incubation
- Add isopropanol to increase yield and place into a Quickspin column
  - Perform this step after gel is dissolved and turns yellow



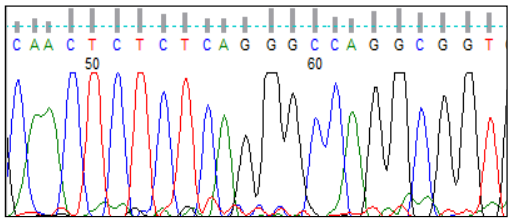
PCR Quickspin Purification

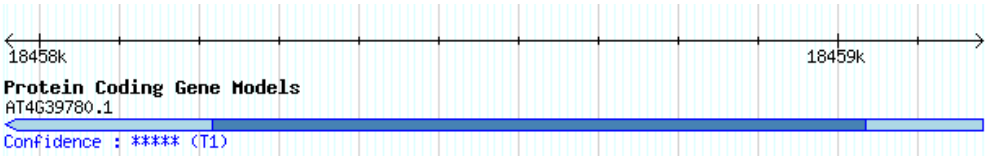
- Reagents
  - PB Buffer
    - DNA binds to the Quickspin membrane and PB brings down cellular compounds
  - PE Buffer
    - Washes out salts and proteins
  - EB Buffer
    - Binds to DNA and provides a stable environment to elute DNA from Quickspin membrane
- Nanodrop
  - Check Nucleotide Concentration

What Does Sequencing Reveal?

The Sanger Sequencing Method is used to determine:

1. The Orientation and location of the T-DNA Insert
2. Your gene of interest and its function
3. The structure of your gene





http://arabidopsis.org/servlets/TairObject?type=gene&id=130865

Bioinformatics

•Analyzes data using statistics and high output computing in a detailed and visual manner

Examples:

1. TAIR: contains information on all genes in the Arabidopsis Genome
2. NCBI: a large database for genes from all species and organisms
3. FinchTV: sequence viewer
4. Seed Gene Network: gives an in depth view on gene expression in Arabidopsis seeds

Phenotyping

•Compare the phenotypes between mutant plants and wild-type plants to determine if there are any differences in phenotype as a result of mutation in a gene

•This comparison can reveal what the gene codes for, where it is expressed, and whether it will cause seed or plant lethality

How can we observe phenotypes?

1. Look for differences between the plants i.e. the leaves, flowers, stems
2. Observe the seeds
3. Look at gene expression

Light Microscopy

•If a plant is heterozygous for the mutant allele there should be one mutant seed for every three green seeds
•Looking for this 3:1 ratio of green to mutant seeds
•Dissect siliques and count seeds to discover the ratio

3:1 Ratio of a Heterozygous Plant

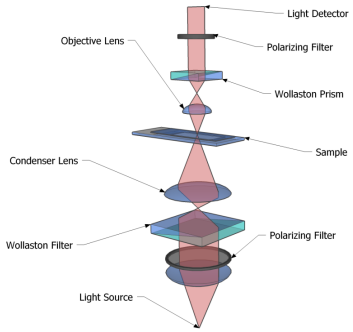
	A	a
A	AA	Aa
a	Aa	aa

What is a Chi-Square Test?

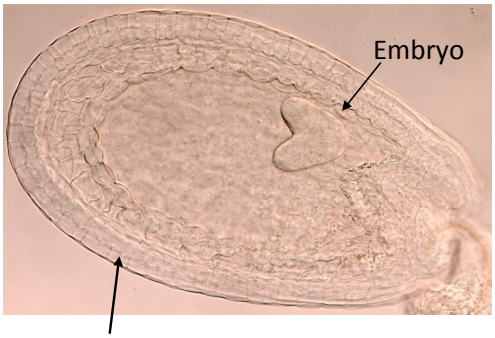
- A statistical test that measures deviation from a null hypothesis
- Tells how much the deviation from the expected 3:1 ratio is due to chance alone
- If a p-value > 0.05, the deviation from the expected ratio is most likely due to chance and not some other factor
- Cannot reject null hypothesis of a 3:1 Ratio if p>0.05 indicating there is no significant difference between the expected and observed results

What can Nomarski Reveal about the Phenotypes of Seeds?

- Allows a more detailed view of the seeds to detect if there is any mutant phenotype not visible by the naked eye as compared to the wild-type seeds



The Nomarski Microscope allows us to see inside the seed so we can see any abnormalities in the embryo or seed coat



http://www.tutorgig.com/ed/Nomarski_interference_contrast

When and Where are Genes Expressed?

Promoter Cloning

How Do You Measure Transcription?

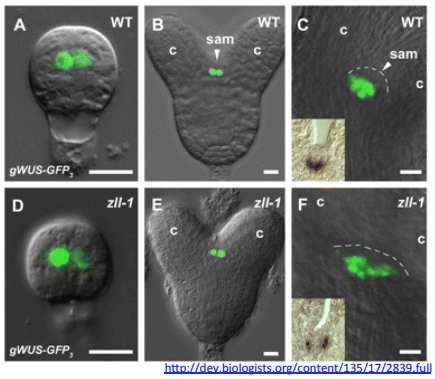
Design an Experiment:

- Transcription is controlled by the promoter, a "switch" that acts independently
- Create a chimeric gene using the Arabidopsis promoter switch and a reporter gene
- Allows the observation of protein expression rather than the accumulation of mRNA in the cytoplasm

What is a Reporter Gene?

GFP

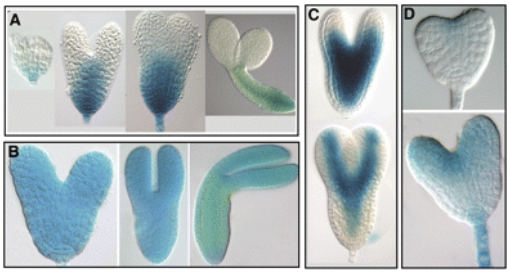
- Green Fluorescent Protein
- Fluoresces green under UV light



http://dev.biologists.org/content/135/17/2839.full

GUS

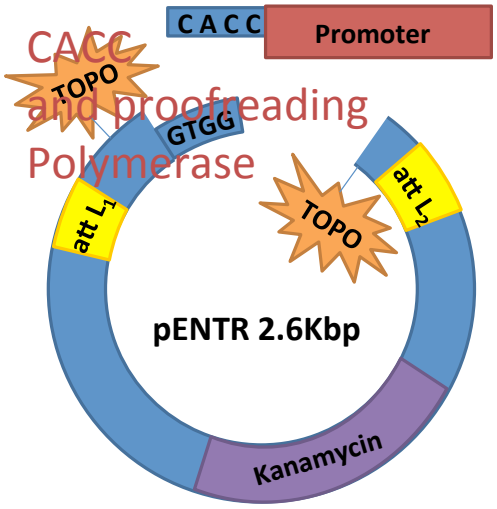
- Beta-glucuronidase
- Enzyme converts x-gluc to a blue precipitate
- More sensitive



http://www.plantphysiol.org/content/156/1/346.full

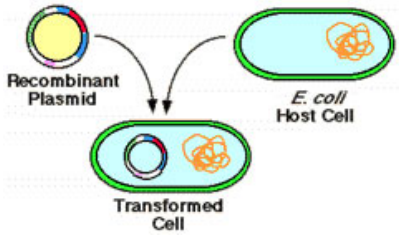
How do you Clone the Promoter Into a Plasmid in the Correct Direction?

- Isolate and amplify the promoter using PCR with primers with a 5' CACC overhang and proofreading DNA Polymerase

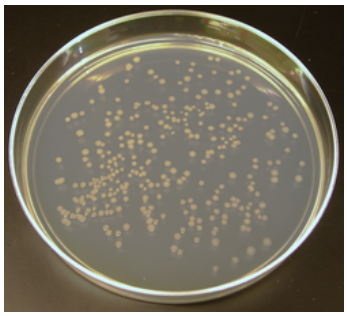


- Ligate PCR product into pENTR/D-TOPO Vector
- pENTR contains:
  - Kanamycin resistance
  - Topoisomerase I
  - GTGG overhang
  - att L_1 + att L_2

Transformation of E. coli Cells and Isolation of Plasmid DNA



- Transform via heat shock
- Grow colonies on Kanamycin plates, selecting for transformed cells
- Select a colony and isolate plasmid DNA

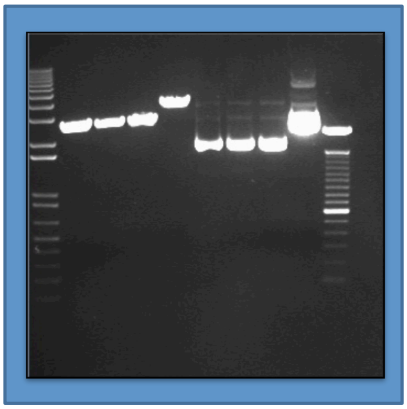


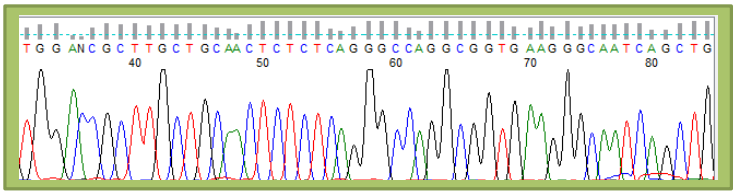
http://www.bio-world.com/productinfo/3_43_287_683/2509/Terrific-Broth-TB-w-Glycerol.html

http://www.sciencelearn.org.nz/Contexts/Enviro-imprints/Sci-Media/Images/E.coli

How Do You Identify an Accurate Recombinant Plasmid?

- Restriction Enzyme Digest
  - Cleave with AscI
  - Gel electrophoresis expected sizes:
    - Without promoter ≈ 2.5Kbp
    - With promoter longer
    - Compare to non-digested plasmids
- Sequence Reaction and BLAST
  - Ensure there are no mutations





When and Where are Genes Expressed?

Next Steps

- Homologous recombination between att sequences flanking the promoter in pENTR and a toxic gene in the T-DNA vector containing GFP, GUS, BastaR, and LB + RB
- Transform *E. coli* cells with T-DNA vector to select and amplify
- Transform *Agrobacterium* cells with T-DNA vector and Ti Helper plasmid containing transfer proteins that package the chimeric gene
- Transform *Arabidopsis* with *Agrobacterium* and observe reporter gene activity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