Detecting a Transposon Tag in Arabidopsis
INTRODUCTION

Throughout the first half of the 20\textsuperscript{th} century, geneticists assumed that a stable genome was a prerequisite for faithfully transmitting genes from one generation to the next. Working at Cold Spring Harbor Laboratory in the post-WWII era, Barbara McClintock found quite a different story in maize (corn). She observed numerous “dissociations” – broken and ring-shaped chromosomes – and traced the source of these mutations to the short arm of chromosome 9. There she identified two related loci, “dissociator” (Ds) and “activator” (Ac).

Equipped only with her maize crosses and a light microscope, she showed that Ac and Ds are mobile genetic elements that affect gene expression when they transpose, or jump, from one position to another. In one classic experiment, McClintock showed that movement of Ds in and out of the colored (C) locus is responsible for purple-speckled kernels. By showing that transposition may inactivate protein expression by inserting into a gene or may reactivate expression by jumping out, McClintock explained color variegation that had intrigued botanists for centuries.

McClintock offered genetic proof that Ac moves independently, but Ds depends on Ac for transposition. Molecular dissection later showed that Ac encodes a transposase that makes double-stranded cuts in DNA to free a transposon to move. Ds is a defective version of Ac, with deletions in the transposase gene.

Today the Ac/Ds system is an important tool in gene discovery, allowing scientists to characterize genes for which no biological role is yet known. In a process known as transposon mutagenesis, Ac and Ds elements are crossed into a corn strain to produce Ds insertions in genes. Backcrossing Ac out of the strain produces stable mutations, each “tagged” with an inserted Ds transposon. Screening a gene library with a probe for a Ds sequence readily identifies a Ds insertion in the gene responsible for an observable mutant phenotype. The Ac/Ds mutagenesis system works well in a number of plants — including tobacco, tomato, and the model plant \textit{Arabidopsis thaliana}.
This laboratory investigates the *CURLY LEAF (CLF)* gene of *Arabidopsis thaliana* to analyze the molecular relationship between genotype and phenotype. *CLF* is involved in homeotic gene regulation, which controls the correct spatial and tissue-specific expression of genes during development. The recessive *clf-2* mutation seen in this lab was created through transposon mutagenesis and produces a dwarf phenotype with curly leaves, early flowers, and fused flower parts.

**PCR amplification of the CLF locus.**

To analyze offspring from a self cross of a *clf-2* heterozygote, DNA is isolated from *Arabidopsis* plants using a rapid method, and the *CLF* locus is amplified using polymerase chain reaction (PCR). Since the *Ds* insertion at the *CLF* locus is too large to amplify across, two sets of primers are used. One set (*CLF1/CLF2*) straddles the *Ds* insertion site and amplifies the wild-type allele. The second set (*CLF1/Ds*) amplifies the insertion allele, with one primer located in the *CLF* gene and one located in the *Ds* transposon. The insertion allele produces a larger DNA product, which can be readily separated from the smaller wild-type allele by agarose gel electrophoresis.

LAB FLOW

I. PLANT ARABIDOPSIS SEED

- PLANT seeds
- WATER seeds
- 3-4 WEEKS

II. ISOLATE DNA FROM ARABIDOPSIS

- ADD leaf tissue
- GRIND
- ADD Edward's buffer
- VORTEX
- CENTRIFUGE
- TRANSFER supernatant
- ADD and MIX Isopropanol
- CENTRIFUGE
- POUR OFF supernatant
- REMOVE supernatant
- DRY
- ADD TE/RNase A buffer
- RESUSPEND DNA
- CENTRIFUGE
- leaf DNA extract

III. AMPLIFY DNA BY PCR

- ADD primer/ loading dye mix
- ADD DNA
- ADD mineral oil (if necessary)
- AMPLIFY in thermal cycler

IV. ANALYZE PCR PRODUCTS BY GEL ELECTROPHORESIS

- POUR gel
- SET
- LOAD gel
- ELECTROPHORESE 130 volts
METHODS

I. PLANT ARABIDOPSIS SEED

To extract DNA from plant tissue, you must plant the Arabidopsis seeds 3–4 weeks prior to DNA isolation and PCR. Depending upon growing conditions, you may observe the phenotypic differences between mutant and wild-type plants in as little as 2 weeks. For further information on cultivation, refer to The Arabidopsis Information Resource (TAIR) at http://www.arabidopsis.org.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Supplies and Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis seeds from heterozygous CLF/clf-2 cross</td>
<td>Planting pots and tray</td>
</tr>
<tr>
<td></td>
<td>Plastic dome lid or plastic wrap</td>
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<tr>
<td></td>
<td>Potting soil</td>
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</table>

For best results, use a potting soil formulated specifically for Arabidopsis.

*Arabidopsis* seeds are very tiny and difficult to handle, so planting is not as simple as it may seem.

1. Moisten the potting soil. Fill planting pots evenly with soil, but do not pack tightly.

2. Fit pots into the tray, but leave one corner space empty to facilitate watering.

3. Carefully scatter seeds evenly on top of the soil.
   a. Fold a 4 X 4 inch sheet of paper in half.
   b. Place the seeds into the fold of the paper, and gently tap them onto the soil.
   c. Provide space between seeds, so they will grow better and plant phenotypes can be readily observed.

4. Cover pots with plastic dome lids or plastic wrap to assist germination. (Remove covers 3–7 days after planting.)

5. Add 1/2 inch of water to tray, using the empty corner space. Water regularly to keep soil damp, but do not allow soil to remain soggy.

6. Grow the plants close to a sunny window at room temperature (20–22°C). For optimum growth, provide a constant (24 hours/day) fluorescent light source about 1 foot directly above the plants.

7. Harvest plant tissue for PCR when the wild-type and *clf*-2 phenotypes become evident. You may wish continue to grow the plants after you have harvested tissue for DNA isolation and amplification. The phenotypic differences between plants become more obvious over time, with the small *clf*-2 mutants flowering well before wild-type plants.

Germination requires a humid environment.

To prevent the soil from drying out, you may keep a small amount of water in the tray at all times.

With 24-hour fluorescent lighting, conditions, phenotypes can be discerned in 2-3 weeks.
II. ISOLATE DNA FROM ARABIDOPSIS

<table>
<thead>
<tr>
<th>Reagents</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Wild-type or clf-2 Arabidopsis plant</td>
<td>Pellet pestles</td>
</tr>
<tr>
<td>Edward’s buffer, 0.5 ml</td>
<td>Permanent marker</td>
</tr>
<tr>
<td>Isopropanol, 0.5 ml</td>
<td>1.5 ml microcentrifuge tubes</td>
</tr>
<tr>
<td>Tris/EDTA (TE) buffer with RNase A, 200 μl</td>
<td>Micropipet and tips (100-1000 μl)</td>
</tr>
<tr>
<td></td>
<td>Microcentrifuge tube racks</td>
</tr>
<tr>
<td></td>
<td>Microcentrifuge</td>
</tr>
<tr>
<td></td>
<td>Vortexer (optional)</td>
</tr>
<tr>
<td></td>
<td>Water bath or heating block (95-100°C)</td>
</tr>
<tr>
<td></td>
<td>Container with cracked or crushed ice</td>
</tr>
</tbody>
</table>

1. Obtain a wild-type or clf-2 mutant Arabidopsis plant.

2. Cut two pieces of tissue approximately 1/4 inch in diameter. Place the leaf tissue in a clean 1.5 ml tube, and label with phenotype and your group number.
   a. If the plant is small, take multiple leaves to make an equivalent amount of leaf tissue.
   b. The clf-2 mutant may be so small that you need to use the entire plant. If so, carefully remove all soil from the roots.

3. Twist a clean pestle against the inner surface of the 1.5 ml tube to forcefully grind the plant tissue for 1 minute.

4. Add 400 μl of Edward’s buffer to the tube. Grind briefly to remove tissue from the pestle and to liquify any remaining pieces of tissue.

5. Vortex the tube for 5 seconds, by hand or machine.

6. Boil the samples for 5 minutes in a water bath or heating block.

7. Place the tube in a balanced configuration in a microcentrifuge, and spin for 2 minutes to pellet any remaining cell debris.

8. Transfer 350 μl of supernatant to a fresh tube. Maintain label for plant phenotype and group number. Be careful not to disturb the pelleted debris when transferring the supernatant. Discard old tube containing the precipitate.

9. Add 400 μl of isopropanol to the tube of supernatant.

10. Mix by inverting the tube several times, and leave at room temperature for 3 minutes.

11. Place the tube in a balanced configuration in a microcentrifuge, and spin for 5 minutes. Align tubes in the rotor with the cap hinges.
The nucleic acid pellet may appear as a tiny teardrop-shaped smear or particles on the tube side. Don't be concerned if you can't see a pellet. A large or greenish pellet is cellular debris carried over from the first centrifugation.

Dry the pellets quickly with a hair dryer! To prevent blowing the pellet away, direct the air across the tube mouth, not into the tube.

You will use 2.5 μl of the DNA extract for the PCR reactions in Part II. The crude DNA extract contains nucleases that will eventually fragment the DNA at room temperature. Keeping the sample cold limits this activity.

pointing outward. Nucleic acids will collect on the tube side under the hinge during centrifugation.

12. Carefully pour off the supernatant from the tube, then completely remove the remaining liquid with a medium pipet set at 100 μl.

13. Air dry the pellet for 10 minutes to evaporate remaining isopropanol.

14. Add 100 μl of TE/RNase A buffer to the tube. Dissolve the nucleic acid pellet by pipetting in and out. Take care to wash down the side of the tube underneath the hinge, where the pellet formed during centrifugation.

15. Incubate TE/RNase A solution at room temperature for 5 minutes.

16. Microcentrifuge the tube for 1 minute to pellet any material that did not go into solution.

17. DNA may be used immediately or stored at –20°C until you are ready to continue with Part III. Keep the DNA on ice during use.

### III. AMPLIFY DNA BY PCR

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>*Wild-type or clf-2 Arabidopsis DNA (from Part II)</td>
<td>Permanent marker</td>
</tr>
<tr>
<td>*CLF1/CLF2 primer/loading dye mix, 30 μl</td>
<td>Micropipet and tips (1-100 μl)</td>
</tr>
<tr>
<td>*CLF1/Ds primer/loading dye mix, 30 μl</td>
<td>Microcentrifuge tube racks</td>
</tr>
<tr>
<td>Ready-To-Go™ PCR Bead</td>
<td>Container with cracked or crushed ice</td>
</tr>
<tr>
<td>Mineral oil, 5 ml (depending on thermal cycler)</td>
<td>Thermal cycler</td>
</tr>
</tbody>
</table>

*Store on ice

1. Set up CLF1/CLF2 reactions:
   a. Obtain a PCR tube containing a Ready-To-Go™ PCR Bead. Label with the primer set and your group number.
   b. Use a micropipet with a fresh tip to add 22.5 μl of the CLF1/CLF2 primer/loading dye mix to your tube. Allow several minutes for bead to dissolve.
   c. Use a micropipet with a fresh tip to add 2.5 μl of wild-type or clf-2 Arabidopsis DNA (from Part II).

The primer loading dye mix will turn purple as the Ready-To-Go™ PCR Bead dissolves.
2. Set up CLF1/Ds reactions:
   a. Obtain a PCR tube containing a Ready-To-Go™ PCR Bead. Label with the primer set and your group number.
   b. Use a micropipet with a fresh tip to add 22.5 µl of the CLF1/Ds primer/loading dye mix to your tube. Allow several minutes for bead to dissolve.
   c. Use a micropipet with a fresh tip to add 2.5 µl of wild-type or clf-2 Arabidopsis DNA (from Part II).

3. If necessary, add one drop of mineral oil to the top of the reactants in the PCR tubes. Be careful not to touch the dropper tip to the tube or reactants, or subsequent reactions will be contaminated with DNA from your preparation.

4. Store samples on ice until you are ready to begin thermal cycling.

5. Program the thermal cycler for 29 cycles of the following profile. The program may be linked to a 4°C hold program after the 29 cycles are completed.

   Denaturing step: 94°C 30 seconds
   Annealing step: 65°C 30 seconds
   Extending step: 72°C 30 seconds

6. After cycling, store the amplified DNA at –20°C until you are ready to continue with Part IV.
IV. ANALYZE PCR PRODUCTS BY GEL ELECTROPHORESIS

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Supplies and Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>* Wild-type or clf-2 Arabidopsis PCR product (from Part III)</td>
<td>Micropipet and tips (1-100 µl)</td>
</tr>
<tr>
<td>*pBR322/BstNI marker, 130 µl</td>
<td>Microcentrifuge tube rack</td>
</tr>
<tr>
<td>1X TBE, 300 ml</td>
<td>1.5 ml microcentrifuge tube</td>
</tr>
<tr>
<td>2% agarose in 1X TBE, 50 ml</td>
<td>Gel electrophoresis chamber</td>
</tr>
<tr>
<td>Ethidium bromide (1 µg/ml), 250 ml</td>
<td>Power supply</td>
</tr>
<tr>
<td>or CarolinaBLU™ gel &amp; buffer stain, 7 ml</td>
<td>Staining trays</td>
</tr>
<tr>
<td>CarolinaBLU™ final stain, 250 ml</td>
<td>Latex gloves</td>
</tr>
<tr>
<td>*Store on ice</td>
<td>UV transilluminator for use with ethidium bromide</td>
</tr>
</tbody>
</table>

1. Seal the ends of the gel-casting tray with masking tape, and insert a well-forming comb.

2. Pour 2% agarose solution to a depth that covers about 1/3 the height of the open teeth of the comb.

3. Allow the gel to solidify completely. This takes approximately 20 minutes.

4. Place the gel into the electrophoresis chamber, and add enough 1X TBE buffer to cover the surface of the gel.

5. Carefully remove the comb, and add additional 1X TBE buffer to just cover and fill in wells, creating a smooth buffer surface.

6. Combine both PCR products (CLF1/CLF2 and CLF1/Ds) together in a fresh 1.5 ml tube. Pipet in and out to mix. (If you used mineral oil during PCR, pierce your pipet tip through the layer of mineral oil to withdraw the PCR products and leave the mineral oil behind in the original tube.)

7. Use a micropipet with a fresh tip to add 25 µl of the combined PCR sample/loading dye mixture into your assigned well of a 2% agarose gel.

8. Load 20 µl of the molecular weight marker (pBR322/BstNI) into one well.

9. Run the gels at 130 V for approximately 30 minutes. Adequate separation will have occurred when the cresol red dye front has moved at least 50 mm from the wells.

The agarose solution should be approximately 60°C - or just cool enough to hold the beaker or flask in your hand.

Avoid pouring an overly thick gel, which is more difficult to visualize. The gel will become cloudy as it solidifies.

Do not add more buffer than necessary. Too much buffer above the gel channels electrical current over the gel, increasing running time.

Expel any air from the tip before loading, and be careful not to push the tip of the pipet through the bottom of the sample well.

100-bp ladder may also be used as a marker.
10. Stain the gel in ethidium bromide or CarolinaBLU™:

a. For ethidium bromide, stain 10-15 minutes. Decant stain back into storage container for reuse, and rinse gel in tap water. *Use gloves when handling ethidium bromide solution and stained gel, or anything that has ethidium bromide on it. Ethidium bromide is a known mutagen and care should be taken when using and disposing of it.*

b. For CarolinaBLU™, follow directions in the Instructor Planning section.

11. View gel using transillumination, and photograph.
RESULTS & DISCUSSION

1. Observe the photograph of the stained gel containing your sample and those of other students. Orient the photograph with wells at the top. Interpret each lane of the gel. Use the sample gel pictured below to help you.

![Image of gel with bands labeled CLF1/Ds and CLF1/CLF2]

<table>
<thead>
<tr>
<th>Genotype</th>
<th>clf-2/clf-2</th>
<th>CLF/clf-2</th>
<th>CLF/CLF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype</td>
<td>clf-2</td>
<td>wt</td>
<td>wt</td>
</tr>
</tbody>
</table>

a. Scan across the photograph of your gel and others as well to get an impression of what you see in each lane. You should notice that virtually all experiment lanes contain one or two prominent bands.

b. Now locate the lane containing the pBR322/BstNI marker on the left hand side of the gel. Working from the well, locate the bands corresponding to each restriction fragment: 1,857 bp, 1,058 bp, 929 bp, 383 bp, and 121 bp (may be faint or not visible at all). Alternatively, locate the lane containing the 100-bp ladder on the right hand side of the gel. These DNA markers increase in size in 100-bp increments starting with the fastest migrating band of 100 bp.

c. The amplification product of the mutant clf-2 allele (539 bp) should align between the 929-bp and the 383-bp fragments of the pBR322/BstNI markers (or between the 500-bp and 600-bp markers of the 100-bp ladder).

d. The amplification product of the wild-type allele (246 bp) should align between the 383-bp and the 121-bp fragments of the pBR322/BstNI markers (or between the 200-bp and 300-bp markers of the 100-bp ladder).
e. It is common to see a second band lower on the gel. This diffuse (fuzzy) band is "primer dimer," an artifact of the PCR reaction that results from the primers overlapping one another and amplifying themselves. Primer dimer is approximately 50 bp, and should be in a position ahead of the 121-bp fragment of the pBR322/BstNI marker (or the 100-bp marker of the 100-bp ladder).

f. Additional faint bands, at other positions on the gel, occur when the primers bind to chromosomal loci other than CLF, giving rise to "nonspecific" amplification products.

2. Why is a second primer set CLF1/Ds used to identify the insertion allele, rather than CLF1/CLF2?
BIOINFORMATICS

Biological information is encoded in the nucleotide sequence of DNA. Bioinformatics is the field that identifies biological information in DNA using computer-based tools. Some bioinformatics algorithms aid the identification of genes, promoters, and other functional elements of DNA. Other algorithms help determine the evolutionary relationships between DNA sequences.

Because of the large number of tools and DNA sequences available on the Internet, experiments done in silico (“in silicon,” or computer) now complement experiments done in vitro (in glass, or test tube). This movement between biochemistry and computation is a key feature of modern biological research.

In Part I you will use the Basic Local Alignment Search Tool (BLAST) to predict the size of the product amplified by the CLF1/CLF2 primer set. In Part II, you will identify the CLF gene and the Ds transposon in biological databases. In Part III, you will identify the function of the CLF protein and its role in development. Part IV summarizes research on homeotic genes and development.

The following primer sets were used in the experiment:

- **CLF1**: 5’-TTAACCCGGACCCGCATTGTTTGGG-3’ (Forward Primer)
- **CLF2**: 5’-AGAGAAAGCTCAAACAAAGCCATCGA-3’ (Reverse Primer)
- **CLF1**: 5’-TTAACCCGGACCCGCATTGTTTGGG-3’ (Forward Primer)
- **Ds**: 5’-GTCGGCGTTCGGCTGGCGCG-3’ (Reverse Primer)

I. **Use BLAST to Predict the Size of the CLF1/CLF2 Amplicon**

1. Initiate a BLAST search.
   b. Click on BLAST in the top speed bar.
   c. Click on Nucleotide-nucleotide BLAST (blastn).
   d. Enter the CLF1/CLF2 primer set into the Search window.
   e. Delete any non-nucleotide characters from the window.
   f. Click on BLAST!.
   g. Click on Format! and wait for your results.
2. The results of the **BLAST** search are displayed in three ways as you scroll down the page:

   a. A graphical overview illustrating how significant hits align with the query sequence,

   b. a list of significant alignments with Geneinfo Identifier (gi) – links, and

   c. a detailed view of the primer sequences *(query)* aligned to the nucleotide sequence of the search hit *(subject)*.

3. What is the predicted length of the product that the primer set would amplify in a PCR reaction *(in vitro)*?

   a. In the list of significant alignments, notice the scores in the **E-Value** column on the right. *The Expectation or E-Value* is the number of alignments with the query sequence that would be expected to occur by chance in the database. The lower the **E-Value** the higher the probability that the hit is related to the query. For example, 3e-4 denotes 3 X 10^-4 or 0.0003. Shorter queries, such as primers, produce higher **E-values**.

   b. Note any significant alignment that has an **E-Value** less than 0.1.

   c. Scroll down to the *Alignments* section, and examine the corresponding alignments with the two primers.

   d. The lowest and highest nucleotide positions in the subject sequence indicate the borders of the amplified sequence. Calculate the length of the amplified sequence

   e. Don't forget to add one nucleotide to your result!

II. **Use BLAST to Identify the Location of the CLF Gene and Insertion site of the Ds Transposon**

1. What portion of the **CLF** gene do the **CLF1/CLF2** primers amplify?

   a. Follow the **gi** link to open a Genbank record, which contains basic information about each DNA sequence submitted to the Genbank database. Accession, number source, references, and other general information is at the top of the page, while the actual DNA sequence is at the bottom. In between are annotations of identifiable features – including gene, mRNA, coding sequence (cds), and repeats. (Follow this link for a clickable record, with descriptions [http://www.ncbi.nlm.nih.gov/Sitemap/samplerecord.html](http://www.ncbi.nlm.nih.gov/Sitemap/samplerecord.html).)
b. Scroll down the feature annotations on the left, until you find the gene that contains or overlaps with the target nucleotide positions identified by the CLF1/CLF2 primers Set A (3.d. above).

c. Examine the mRNA feature that follows. “Join” denotes the start and end nucleotides for each coding exon of the gene (axxx…bxxx).

d. How would you describe the region of the CLF gene that is amplified by the CLF1/CLF2 primers?

2. Copy the Arabidopsis thaliana gene ID (At2g23380) from the gene feature.

a. In Search window at the top of the page, change Nucleotide to Genome, paste the ID, and click Go.

b. Follow the link to see a map of Arabidopsis Chromosome 2.

c. Enter the gene ID into the Search window at the top of the page, and click Find in This View.

d. Use the Zoom feature on the left to get a closer view.

e. How many exons and introns does the CLF gene have?

f. (For a different trip to the same location: Return to the NCBI home page, www.ncbi.nlm.nih.gov/. Click on Map Viewer from Hot Spots list on the right. > Click on Arabidopsis thaliana in the Plants list. > In Search window at the top of the page, paste the ID, and click Find. > Click on the link to the highlighted chromosome.)

3. Repeat the electronic PCR with the CLF/Ds primer set (directions in 1 above).

a. In the list of significant alignments, note any alignment that has an E-Value of less than 0.1.

b. Scroll down to the Alignments section. Does any hit contain matches to both primers? What does this mean?

c. Follow gi - links to learn more about some of the significant hits.

d. What is different about the matches with the forward and reverse primers?

4. Approximately where in the CLF gene is the Ds insertion?

a. On the picture in the RESULTS & DISCUSSION section the CLF1/Ds amplicon migrates between the 500-bp and 600-bp markers of the 100-bp ladder; exact mapping reveals it to be 539 bp long.
b. The 539-bp amplicon consists of sequences from the CLF gene and sequences from the Ds transposon (as evident from the results of the blastn search with the CLF1/Ds primer pair above).

c. To determine the length of the Ds portion of the amplicon, identify in the CLF1/Ds blastn results a hit that lists "Ds2(D1)" in its title.

d. Scroll down to the sequence alignment (Query/Subject) for this hit. The largest number in Subject is the length of the Ds portion of the CLF1/Ds amplicon (426 bp).

e. To determine the CLF portion of the amplicon, subtract the length of its Ds portion from the length of the entire amplicon (539 bp).

f. Add the length of CLF portion to the point where the CLF1 primer matches the CLF gene. The result is the exact position where the Ds element interrupts the CLF gene.

g. Compare this position with the map for the introns and exons in the CLF gene (mRNA feature in II.1.c above) and determine whether the Ds element is inserted in an exon or intron.

III. Use BLAST to Determine the Function of the CLF Protein


2. Obtain the Genbank record for the CLF gene by pasting the CLF gene ID (At2g23380) into the Search window at the top of the page. Change All Databases to Nucleotide, and click Go.

3. Follow the Curly Leaf link to the Genbank record.

4. Scroll down to translation, a sequence of letter abbreviations for the amino acids. Extract (highlight and copy) the protein sequence for the CLF gene.


6. Click on BLAST in the top speed bar.

7. Click on Protein-protein BLAST (blastp).

8. Paste the CLF protein sequence into the Search window.

9. Click on BLAST!.

10. An algorithm quickly scans the query sequence to identify any functional domain that is conserved in different organisms. Click on a domain box to go to a Conserved Domain page.
11. Move your cursor over one of the boxes, and a window will display a summary of the domain and its characteristics. (For more detailed information follow the gnl – links for one of the matches.)

12. Go back to the search page and click on Format!.

13. In the list of significant alignments, notice the scores in the E-Value column on the right. As in nucleotide searches, the lower the E-Value the higher the probability that the hit is related to the query. For example, 3e-4 denotes 3x10^-4 or 0.0003.

14. In what organisms can you find hits with significant E-values (less than 0.1)?

15. Note the variety of proteins in different species that are related (homologous) to the Arabidopsis CLF protein.

16. Dig more deeply into the function of CLF and related proteins.
   a. Follow the gi - link to reports for CLF and other sequences with low E-values.
   b. In the right hand corner of the report, click on Links and select Pubmed.
   c. Pubmed provides a link to the research article relevant to the selected protein. Click on Related Articles for many more.
   d. For an animated introduction to pathfinding research on homeotic genes and development, see “Master genes control basic body plans;” at DNA from the Beginning: http://www.dnaftb.org/dnaftb/37/concept/index.html.

IV. Homeotic Genes and Development

The CLF gene is a Polycomb-Group gene that regulates homeotic gene expression in Arabidopsis. Homeotic genes were first identified in Drosophila but have since been found in vertebrates and plants. Homeotic genes are required for the correct spatial and tissue-specific expression of target genes that control early development of an organism. During development in Drosophila, the embryo is divided into smaller and smaller domains through the action of a hierarchy of genes. The first genes in this hierarchy establish the anterior/posterior and dorsal/ventral axes of the embryo. The next three series of genes – the gap, pair rule, and segment polarity genes – divide the anterior/posterior axis into segments. The last set of genes expressed are homeotic genes that are required throughout the rest of Drosophila development and adult life.
The combinatorial activities of the homeotic gene products specify the identities of the Drosophila segments: head, thorax, and abdomen. In homeotic gene mutants, the identity of one body part is exchanged for a different one. For example, the Drosophila Antennapedia mutant has legs in the place of antennae. Correct patterns of homeotic gene expression must be maintained through multiple mitotic divisions during development, and, so, can be thought of as a molecular memory system. Genes related to Antennapedia and Bithorax establish initial patterns of homeotic gene expression, which are maintained later in development by genes of the Polycomb-Group (Pc-G) and their antagonists of the trithorax-Group (trx-G).

Arabidopsis flowers contain four organs – sepals, petals, stamens, and carpels – that are organized into concentric whorls of organs when viewed from the top of the flower (figure 1). Similar to Drosophila segment identity, the identities of these whorls are established by the combined action of plant homeotic genes.

The Arabidopsis homeotic gene AGAMOUS is normally expressed in whorls 3 and 4 of the flower, and is required for correct development of stamens and carpels. It is not expressed in whorls 1 and 2 of the flower, which develop into sepals and petals. AGAMOUS expression is normally repressed in whorls 1 and 2 by the CLF protein. However, plants that are homozygous for the clf-2 mutation fail to repress AGAMOUS and exhibit partial homeotic transformation of sepals and petals into stamens and carpels.

The CLF protein is a homolog of Drosophila Enhancer of Zeste protein that belongs to the Polycomb-Group. Thus, CLF helps maintain patterns of homeotic gene expression after whorl identity has already been established – hence the partial confusion of flower parts in clf-2 mutants.

![Arrangement of flower organs.](image-url)
Instructor Information

REAGENTS, SUPPLIES & EQUIPMENT CHECK LIST

This experiment is available as a DNA Learning Center Kit, available ready-to-use from Carolina Biological Supply Company, catalog numbers 21–1290 through 21–1295. All kits include materials needed for DNA extraction and PCR amplification (for 0.2 ml or 0.5 ml PCR tubes); some kits contain additional materials for gel electrophoresis and staining with ethidium bromide or CarolinaBLU™. Visit the Carolina Biological Internet site at http://www.carolina.com/ or call 800-334-5551.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Supplies and Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Arabidopsis seed from self cross of a clf-2 heterozygotic</em></td>
<td>Planting pots and tray</td>
</tr>
<tr>
<td>Edward's buffer</td>
<td>Plastic dome lid or plastic wrap</td>
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<tr>
<td>100% Isopropanol</td>
<td>Potting soil (optimized for <em>Arabidopsis</em>)</td>
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<tr>
<td>Tris/EDTA (TE) buffer with RNase A</td>
<td>Micropipets and tips (1 – 1,000 μl)</td>
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<tr>
<td><em>CLF1/CLF2 primer/loading dye mix</em></td>
<td>1.5-ml microcentrifuge tubes</td>
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<tr>
<td><em>CLF1/Ds primer/loading dye mix</em></td>
<td>Microcentrifuge tube racks</td>
</tr>
<tr>
<td>Ready-to-Go™ PCR Beads</td>
<td>Microcentrifuge for 1.5-ml tubes</td>
</tr>
<tr>
<td>Mineral oil (depending on thermal cycler)</td>
<td>Pellet pestles</td>
</tr>
<tr>
<td>DNA marker pBR322/BstNI (0.075 μg/μl)*</td>
<td>Water bath or heating block (95 – 100°C)</td>
</tr>
<tr>
<td>Agarose</td>
<td>Thermal cycler</td>
</tr>
<tr>
<td>1x TBE electrophoresis buffer</td>
<td>Electrophoresis chambers</td>
</tr>
<tr>
<td>Ethidium bromide solution, 1 μg/ml OR CarolinaBLU™ gel &amp; buffer stain</td>
<td>Electrophoresis power supplies</td>
</tr>
<tr>
<td>CarolinaBLU™ final stain</td>
<td>Latex gloves</td>
</tr>
<tr>
<td></td>
<td>Staining trays</td>
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<tr>
<td></td>
<td>UV transilluminator (ethidium bromide staining)</td>
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<td></td>
<td>White light box (CarolinaBLU™ staining)</td>
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<td></td>
<td>Camera or photo-documentary system</td>
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<td></td>
<td>Permanent markers</td>
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<td></td>
<td>Container with cracked or crushed ice</td>
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<td></td>
<td>Vortexer (optional)</td>
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<tr>
<td></td>
<td>Waterbath for agarose (60°C)</td>
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</tbody>
</table>

*Store at −20°C

Ready-to-Go™ PCR Beads incorporate Taq polymerase, dNTPs, and MgCl₂. Each bead is supplied in an individual 0.5-ml tube or a 0.2-ml tube.
CONCEPTS AND METHODS

This laboratory can help students understand several important concepts of modern biology:

- The relationship between genotype and phenotype.
- The use of transposable elements to mutagenize and tag genes.
- The role of homeotic genes in plant and animal development.
- The movement between in vitro experimentation and in silico computation.

The laboratory uses several methods for modern biological research:

- DNA extraction and purification.
- Polymerase chain reaction (PCR).
- Gel electrophoresis.
- Bioinformatics.

INSTRUCTOR PLANNING AND PREPARATION

The following table will help you to plan and integrate the four parts of the experiment.

<table>
<thead>
<tr>
<th>Part</th>
<th>Day</th>
<th>Time</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Plant Arabidopsis seeds 3-4 weeks before lab</td>
<td>15-30 min.</td>
<td>Plant Arabidopsis seeds</td>
</tr>
<tr>
<td>II.</td>
<td>Isolate DNA</td>
<td>1</td>
<td>30 min.</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>30-60 min.</td>
</tr>
<tr>
<td>III.</td>
<td>Amplify DNA by PCR 2</td>
<td>30-60 min.</td>
<td>Pre-lab: Set up student stations</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15-30 min.</td>
<td>Isolate Arabidopsis DNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70+ min.</td>
<td>Pre-lab: Set up student stations</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Set up PCR reactions</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post-lab: Amplify DNA in thermal cycler</td>
</tr>
<tr>
<td>IV.</td>
<td>Analyze Amplified DNA by Gel 4</td>
<td>30 min.</td>
<td>Prepare agarose gel solution and cast gels</td>
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<td></td>
<td></td>
<td>30 min.</td>
<td>Load DNA samples into gels</td>
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<td></td>
<td></td>
<td>30+ min.</td>
<td>Electrophorese samples</td>
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<td></td>
<td></td>
<td>20+ min.</td>
<td>Post-lab: Stain gels</td>
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<tr>
<td></td>
<td></td>
<td>20 min. to overnight</td>
<td>Post-lab: De-stain gels</td>
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<td></td>
<td></td>
<td>20 min.</td>
<td>Post-lab: Photograph gels</td>
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</tbody>
</table>

I. Plant Arabidopsis Seed

Arabidopsis seeds must be planted 3-4 weeks before the date anticipated for DNA extraction and amplification by PCR. Two 1/4 inch diameter leaf disks are required for each experiment, but multiple small leaves and even whole plantlets can be used.

Fluorescent light fixtures for growing Arabidopsis should be fitted with at least two 40-watt "daylight" bulbs (not cool white). The following products suggested for Arabidopsis cultivation are available from Carolina Biological Supply Company (www.carolina.com). Products from garden stores may be substituted.

- Standard Poly-Tray without Holes (54 x 27 x 6 cm tray)
  - item number 66-5666
- Poly-Flats (6-cm deep cells that can be separated into individual pots)
  - 8-Cell Tray
    - item number 66-5668
  - 24-Cell Tray
    - item number 66-5669
  - 36-Cell Tray
    - item number 66-5670
- Redi-Earth Soil (8 lb. Bag)
  - item number 15-9701
II. Isolate DNA from Arabidopsis

Make sure that different student teams extract DNA from both wild-type and clf-2 mutant plants. Assign each team a number at the outset of the experiment. This will make it easier to mark and identify the several types of small tubes used in the experiment.

The cells walls of living plant tissue typically are broken up by grinding with a mortar and pestle. This can be accomplished directly in a 1.5 ml tube using a plastic pestle. An no-cost pestle can be made by heating a 1,000 µl pipet tip in a gas flame until it just melts. Then force the melted tip into a 1.5 ml tube, and twist to obtain a smooth surface.

Pre-lab Set Up (per student team)

- Wild-type or clf-2 Arabidopsis plant
- Edward’s buffer, 0.5 ml
- Isopropanol, 0.5 ml
- Tris/EDTA (TE) buffer with RNase A, 200 µl (thaw and store on ice)

- 1.5 ml microcentrifuge tubes
- Permanent marker
- Pellet pestles
- Micropipet and tips (100-1000 µl)
- Microcentrifuge tube rack
- Container with cracked or crushed ice

Shared Items

- Microcentrifuge
- Water bath or heating block (95-100°C)
- Vortexer (optional)

III. Amplify DNA by PCR

The Ds insertion at the CLF locus is too large to amplify across, so a single primer set cannot amplify both the wild-type and insertion alleles. Thus, this experiment amplifies the wild-type and insertion alleles in separate PCR reactions using two different sets of primers. One primer set (CLF1/CLF2) spans the Ds insertion site and amplifies the wild-type CLF allele. The CLF2 primer is moved out of amplifiable range in chromosomes with a Ds insertion. So, to amplify the insertion allele, the CLF1 primer is paired with a second primer, Ds, which is located within the Ds transposon. To simplify analysis of the three genotypes (CLF/CLF, CLF/clf-2, and clf-2/clf-2), the results of the two PCR reactions are mixed prior to electrophoresis.

Each Ready-To-Go™ PCR Bead contains reagents so that when brought to a final volume of 25 µl the reaction contains 2.5 units of Taq DNA polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, and 200 µM of each dNTP.

The lyophilized Taq DNA polymerase in the Ready-To-Go™ PCR Bead becomes active immediately upon addition of the primer/loading dye mix. In the absence of thermal cycling, "nonspecific priming" at room temperature allows the polymerase to begin generating erroneous products, which can show up as extra bands in gel analysis. Therefore, work quickly. Be sure the thermal cycler is set and have all experimenters set up their PCR reactions as a coordinated effort. Add primer/loading dye mix to all reaction tubes, then add each student template, and begin thermal cycling as quickly as possible. Hold reactions on ice until all are ready to load into the thermal cycler.
Each primer/loading dye mix incorporates the appropriate primer pair (0.25 picomoles/μl of each primer), 13.9% sucrose, and 0.0082% cresol red. The inclusion of loading dye components, sucrose and cresol red, allows the amplified product to be directly loaded into an agarose gel for electrophoresis. The primer/loading dye mix may collect in the tube caps during shipping; pool the reagent by spinning the tubes briefly in a microcentrifuge or by tapping the tube ends on the desktop.

PCR amplification from crude cell extracts is biochemically demanding, and requires the precision of automated thermal cycling. However, amplification of the CLF locus is not complicated by the presence of repeated units. Therefore, the recommended amplification times and temperatures will work adequately for all types of thermal cyclers.

**Pre-lab Set Up (per student team)**

- Wild-type or clf-2 Arabidopsis DNA, from Part II (store on ice)
- 30 μl CLF1/CLF2 primer/loading dye mix (thaw and store on ice)
- 30 μl CLF1/Ds primer/loading dye mix (thaw and store on ice)
- 4 Ready-To-Go™ PCR Beads (in PCR tubes)
- Permanent marker
- Micropipet and tips (1-100 μl)
- Microcentrifuge tube rack
- Mineral oil, 5 ml (depending on thermal cycler)
- Container with cracked or crushed ice

**Shared Item**

- Thermal cycler

**IV. ANALYZE AMPLIFIED DNA BY GEL ELECTROPHORESIS**

Prepare a 1X concentration of TBE by adding 75 ml of 20X concentrated stock into 1,425 ml of deionized or distilled water. Mix thoroughly.

Prepare a 2% agarose solution by adding 2 g of agarose to 100 ml of 1X TBE in a 500 ml flask or beaker. Heat the flask or beaker in a boiling water bath (approximately 15 minutes) or in a microwave oven (approximately 4 minutes) until the agarose is completely dissolved. You should no longer see agarose particles floating in solution when the beaker is swirled. Allow the agarose to cool to approximately 60°C, and hold at this temperature in a hot water bath. Cover beaker or flask with aluminum foil, and skim any polymerized "skin" off the top of the solution before pouring.

The cresol red and sucrose in the primer mix function as loading dye, so that amplified samples can be loaded directly into an agarose gel. This is a nice time saver. However, since it has relatively little sugar and cresol red, this loading dye is more difficult to use than typical loading dyes. So, encourage students to load carefully.

Plasmid pBR322 digested with the restriction endonuclease BstNI is an inexpensive marker and produces fragments that are useful as size markers in this experiment. The size of the DNA fragments in the marker are 1,857 bp, 1,058 bp, 929 bp, 383 bp, and 121 bp. Use 20 μl of a 0.075 μg/μl stock solution of this DNA ladder per gel. Other markers or a 100 bp ladder may be substituted.

View and photograph gels as soon as possible after appropriate staining/destaining. Over time, at room temperature, the small-sized PCR products will diffuse through the gel and lose sharpness.
Pre-lab Set Up (per student lab team)

Wild-type or clf-2 Arabidopsis PCR products from Part III (store on ice)
pBR322/BstNI markers (thaw and store on ice)
2% agarose in 1X TBE (hold at 60°C), 50 ml per gel
1X TBE buffer, 300 ml per gel
Ethidium bromide (1 µg/ml), 250 ml
or
CarolinaBLU™ gel & buffer stain, 7 ml
CarolinaBLU™ final stain, 250 ml

1.5 ml microcentrifuge tubes
Micropipet and tips (1-100 µl)
Microcentrifuge tube rack
Electrophoresis chamber and power supply
Latex gloves
Staining tray
Container with cracked or crushed ice

Shared Items
Water bath for agarose solution (60°C)
Transilluminator with camera
**CarolinaBLU™ STAINING**

**Post-Staining**

1. Cover the electrophoresed gel with the CarolinaBLU™ Final Stain, and let sit for 20–30 minutes. Agitate gently (optional).

2. After staining, pour the stain back into the bottle for future use. (The stain can be used 6–8 times.)

3. Cover the gel with deionized or distilled water to destain. (Chloride ions in tap water can partially remove the stain from the DNA bands and will cause the staining to fade.)

4. Change the water 3–4 times over the course of 30–40 minutes. Agitate the gel occasionally.

5. Bands that are not immediately present will become more apparent with time and will reach their maximum visibility if the gel is left to stain overnight in just enough stain to cover the gel. Gels left overnight in a large volume of water may destain too much.

**Pre-Staining**

CarolinaBLU™ can also be used to stain the DNA while it is being electrophoresed. Pre-staining will allow students to visualize their results prior to the end of the gel run. However, post-staining is still required for optimum viewing.

To pre-stain the gel during electrophoresis, add CarolinaBLU™ Gel and Buffer Stain in the amounts indicated in the table below. Note that the amount of stain added is dependent upon the voltage used for electrophoresis. Do not use more stain than recommended. This may precipitate the DNA in the wells and create artifact bands.

Gels containing CarolinaBLU™ may be prepared one day ahead of the lab day, if necessary. However, gels stored longer tend to fade and lose their ability to stain DNA bands during electrophoresis.

Use the following table to add the appropriate volume of CarolinaBLU™ stain to the agarose gel:

<table>
<thead>
<tr>
<th>Voltage</th>
<th>Agarose Volume</th>
<th>Stain volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;50 Volts</td>
<td>30 ml</td>
<td>40 µl (1 drop)</td>
</tr>
<tr>
<td></td>
<td>200 ml</td>
<td>240 µl (6 drops)</td>
</tr>
<tr>
<td></td>
<td>400 ml</td>
<td>520 µl (13 drops)</td>
</tr>
<tr>
<td>&gt;50 Volts</td>
<td>50 ml</td>
<td>80 µl (2 drop)</td>
</tr>
<tr>
<td></td>
<td>300 ml</td>
<td>480 µl (12 drops)</td>
</tr>
<tr>
<td></td>
<td>400 ml</td>
<td>640 µl (16 drops)</td>
</tr>
</tbody>
</table>

Use the table below to add the appropriate volume of CarolinaBLU™ stain to 1X TBE buffer:

<table>
<thead>
<tr>
<th>Voltage</th>
<th>Agarose Volume</th>
<th>Stain volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;50 Volts</td>
<td>500 ml</td>
<td>480 µl (12 drop)</td>
</tr>
<tr>
<td></td>
<td>3000 ml</td>
<td>3 ml (72 drops)</td>
</tr>
<tr>
<td>&gt;50 Volts</td>
<td>500 ml</td>
<td>960 µl (24 drop)</td>
</tr>
<tr>
<td></td>
<td>2600 ml</td>
<td>5 ml (125 drops)</td>
</tr>
</tbody>
</table>
ANSWERS TO DISCUSSION QUESTIONS

2. Why is a second primer set $CLF1/Ds$ used to identify the insertion allele, rather than $CLF1/CLF2$?

The $Ds$ insertion at the $CLF$ locus is too large to amplify across, so a single primer set cannot amplify both the wild-type and insertion alleles. Thus, this experiment amplifies the wild-type and insertion alleles in separate PCR reactions using two different sets of primers. One primer set ($CLF1/CLF2$) spans the $Ds$ insertion site and amplifies the wild-type $CLF$ allele. The $CLF2$ primer is moved out of amplifiable range in chromosomes with a $Ds$ insertion. So, to amplify the $clf-2$ insertion allele, the $CLF1$ primer is paired with a second primer, $Ds$, which is located within the $Ds$ transposon.