



Dolan DNA Learning Center
Cold Spring Harbor Laboratory

Detecting a Transposon Tag in *Arabidopsis*



Funded by NSF

INTRODUCTION

Throughout the first half of the 20th 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.

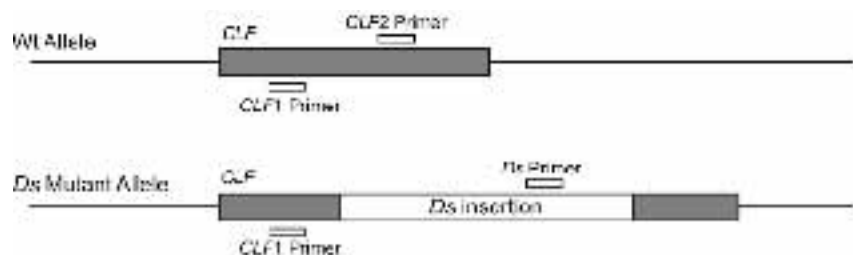
McClintock offered genetic proof that *Ac* moves independently, but *Ds* depends on *Ac* for transposition. Molecular dissection later showed that *Ac* encodes a transposase that makes double-stranded cuts in DNA to free a transposon to move. *Ds* is a defective version of *Ac*, with deletions in the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



Wild-type (left) and *clf-2* mutant (right) *Arabidopsis* plants.

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.

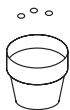
To analyze offspring from a self cross of a *clf-2* heterozygote, DNA is isolated from *Arabidopsis* plants using a rapid method, and the *CLF* locus is amplified using polymerase chain reaction (PCR). Since the *Ds* insertion at the *CLF* locus is too large to amplify across, two sets of primers are 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.

- Edwards, K., Johnstone, C., and Thompson, C. (1991). A Simple and Rapid Method for the Preparation of Plant Genomic DNA for PCR Analysis. *Nucleic Acids Res.* **19**:1349.
- Fedoroff, N., Wessler, S., and McClure, M. (1983). Isolation of the Transposable Maize Controlling Elements *Ac* and *Ds*. *Cell* **35**:235–242.
- Goodrich, J., Puangsomlee, P., Martin, M., Long, D., Meyerowitz, E.M., and Coupland, G. (1997). A Polycomb-Group Gene Regulates Homeotic Gene Expression in *Arabidopsis*. *Nature* **386**:44–51.
- Hsieh, T-F, Hakim, O., Ohad, N., and Fischer, R.L. (2003). From Flour to Flower: How Polycomb-Group Proteins Influence Multiple Aspects of Plant Development. *Trends Plant Sci.* **8**:439–445.
- Kim, G-T, Tsukaya, H., and Uchimiya, H. (1998). The *CURLY LEAF* Gene Controls Both Division and Elongation of Cells During the Expansion of the Leaf Blade in *Arabidopsis thaliana*. *Planta* **206**:175–183.
- McClintock, B. (1951). Chromosome Organization and Genic Expression. *Cold Spring Harbor Symp. Quant. Biol.* **16**:13–47.

LAB FLOW

I. PLANT *ARABIDOPSIS* SEED

PLANT
seeds



WATER
seeds



3-4 WEEKS

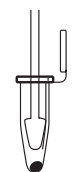


II. ISOLATE DNA FROM *ARABIDOPSIS*

ADD
leaf
tissue



GRIND



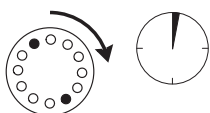
ADD
Edward's
buffer



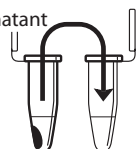
VORTEX



CENTRIFUGE



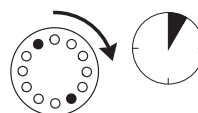
TRANSFER
supernatant



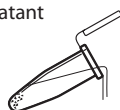
ADD and MIX
Isopropanol



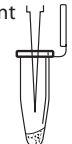
CENTRIFUGE



POUR OFF
supernatant



REMOVE
supernatant



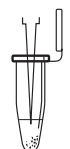
DRY



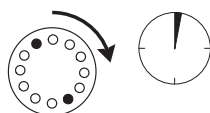
ADD
TE/RNase A
buffer



RESUSPEND
DNA



CENTRIFUGE



leaf
DNA
extract

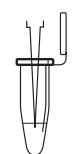


III. AMPLIFY DNA BY PCR

ADD
primer/
loading
dye mix



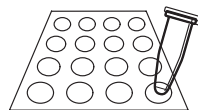
ADD
DNA



ADD
mineral oil
(if necessary)

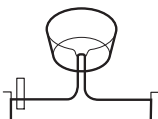


AMPLIFY
in thermal
cycler



IV. ANALYZE PCR PRODUCTS BY GEL ELECTROPHORESIS

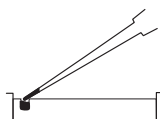
POUR
gel



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>.

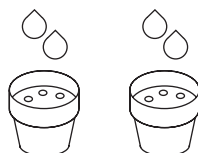
Reagents	Supplies and Equipment
<i>Arabidopsis</i> seeds from heterozygous CLF/clf-2 cross	Planting pots and tray Plastic dome lid or plastic wrap Potting soil

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.

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.

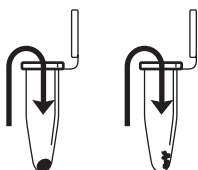


1. Moisten the potting soil. Fill planting pots evenly with soil, but do not pack tightly.
2. Fit pots into the tray, but leave one corner space empty to facilitate watering.
3. Carefully scatter seeds evenly on top of the soil.
 - a. Fold a 4 X 4 inch sheet of paper in half.
 - b. Place the seeds into 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.

II. ISOLATE DNA FROM *ARABIDOPSIS*

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

The large end of a 1,000 µl pipet tip will punch disks of this size.



After grinding, most of the tissue should be converted to a green liquid.

This step denatures proteins, including DNA-digesting enzymes

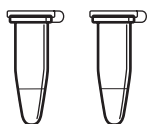
This step pellets insoluble material at the bottom of the tube.

This step precipitates nucleic acids, including DNA.

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.



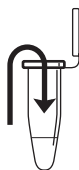
You will use 2.5 μ l of the DNA extract for the PCR reactions in Part II. The crude DNA extract contains nucleases that will eventually fragment the DNA at room temperature. Keeping the sample cold limits this activity.

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 μ l.
13. Air dry the pellet for 10 minutes to evaporate remaining isopropanol.
14. Add 100 μ l 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.

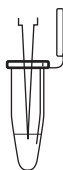
III. AMPLIFY DNA BY PCR

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



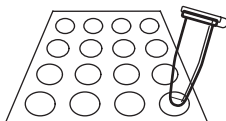
The primer loading dye mix will turn purple as the Ready-To-Go™ PCR Bead dissolves.

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 μ l 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 μ l of wild-type or *clf-2 Arabidopsis* DNA (from Part II).



If the reagents become splattered on the wall of the tube, pool them by pulsing in a microcentrifuge or by sharply tapping the tube bottom on the lab bench.

The mineral oil prevents the PCR mix from evaporating and condensing on the tube cap during cycling. Most modern thermal cyclers have heated lids that prevent condensing and DO NOT require the addition of mineral oil.



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

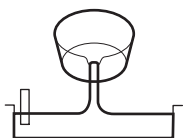
Reagents

* Wild-type or *clf-2 Arabidopsis* PCR product (from Part III)
 *pBR322/*Bst*NI marker, 130 μ l
 1X TBE, 300 ml
 2% agarose in 1X TBE, 50 ml
 Ethidium bromide (1 μ g/ml), 250 ml
 or
CarolinaBLU[™] gel & buffer stain, 7 ml
CarolinaBLU[™] final stain, 250 ml

*Store on ice

Supplies and Equipment

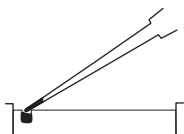
Micropipet and tips (1-100 μ l)
 Microcentrifuge tube rack
 1.5 ml microcentrifuge tube
 Gel electrophoresis chamber
 Power supply
 Staining trays
 Latex gloves
 UV transilluminator for use with ethidium bromide)
 White light transilluminator (for use with *CarolinaBLU*[™])
 Camera (optional)
 Water bath (60°C)
 Container with cracked or crushed ice



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.



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 μ l of the combined PCR sample/loading dye mixture into your assigned well of a 2% agarose gel.
8. Load 20 μ l of the molecular weight marker (pBR322/*Bst*NI) 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.

10. Stain the gel in ethidium bromide or *CarolinaBLU*[™]:

Destaining the gel for 5-10 minutes in tap water leeches unbound ethidium bromide from the gel, decreasing background and increasing contrast of the stained DNA.

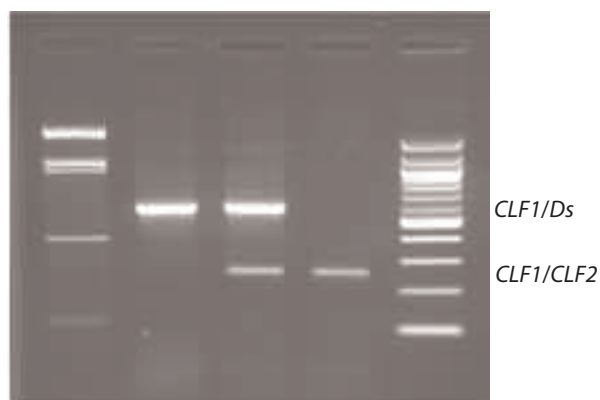
- a. For ethidium bromide, stain 10-15 minutes. Decant stain back into storage container for reuse, and rinse gel in tap water. *Use gloves when handling ethidium bromide solution and stained gel, or anything that has ethidium bromide on it. Ethidium bromide is a known mutagen and care should be taken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

Transillumination, where the light source is below the gel, increases brightness and contrast.

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.



Genotype	<i>clf-2/clf-2</i>	<i>CLF/clf-2</i>	<i>CLF/CLF</i>
Phenotype	<i>clf-2</i>	wt	wt

- 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/*Bst*NI 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/*Bst*NI 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/*Bst*NI 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/*Bst*NI 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:

<i>CLF1</i>	5'-TTAACCCGGACCCGCATTGTTTCGG-3' (Forward Primer)
<i>CLF2</i>	5'-AGAGAAGCTCAAACAAGCCATCGA-3' (Reverse Primer)
<i>CLF1</i>	5'-TTAACCCGGACCCGCATTGTTTCGG-3' (Forward Primer)
<i>Ds</i>	5'-GTCGGCGTGCGGCTGGCGGCG-3' (Reverse Primer)

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

1. Initiate a *BLAST* search.
 - a. Open the Internet site of the National Center for Biotechnology Information (NCBI), www.ncbi.nlm.nih.gov/.
 - 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 3×10^{-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>.)

- 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

1. Open the NCBI Internet site, www.ncbi.nlm.nih.gov/.
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.
5. Return to the NCBI homepage, www.ncbi.nlm.nih.gov/.
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 3×10^{-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

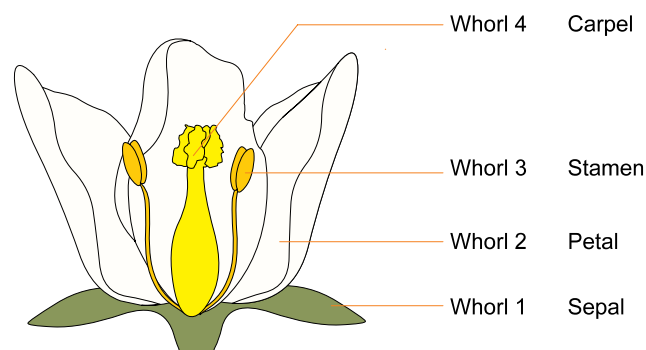
The *CLF* gene is a Polycomb-Group gene that regulates homeotic gene expression in *Arabidopsis*. Homeotic genes were first identified in *Drosophila* but have since been found in vertebrates and plants. Homeotic genes are required for the correct spatial and tissue-specific expression of target genes 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.

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.

Reagents

Arabidopsis seed from self cross of a *clf-2*
heterozygote
Edward's buffer
100% Isopropanol
Tris/EDTA (TE) buffer with RNase A
CLF1/CLF2 primer/loading dye mix*
CLF1/Ds primer/loading dye mix*
Ready-to-Go™ PCR Beads
Mineral oil (depending on thermal cycler)
DNA marker pBR322/*Bst*NI (0.075 µg/µl)*
Agarose
1x TBE electrophoresis buffer
Ethidium bromide solution, 1 µg/ml OR
CarolinaBLU™ gel & buffer stain
CarolinaBLU™ final stain

Supplies and Equipment

Planting pots and tray
Plastic dome lid or plastic wrap
Potting soil (optimized for *Arabidopsis*)
Micropipets and tips (1 – 1,000 µl)
1.5-ml microcentrifuge tubes
Microcentrifuge tube racks
Microcentrifuge for 1.5-ml tubes
Pellet pestles
Water bath or heating block (95 – 100°C)
Thermal cycler
Electrophoresis chambers
Electrophoresis power supplies
Latex gloves
Staining trays
UV transilluminator (ethidium bromide staining)
White light box (*CarolinaBLU™* staining)
Camera or photo-documentary system
Permanent markers
Container with cracked or crushed ice
Vortexer (optional)
Waterbath for agarose (60°C)

*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.

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

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 μ l 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 μ l (thaw and store on ice)

1.5 ml microcentrifuge tubes
Permanent marker
Pellet pestles
Micropipet and tips (100-1000 μ l)
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 μ l 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 μ M 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 cyclers are 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 cyclers.*

Each primer/loading dye mix incorporates the appropriate primer pair (0.25 picomoles/ μ l 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 μ l CLF1/CLF2 primer/loading dye mix (thaw and store on ice)
- 30 μ l 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 μ l)
- 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 *Bst*NI 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 μ l of a 0.075 μ g/ μ l 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/*Bst*NI 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:

Voltage	Agarose Volume	Stain volume
<50 Volts	30 ml	40 µl (1 drop)
	200 ml	240 µl (6 drops)
	400 ml	520 µl (13 drops)
>50 Volts	50 ml	80 µl (2 drop)
	300 ml	480 µl (12 drops)
	400 ml	640 µl (16 drops)

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

Voltage	Agarose Volume	Stain volume
<50 Volts	500 ml	480 µl (12 drop)
	3000 ml	3 ml (72 drops)
>50 Volts	500 ml	960 µl (24 drop)
	2600 ml	5 ml (125 drops)

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 .