



Dolan DNA Learning Center
Cold Spring Harbor Laboratory

Linkage Mapping a Mutation in *Arabidopsis*



Funded by NSF

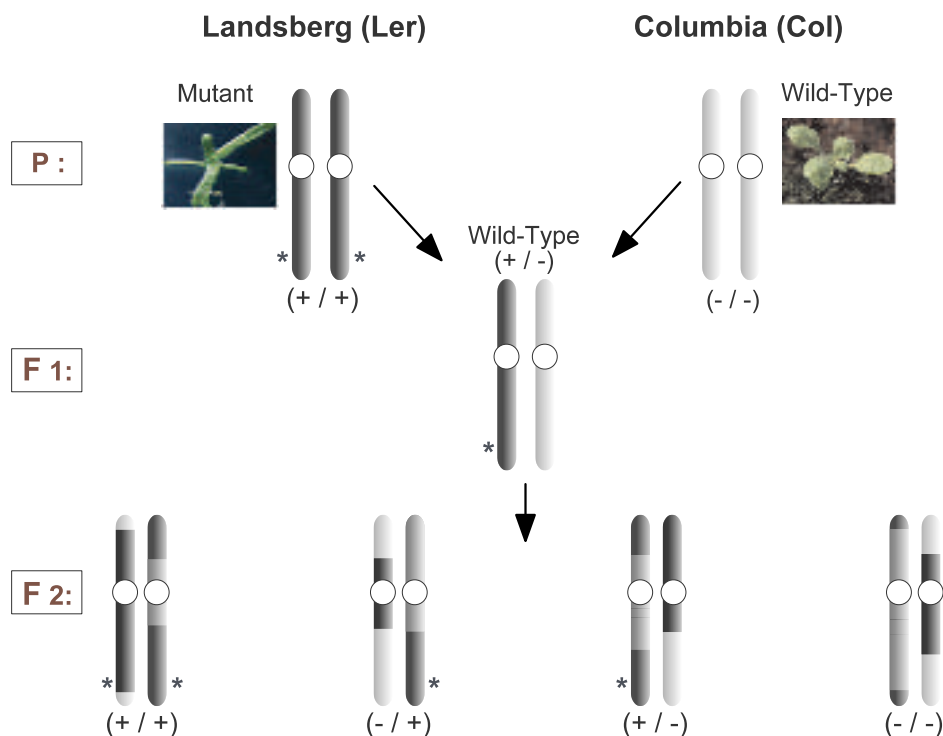
INTRODUCTION

Since Alfred Sturtevant constructed the first genetic map of a *Drosophila* chromosome in 1913, new mutations have been mapped using his method of linkage analysis. Determining the map position of a new mutation – and its corresponding gene – consists of testing for linkage with a number of previously mapped genes or DNA markers. Linkage is the principle that the closer two genes or markers are located to one another on a chromosome, the greater the chance that they will be inherited together as a unit (linked). Conversely, locations farther apart on the chromosome are more likely to be separated by chromosome recombination during meiosis. Thus, the frequency of recombination with previously mapped genes or markers allows one to determine the map position of a gene of interest.

The increasing availability of whole genome sequences and sophisticated computer software has made it possible to map genes using bioinformatic approaches. However, traditional mapping techniques are still used to map genes for which no sequence information is available – for example, mutant phenotypes produced by chemical mutagenesis. Although early gene maps relied on genes and mutations with observable phenotypes, modern gene maps are populated with DNA polymorphisms that are detected by molecular methods. In *Arabidopsis*, molecular markers exploit the natural differences between distinct ecotypes, such as the widely used Landsberg (Ler) and Columbia (Col), which differ about 1% at the DNA level.

This laboratory uses linkage to map *ago1* (*argonaute*), a Chromosome 1 mutation that produces a dwarf phenotype and serrated leaves. In preparation for this experiment, a Ler plant homozygous for the *ago1* mutation (+/+) was crossed to a wild-type Col plant (-/-). A heterozygous F₁ plant was then allowed to self-pollinate. The resulting F₂ progeny conform to the expected 1:2:1 genotypic ratio of homozygous wild-type (-/-) to heterozygous (+/-) to homozygous mutant (+/+). However, due to recombination events during gamete formation, each of the F₂ chromosomes is a mixture of the Ler and Col ecotypes.

F₂ plants that are homozygous for the *ago1* mutation are used for mapping. Since *ago1* is always in a Ler background, the number of recombination events is equivalent to the number of times the Col ecotype is found on the chromosome. Recombination events are scored by evaluating a panel of four DNA polymorphisms called CAPS (cleaved amplified polymorphic sequences) markers that identify homologous regions of the Ler and Col chromosomes. At each CAPS location (locus), a point mutation creates a restriction site in either the Ler or Col ecotype.



Development of F₂ plants to map a mutation (*).

Each marker region is amplified by polymerase chain reaction (PCR), and then cut with a specific restriction enzyme to identify a restriction fragment length polymorphism (RFLP) that distinguishes the two ecotypes. After agarose gel electrophoresis, the banding pattern indicates the ecotype source of each of the paired chromosomes: homozygous Ler/Ler, heterozygous Ler/Col, or homozygous Col/Col. By pooling class data, recombination frequencies can be calculated between *ago1* and each of the CAPS markers – allowing one to map *ago1*.

This laboratory is adapted from: “*Arabidopsis* Molecular Genetics, Course Manual,” Cold Spring Harbor Laboratory and “EMBO COURSE, Practical Course on Genetics, Genetic and Molecular Analysis of *Arabidopsis*, Module 2: Mapping mutations using molecular markers.”

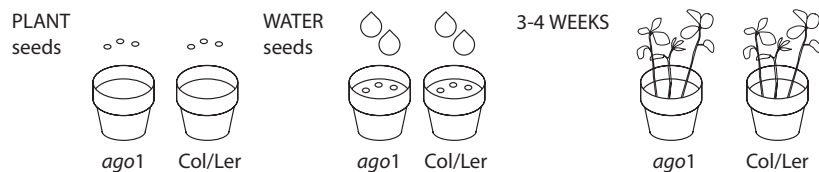
<http://www.isv.cnrs-gif.fr/embo99/manuals/>

- Bohmert, K., Camus, I., Bellini, C., Bouchez, D., Caboche, M., and Benning, C. (1998). AGO1 defines novel locus of *Arabidopsis* controlling leaf development. *EMBO J.* **17**:170–180.
- 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.
- Konieczny, A., and Ausbel, F.M. (1993). A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. *Plant J.* **4**:403–410.
- Korneef, M., Alonso-Blanco, C., and Stam, P. (1998). Martinez-Zapater, J., Salinas, J. (eds). Genetic Analysis. *Methods in Molecular Biology*. Vol. **82**:105–117.

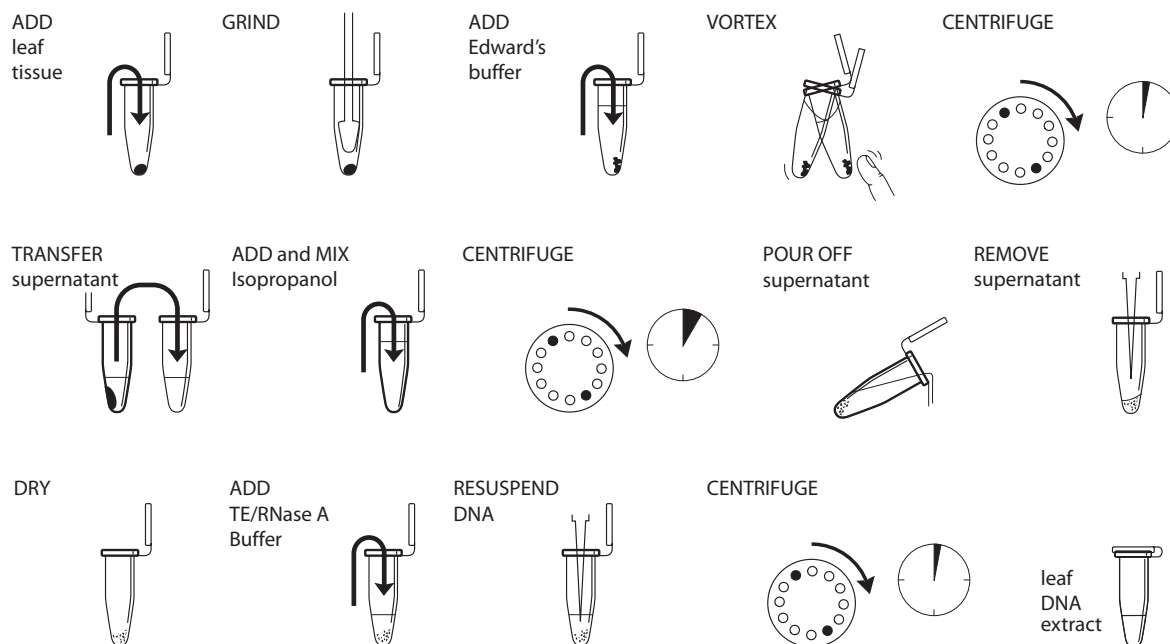
Sturtevant’s linkage experiments explained as an animation in *DNA from the Beginning*, concept 11: <http://www.dnafb.org/dnafb/11/concept/index.html>.

LAB FLOW

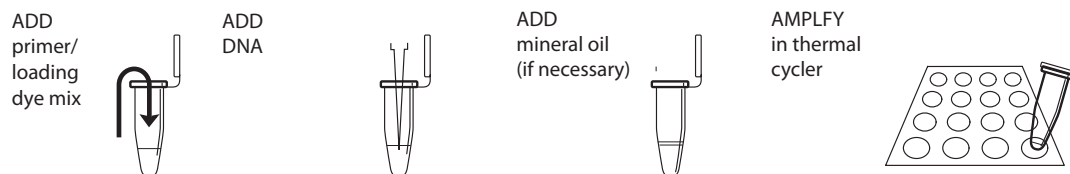
I. PLANT *ARABIDOPSIS* SEED



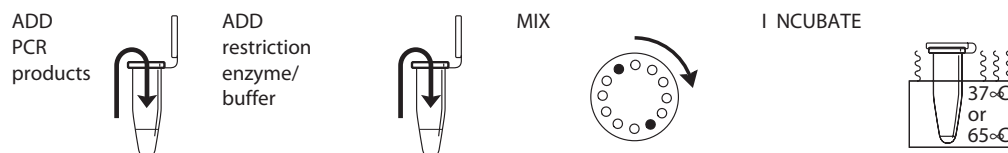
II. ISOLATE DNA FROM *ARABIDOPSIS*



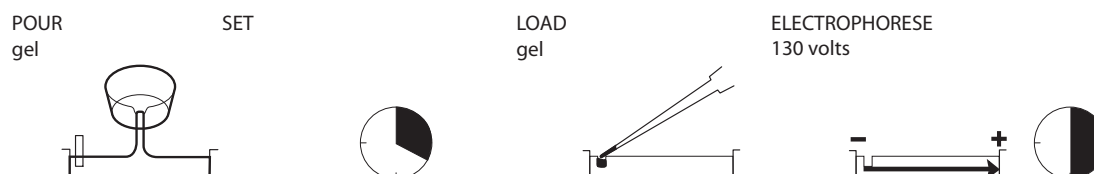
III. AMPLIFY DNA BY PCR



IV. RESTRICT PCR PRODUCTS TO PRODUCE CAPS MARKERS



V. ANALYZE CAPS MARKERS BY GEL ELECTROPHORESIS



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>.

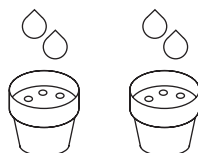
Reagents	Supplies and Equipment
<i>Arabidopsis</i> seeds from self cross of an <i>ago1</i> heterozygote Col and Ler control	Planting pots and tray Plastic dome lid or plastic wrap Potting soil

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 water in the tray at all times.

With 24-hour fluorescent lighting conditions, phenotypes can be discerned in 2-3 weeks.



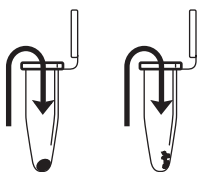
1. Moisten the potting soil. Fill planting pots evenly with soil, but do not pack tightly.
2. Label each pot with plant type.
3. Fit pots into the tray, but leave one corner space empty to facilitate watering.
4. Carefully scatter seeds evenly on top of the soil.
 - a. Fold a 4 X 4 inch sheet of paper 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
5. Cover pots with plastic dome lids or plastic wrap to assist germination. (Remove covers 3-7 days after planting.)
6. 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.
7. 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.
8. Harvest plant tissue for PCR when the wild-type and *ago1* phenotypes become evident. The dwarf *ago1* mutants are very small and have serrated leaves. 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.

II. ISOLATE DNA FROM *ARABIDOPSIS*

Reagents	Supplies and Equipment
<i>Arabidopsis</i> plants <i>ago1</i> and Col or Ler control	Container with cracked or crushed ice
Edward's buffer, 1 ml	Microcentrifuge
Isopropanol, 1 ml	Microcentrifuge tube racks
Tris/EDTA (TE) buffer with RNase A, 400 μ l	1.5 ml microcentrifuge tubes
	Micropipet and tips (100-1000 μ l)
	Pellet pestles
	Water bath or heating block (96-100°C)
	Permanent marker
	Vortexer (optional)

Each lab team should be assigned a Col or Ler control, with two teams sharing results to be evaluated with their *ago1* mutants.

The large end of a 1,000 μ L 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 one *ago1* mutant and one Col or Ler *Arabidopsis* plant.
2. From each plant, cut two pieces of tissue approximately 1/4 inch in diameter. Place the leaf tissue into clean 1.5 ml tubes, and label each 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 *argonaute* 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 each 1.5 ml tube to *forcefully* grind the plant tissue for 1 minute.
4. Add 400 μ l of Edward's buffer to each tube. Grind briefly to remove tissue from the pestle and to liquify any remaining pieces of tissue.
5. Vortex the tubes for 5 seconds, by hand or machine.
6. Boil the samples for 5 minutes in a water bath or heating block.
7. Place the tubes in a balanced configuration in a microcentrifuge, and spin for 2 minutes to pellet any remaining cell debris.
8. Transfer 350 μ l of each supernatant to a fresh tube. Maintain labels for plant phenotype and group number. Be careful not to disturb the pelleted debris when transferring the supernatant. Discard old tubes containing the precipitate.
9. Add 400 μ l of isopropanol to each tube of supernatant.
10. Mix by inverting the tubes several times, and leave at room temperature for 3 minutes.
11. Place the tubes in a balanced configuration in a microcentrifuge, and spin for 5 minutes. Align tubes in the rotor with the cap hinges pointing outward. Nucleic acids will collect on the tube side under the hinge during centrifugation.

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 III. The crude DNA extract contains nucleases that will eventually fragment the DNA at room temperature. Keeping the sample cold limits this activity.

12. Carefully pour off the supernatant from each tube, then *completely* remove the remaining liquid with a medium pipet set at 100 µl.
13. Air dry the pellets for 10 minutes to evaporate remaining isopropanol.
14. Add 100 µl of TE/RNase A buffer to each 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 solutions at room temperature for 5 minutes.
16. Microcentrifuge the tubes 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
<i>Arabidopsis</i> DNA (from Part II)*	Container with cracked or crushed ice
<i>ago1</i> and Col or Ler control	Micropipet and tips (1-100 µl)
Mineral oil, 5 ml (depending on thermal cycler)	Microcentrifuge tube rack
g4026 primer/loading dye mix, 50 µl*	Permanent marker
H77224 primer/loading dye mix, 50 µl*	Thermal cycler
m235 primer/loading dye mix, 50 µl*	
UFO primer/loading dye mix, 50 µl*	
Ready-To-Go™ PCR beads	
*Store on ice	

You will set up *ago1* and control Col or Ler reactions for each of the four CAPS markers.

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

1. Obtain 8 PCR tubes containing Ready-To-Go™ PCR Beads. Label each tube with your group number and the initials as shown below:

Tube	g4026	H77224	m235	UFO	<i>ago1</i>	Col/Ler
GA	22.5 µl				2.5 µl	
GC/GL	22.5 µl					2.5 µl
HA		22.5 µl			2.5 µl	
HC/HL		22.5 µl				2.5 µl
MA			22.5 µl		2.5 µl	
MC/ML			22.5 µl			2.5 µl
UA				22.5 µl	2.5 µl	
UC/UL				22.5 µl		2.5 µl

Add the DNA directly into the PCR reaction solution.

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. Use a micropipet with a fresh tip to add 22.5 μ l of the appropriate CAPS primer/loading dye mix to each pair of tube. Allow several minutes for beads to dissolve.
3. Use a micropipet with a fresh tip to add 2.5 μ l of *ago1* DNA (from Part II) to the appropriate reactions. Change tips for each reaction.
4. Use a micropipet with a fresh tip to add 2.5 μ l of Col/Ler DNA (from Part II) to the appropriate reactions. Change tips for each reaction.
5. 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.
6. Store samples on ice until you are ready to begin thermal cycling.
7. Program the thermal cycler for 30 cycles of the following profile. The program may be linked to a 4°C hold program after the 30 cycles are completed.

Denaturing step:	94°C	30 seconds
Annealing step:	55°C	30 seconds
Extending step:	72°C	30 seconds

8. After cycling, store the amplified DNA at –20°C until you are ready to continue with Part IV.

IV. Digest PCR PRODUCTS TO PRODUCE CAPS MARKERS

Reagents	Supplies and Equipment
<i>Arabidopsis ago1</i> and Col orLer PCR products (from Part III)	Container with cracked or crushed ice
g4026 (GA and GC or GL), 25 μ l*	Microcentrifuge tube rack
H77224 (HA and HC or HL), 25 μ l*	1.5 ml microcentrifuge tubes
m235 (MA and MC or ML), 25 μ l*	Micropipet and tips (1-100 μ l)
UFO (UA and UC or UL), 25 μ l*	Permanent marker
<i>Hind</i> III enzyme/buffer mix, 25 μ l*	Water bath or heating block (37°C)
<i>Rsa</i> I enzyme/buffer mix, 25 μ l*	Water bath or heating block (65°C)
<i>Taq</i> I enzyme/buffer mix, 50 μ l*	
*Store on ice	

Note that *RsaI* and *HindIII* reactions are incubated at 37°C, while the *TaqI* reaction is incubated at 65°C. This is because *TaqI* is isolated from the thermophile (heat-loving) bacterium *Thermus aquaticus*, which inhabits hot springs.

1. Label 8 1.5 ml tubes with your group number and the initials for PCR products from Part III, as shown below:

Tube	PCR Product	<i>RsaI</i> buffer mix	<i>TaqI</i> buffer mix	<i>HindIII</i> buffer mix	Incubate
GA	10 µl	10 µl			37°C
GC/GL	10 µl	10 µl			37°C
HA	10 µl		10 µl		65°C
HC/HL	10 µl		10 µl		65°C
MA	10 µl			10 µl	37°C
MC/ML	10 µl			10 µl	37°C
UA	10 µl		10 µl		65°C
UC/UL	10 µl		10 µl		65°C

A lengthy incubation helps insure that the restriction enzymes digest the CAPS markers completely. A partial digest can make a homozygous plant appear heterozygous, confounding analysis.

2. Use a micropipet with a fresh tip to add 10 µl of the appropriate restriction enzyme/buffer mix to the appropriate 1.5-ml tube.
3. Use a micropipet with a fresh tip to transfer 10 µl of each PCR product into the appropriate reaction. Change tips for each reaction. Store remaining PCR products at –20°C, in case you need to repeat any restriction reactions.
4. Tap tubes with finger to mix, or pulse spin in microcentrifuge.
5. Place tubes in the appropriate water bath (37°C or 65°C), and incubate for at least 1 hour.
6. Store restriction digests at –20°C until you are ready to continue with Part V.

V. ANALYZE CAPS MARKERS BY GEL ELECTROPHORESIS

Reagents	Supplies and Equipment
<i>Arabidopsis</i> CAPS markers (from Part IV)	Camera (optional)
g4026 (GA and GC or GL), 20 µl*	Container with cracked or crushed ice
H77224 (HA and HC or HL), 20 µl*	Gel electrophoresis chamber
m235 (MA and MC or ML), 20 µl*	Latex gloves
UFO (UA and UC or UL), 20 µl*	Microcentrifuge tube rack
*pBR322/ <i>Bst</i> NI marker, 130µl	1.5 ml microcentrifuge tubes
1X TBE, 300 ml	Micropipet and tips (1-100 µl)
2% agarose in 1X TBE, 50 ml	Power supply
Ethidium bromide (1 µg/ml), 250 ml	Staining trays
or	UV transilluminator for use with ethidium bromide)
CarolinaBLU™ gel & buffer stain, 7 ml	White light transilluminator (for use with CarolinaBLU™)
CarolinaBLU™ final stain, 250 ml	Water bath (60°C)
*Store on 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.

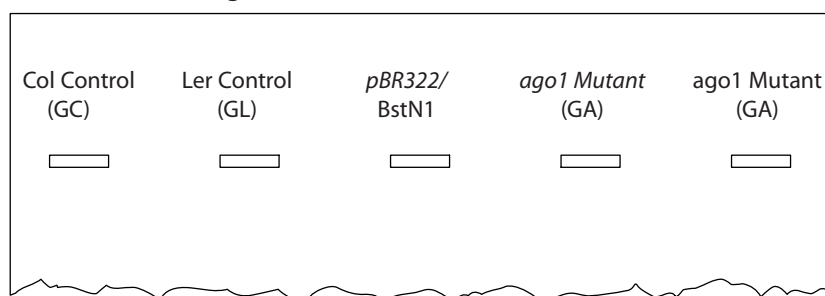
Expel any air from the tip before loading, and be careful not to push the tip of the pipet through the bottom of the sample well.

You may double comb the gel so that two CAPS panels are loaded in a single gel. A 100-bp ladder may also be used as a marker.

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.

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

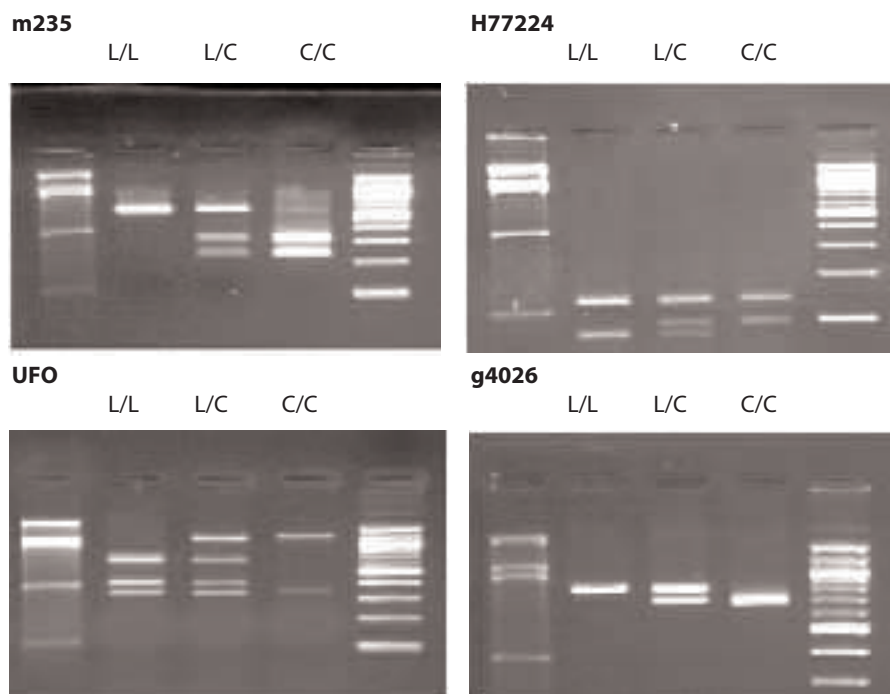
1. Seal the ends of the gel-casting tray with masking tape, and insert a well-forming comb.
2. Pour 2% agarose solution to a depth that covers about 1/3 the height of the open teeth of the comb.
3. Allow the gel to solidify completely. This takes approximately 20 minutes.
4. Place the gel into the electrophoresis 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. Use a micropipet with a fresh tip to add 20 µl of each digested CAPS marker into different wells of a 2% agarose gel. Load each of the four CAPS panels according to the scheme shown below for g4026. (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. Load 20 µl of the molecular weight marker (pBR322/BstNI) into one well.
8. 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.
9. 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.
10. View gel using transillumination, and photograph.

RESULTS & DISCUSSION

1. Observe the photograph of the stained gels containing your samples and those of other students. Orient the photograph with wells at the top. Interpret each lane of the gel. The sample gels below illustrate the CAPS genotypes of chromosomes analyzed in this experiment:

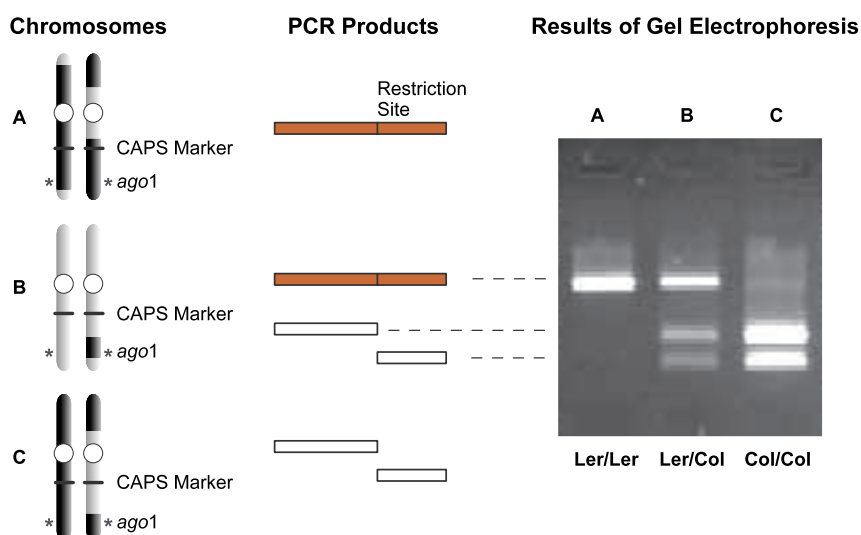


- a. Locate the lane containing the pBR322/*Bst*NI markers on the left hand side of each gel. Working from the well, locate the bands corresponding to each restriction fragment: 1,857 bp, 1,058 bp, 929 bp, 383 bp, and 121 bp (may be faint or not visible at all). Alternatively, locate the lane containing the 100-bp ladder on the right hand side of each gel. These DNA markers increase in size in 100-bp increments starting with the fastest migrating band of 100 bp.
- b. Identify each of the following fragments in the Ler (L/L) and Col (C/C) controls:

Cap Marker	Col Fragments (bp)	Ler Fragments (bp)
g4026	668, 94*, 80*, 2*	762, 80*, 2*
H77224	135, 87	135, 69, 18*
m235	305, 226	531
UFO	978, 316	600, 378, 316

*May not be visible

- c. Compare the bands present in your *ago1* plant with the Ler and Col controls, then score the genotype for each of the four CAPS markers as: Ler/Ler, Ler/Col, or Col/Col.
 - d. The incomplete digest of a homozygous marker may leave an additional faint band in position of the larger parent fragment (higher on the gel), which might be mistaken as a heterozygous result. This is seen the Col/Col (C/C) lane for the m235 marker in the top-left panel above.
2. Refer to the diagrams in the introduction and below to answer the following questions:



- a. From which *Arabidopsis* parent (P) ecotype did all of the mutants analyzed in this experiment inherit the *ago1* mutation?
 - b. In what type of chromosome background is the *ago1* mutation always found?
 - c. How many chromosomes are assayed in each CAPS experiment?
 - d. What is the significance of each of the following genotypes for a given CAPS marker?
 - Ler/Ler
 - Col/Col
 - Ler/Col
3. Calculate the recombination frequency (r) between *ago1* and each of the four CAPS markers.
- a. Compile class results for each of the four CAPS markers.

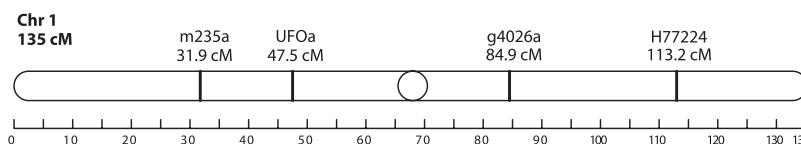
- b. Add up the total number of Col alleles for each marker.
- c. The recombination frequency (r) between each CAPS marker and *ago1* is proportional to the number of chromosomes with the Col allele, or the Col allele frequency. The recombination frequency (%) is calculated using the following formula:

$$r = 100 (\text{N Col Alleles} / \text{Total Alleles})$$

- d. Next, convert the recombination frequency (%) for each CAPS marker into a map distance (D , in cM) from the *ago1* locus. In *Arabidopsis*, a reasonable estimate of map distance is given by the Kosambi function (\ln = natural log):

$$D = 25 \times \ln [(100 + 2r) / (100 - 2r)]$$

- e. The following map shows the locations of the four CAPS makers on Arabidopsis Chromosome 1. Use the map distances (D) to locate *ago1* on Chromosome 1.



BIOINFORMATICS

Biological information is encoded in the nucleotide sequence of DNA. Bioinformatics is the field that identifies the 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 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 computers) complement experiments done *in vitro* (in glass, or test tubes). This integration of 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 products amplified by the four CAPS primer sets. In Part II you will extract the predicted sequences of the CAPS markers and store them for further manipulations. In Part III you will use an electronic tool to predict the products of restriction digests of the PCR products. In Part IV you will identify the *AGO1* gene and learn about the function of the *argonaute* protein. In Part V you will electronically map *ago1* and the four CAPS markers on *Arabidopsis* Chromosome 1.

I. Use **BLAST** to Predict the Size of the CAPS Amplicons

The following primer sets were used in the experiment:

g4026	5'-GGGGTCAGTTACATTACTAGC-3'	(Forward Primer)
	5'-GTACGGTCTCTTCCCTTA-3'	(Reverse Primer)
H77224	5'-GGATTGGGGAAGAGGAAGTAA-3'	(Forward Primer)
	5'-TCCTTAGCCTTGCTTGATAGT-3'	(Reverse Primer)
m235	5'-GAATCTGTTTCGCCTAACGC-3'	(Forward Primer)
	5'-AGTCCACAACAATTGCAGCC-3'	(Reverse Primer)
UFO	5'-GTGGCGGTTTCAGACGGAGAGG-3'	(Forward Primer)
	5'-AAGGCATCATGACTGTGGTTTTTC-3'	(Reverse Primer)

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 g4026 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 (*sbjct*).
 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 equals 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. Record these start and stop positions in the table below.

Marker	Start Position	Stop Position	Amplicon (bp)	gi-number
g4026				gi-2252639
H77224				gi-18410209
m235				gi-2098816
UFO				gi-9690193

- e. Calculate the predicted length of the amplicon and record in the table. Don't forget to add one nucleotide to your result!
- f. Repeat Steps a-e above for the remaining three CAPS primer sets.

II. Extract and Store Predicted CAPS Marker Sequences

1. Follow the *gi* – link for the g4026 marker 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>.)
2. Scroll down to the sequence record. Extract the predicted CAPS marker by highlighting and copying the region between the start and stop positions listed on your chart.
3. Paste the predicted CAPS marker into a text file, such as MS Word, Wordpad, or Notepad.
4. Repeat steps 1–3 with the remaining three primer sets, and save the file. This sequence repository will be used in the following sections.

III. Digest CAPS Markers Electronically (*In Silico*)

1. Open
http://www.dnalc.org/bioinformatics/nucleotide_analyzer.htm#clipper.
2. Copy the g4026 marker from your sequence repository, and paste it into the sequence window.
3. Enter the name of the restriction enzyme used to produce the CAPS marker in the experiment.
4. Record the length of the predicted restriction fragments in the table below.

Marker	Restriction Enzyme	Amplicon (bp)	Restriction Fragments (bp)
g4026	<i>RsaI</i>		
H77224	<i>TaqI</i>		
m235	<i>HindIII</i>		
UFO	<i>TaqI</i>		

5. Repeat steps 1–4 with the remaining three markers.
6. Compare the results of your electronic (*in silico*) and electrophoretic (*in vitro*) analyses. Which fragments are missing and why?
7. Which *Arabidopsis* ecotype was used for the *Arabidopsis* Genome Sequencing Project?

IV. Use *BLAST* to Identify the *AGO1* Gene

1. Open the NCBI Internet site, www.ncbi.nlm.nih.gov/.
2. Click on *Map Viewer from the Hot Spots* list on the right.
3. In *Search* window at the top of the page, select *Arabidopsis thaliana* (*thale cress*), enter *ago1*, and click on *Go!*
4. Click on the link to the chromosome that shows a hit.
5. Click on the *genes seq* link or use the *zoom* feature on the left to enlarge the structure of the *AGO1* gene. How long is the gene, and how many exons and introns does it have?
6. Follow links in the pink speed bar to learn about the function of the argonaute protein and its functional domains.
7. Click on At2g23380 (*Arabidopsis thaliana* gene ID).
8. Scroll down the page to Related Sequences, and click on *mRNA U91995*.
9. Extract (highlight and copy) the coding sequence at the bottom of the page. Then paste this *AGO1* gene sequence into the sequence repository you started in Part II.

V. Map *AGO1* and CAPS Markers Electronically

1. Open the NCBI Map Viewer, <http://www.ncbi.nlm.nih.gov/mapview/>.
2. Click on the *BLAST* link to the left of *Arabidopsis thaliana* (*thale cress*) in the *Plants list*.
3. Copy all of the CAPS markers and the *AGO1* gene from your sequence repository, and paste them into the sequence window.
4. Click on *Begin Search*.
5. Click on *Format!* and wait for your results.
6. Click *Genome View*.
7. Click on the link to the chromosome that shows the hits.
8. Click on the pink *Blast hit* link to identify the locations of the *AGO1*.

9. Use the chart below to record the answers to the following questions:

- a. What is the order of the five search sequences on the chromosome?
- b. Use the ruler on the left to determine the map positions for the CAPS markers and the *AGO1* gene.

M = Mb = megabasepairs = 1 millions basepairs

Locus	Chromosome Order	Map Position (Mb)
AGO1		
g4026		
H77224		
m235		
UFO		

10. Which CAPS marker is located most closely to *ago1*? How many Mb separate them?

Instructor Information

REAGENTS, SUPPLIES & EQUIPMENT CHECK LIST

Reagents

Arabidopsis seed
ago1 heterozygous cross
 Col and/or Ler control
 Edward's buffer
 100% Isopropanol
 Tris/EDTA (TE) buffer with RNase A
 g4026 primer/loading dye mix*
 H77224 primer/loading dye mix*
 m235 primer/loading dye mix*
 UFO primer/loading dye mix*
 Ready-to-Go™ PCR Beads
 Mineral oil (depending on thermal cycler)
*HindIII**
*RsaI**
*TaqI**
 10X BSA*
 10X *HindIII* restriction buffer*
 10X *RsaI* restriction buffer*
 10X *TaqI* restriction buffer*
 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 microcentrifuge tubes
 Microcentrifuge tube racks
 Microcentrifuge for 1.5-ml tubes
 Pellet pestles
 Two Water baths or heating blocks (37-100°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)

*Store at -20°C

Ready-to-Go™ PCR Beads incorporate *Taq* DNA 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:

- Linkage mapping, genetic linkage, and recombination.
- Restriction fragment length polymorphisms as molecular markers.
- 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).
- Restriction analysis.
- 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. Digest PCR Products	3	30-60 min. 15-30 min. 60+ min.	Pre-lab: Make restriction enzyme/buffer mixes; Set up student stations Set up restriction reactions
V. Analyze Amplified DNA by Gel Electrophoresis	4	30 min.	Post-lab: Incubate restriction reactions Prepare agarose gel solution and cast gels
	5	30 min. 30+ min. 20+ min. 20 min. to overnight 20 min.	Load DNA samples into gels Electrophorese samples Post-lab: Stain gels Post-lab: De-stain gels Post-lab: Photograph gels

I. Plant *Arabidopsis* Seed

Obtain seeds from plants that are homozygous for the *ago1* mutation (in a Landsberg background). Also obtain seeds from Columbia (Col) and Landsberg (Ler) plants to use as controls. *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)	<i>item number 66-5666</i>
Poly-Flats (6-cm deep cells that can be separated into individual pots)	
8-Cell Tray	<i>item number 66-5668</i>
24-Cell Tray	<i>item number 66-5669</i>
36-Cell Tray	<i>item number 66-5670</i>
Redi-Earth Soil (8 lb. Bag)	<i>item number 15-9701</i>

II. Isolate DNA from *Arabidopsis*

This experiment is set up so that each team extracts DNA from an *ago1* mutant plant – and either a Col or Ler control. Thus two teams ultimately share Col and Ler controls for analysis on agarose gels. Four PCR reactions must be set up for each plant analyzed. The number of PCR reactions can be further reduced, depending on the number of lanes available in the agarose gels cast by your apparatus. The electrophoretic panel for each CAPS marker should include 1 Col and 1 Ler control, and 1 size marker. The rest of the available lanes in the gel can be filled with the *ago1* mutants of different lab teams. So, up to five teams could share 1 Col and 1 Ler control on a typical mini-gel with 8 lanes.

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 tube using a plastic pestle. A 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)

Arabidopsis plants
 ago1 mutant and Col or Ler control
Edward's buffer, 1 ml
Isopropanol, 1 ml
Tris/EDTA (TE) buffer with RNase A, 400 μ l (thaw and store on ice)

Container with cracked or crushed ice
Microcentrifuge tube rack
1.5 ml microcentrifuge tubes
Micropipet and tips (100-1,000 μ l)
Pellet pestles
Permanent marker

Shared Items

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

III. AMPLIFY DNA BY PCR

This method uses four PCR reactions to analyze each plant. Each PCR reaction requires a specific primer set to amplify one of four CAPS markers on chromosome 1: g4026, H77224, m235, and UFO. The table below shows the expected PCR product size for each CAPS marker.

CAPS Marker	g4026	H77224	m235	UFO
PCR product (bp)	844	222	531	1294

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 *ago1* 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)

ago1 and *Col* or *Ler Arabidopsis* DNA, from Part II (store on ice)
50 µl g4026 primer/loading dye mix (thaw and store on ice)
50 µl H77224 primer/loading dye mix (thaw and store on ice)
50 µl m235 primer/loading dye mix (thaw and store on ice)
50 µl UFO primer/loading dye mix (thaw and store on ice)
8 Ready-To-Go™ PCR Beads (in PCR tubes)

Container with cracked or crushed ice
Permanent marker
Microcentrifuge tube rack
Micropipet and tips (1-100 µl)
Mineral oil, 5 ml (depending on thermal cycler)

Shared Item

Thermal cycler

IV. Digest PCR PRODUCTS TO PRODUCE CAPS MARKERS

It is always prudent to analyze PCR products by agarose gel electrophoresis to confirm that the amplification has produced the expected amplicons. However, gel analysis adds one or two additional lab periods to the experiment. So, as a time saver, this step has been omitted from this method. If you like, you may have students electrophorese 5 µl of each PCR product on a 2% agarose gel. Save the remaining 20 µl of each PCR product for Part IV.

In Part IV, restriction digests of PCR products (from Part II) generate RFLPs that differentiate chromosomes of the *Col* and *Ler* ecotypes. Students will set up separate restriction digests for each CAPS marker, using DNA isolated from an *ago1* mutant and a *Col* or *Ler* control. It is imperative to use the correct reaction conditions

for each CAPS marker. Each of three restriction enzymes requires a different buffer, and *TaqI* requires incubation at 65°C. To simplify the method and minimize error, we recommend that a combination restriction enzyme/buffer mix be made up before class, according to the proportions below. The restriction enzyme/buffer mix can be stored on ice for several hours before use. Do not freeze, as the water in the mix will form ice crystals that will destroy enzyme activity.

1 µl of 10X BSA
 1 µl of 10X restriction buffer (from supplier)
 7 µl of distilled water
 1 µl of restriction enzyme

The table below shows the restriction enzyme specific for each CAPS marker, incubation temperature, and predicted sizes of resulting RFLPs. Note that *HindIII* and *RsaI* are incubated at 37°C, while *TaqI* is incubated at 65°C. Incubate reactions for 60 minutes or more to insure complete cutting. If you like, reactions can be incubated for several hours or even overnight. After several hours at incubation temperature, the enzymes denature and the reaction halts.

CAPS Marker	Restriction Enzyme	Incubation Temperature	Col Fragments (bp)	Ler Fragments (bp)
g4026	<i>RsaI</i>	37°C	668, 94*, 80*, 2*	762, 80*, 2*
H7722	<i>TaqI</i>	65°C	135, 87	135, 69, 18*
m235	<i>HindIII</i>	37°C	305, 226	531
UFO	<i>TaqI</i>	65°C	978, 316	600, 378, 316

*May not be visible

Pre-lab Set Up (per student lab team)

g4026 *ago1* PCR product, from Part III (thaw and store on ice)
 H77224 *ago1* PCR product, from Part III (thaw and store on ice)
 m235 *ago1* PCR product, from Part III (thaw and store on ice)
 UFO *ago1* PCR product, from Part III (thaw and store on ice)
 g4026 Col or Ler PCR product, from Part III (thaw and store on ice)
 H77224 Col or Ler PCR product, from Part III (thaw and store on ice)
 m235 Col or Ler PCR product, from Part III (thaw and store on ice)
 UFO Col or Ler PCR product, from Part III (thaw and store on ice)
HindIII enzyme/buffer mix, 25 µl (on ice)
TaqI enzyme/buffer mix, 50 µl (on ice)
RsaI enzyme/buffer mix, 25 µl (on ice)

Container with cracked or crushed ice
 Microcentrifuge tube rack
 1.5 ml microcentrifuge tubes
 Micropipet and tips (1–100 µl)
 Permanent marker

Shared Items

37°C water bath or heating block
 65°C water bath or heating block

V. ANALYZE CAPS MARKERS 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 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)

Arabidopsis restriction products from Part IV (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
*Carolina*BLU™ gel & buffer stain, 7 ml
*Carolina*BLU™ final stain, 250 ml

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

Shared Items

Transilluminator with camera
Water bath for agarose solution (60°C)

***Carolina*BLU™ STAINING**

Post-Staining

1. Cover the electrophoresed gel with the *Carolina*BLU™ 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

*Carolina*BLU™ 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 *Carolina*BLU™ 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 *Carolina*BLU™ 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.

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 drops)
	300 ml	480 µl (12 drops)
	400 ml	640 µl (16 drops)

Use the table below to add the appropriate volume of *Carolina*BLU™ stain to the agarose gel:

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 drops)
	2600 ml	5 ml (125 drops)

ANSWERS TO DISCUSSION QUESTIONS

2.

- a. From which *Arabidopsis* parent (P) ecotype did all of the mutants analyzed in this experiment inherit the ago1 mutation? Ler
- b. In what type of chromosome background is the ago1 mutation always found? Ler
- c. How many chromosomes are assayed in each CAPS experiment? Two
- d. What is the significance of each of the following genotypes for a given CAPS marker?

Ler/Ler: the ago1 mutation has remained linked to CAPS marker on each of the two chromosomes.

Col/Col: there has been recombination between ago1 and the CAPS marker on each of the two chromosomes.

Ler/Col: the ago1 mutation is linked to the CAPS marker on one chromosome and has recombined.