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Using SNPs to Map Genes that Control Maize Leaf Angle

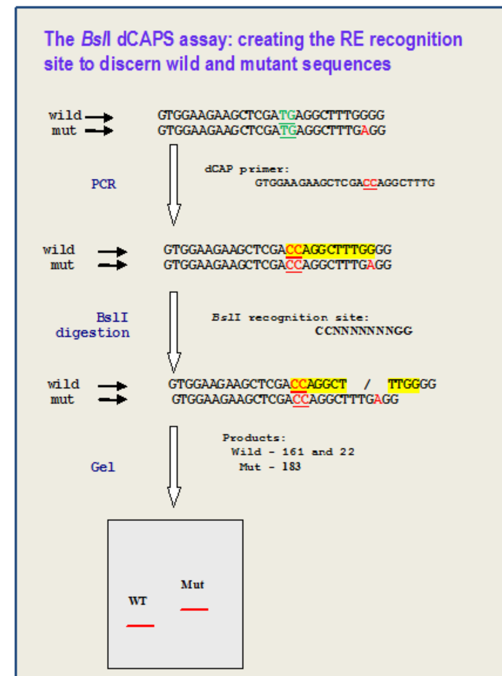
Section 3: Genotyping SNPs

The Genotype:

In this activity, the individual genotypes of six maize lines will be determined and evaluated. Here, students will use PCR to amplify a piece of DNA from certain maize genotypes that harbor a variable region of the genome. They will use a restriction enzyme designed to cut the amplified sequence at a recognition domain ONLY if a certain allele is present. Performing the restriction digest followed by visualization on an electrophoresis gel will enable dissection of an individual's genotype at the amplified locus.

Polymerase chain reaction (PCR) is a method widely used in molecular biology to make many copies of a specific DNA segment.

Derived Cleaved Amplified Polymorphic Sequences (dCAPS) assay is a technique used for detection of Single Nucleotide Polymorphisms (SNPs).
In a dCAPS assay, one or more mismatches in a PCR primer are used to create a restriction endonuclease-sensitive polymorphism based on the target SNP. The PCR product modified in this manner is then subjected to restriction enzyme digestion, and the presence or absence of the SNP is determined by the resulting restriction pattern via agarose gel electrophoresis.



dCAPS assays are useful for genotyping known SNPs and genetic mapping of isolated DNAs.

Restriction endonuclease is an enzyme that cleaves DNA into fragments at or near specific recognition sites.

Agarose gel electrophoresis is a method used to separate a mixed population of biomolecules, such as DNA, in a matrix of agarose. By applying an electric field to move the charged molecules through an agarose matrix, the biomolecules are separated by size. In this case, DNA molecules will be separated by length.

Before Your Class…

Set up the program in PCR machine (thermal cycler)

If you use the PCR machine provide by Donald Danforth Plant Science Center, the PCR reaction and Restriction Endonuclease reaction have already been preprogramed in the machine as "pcr reaction" and "re reaction", respectively. Connect the power core of PCR machine with power

and turn it on. Select the "*protocol library*" and find "*pcr reaction*" or "*re reaction*" for the corresponding part.

You also can set up the program in your PCR machine.

Program your PCR machine for *PCR reaction* as follows:

Step	Temperature	Time	Number of Cycles
Initial Denaturation	95°C	2 minutes	1 cycle
Denaturation	95°C	0.5minute	34 cycles
Annealing	60°C	0.5minute	
Extension	72°C	0.2min	
Final Extension	72°C	5 minutes	1 cycle
Soak	4°C	Indefinite	1 cycle

Program your PCR machine for *Restriction Endonuclease reaction* as follows:

Step	Temperature	Time	Number of Cycles
Incubation	37°C	180 minutes	1 cycle
Soak	4°C	Indefinite	1 cycle

Make 1XTAE buffer

1. Measure out 14ml of 50xTAE buffer using a 15ml purple cap tube and pour the buffer into a 1L bottle.
2. Fill the bottle with distilled water to 700ml line.
3. Close the cap of the bottle and mix the buffer by shaking the bottle.

dCAP marker information for genotyping corn seedling leaf angles

Marker	Primer pair	RE	Recognition site	SNPs				Allele will be digested
				Chromosome	Position	Names	Alleles	
Marker2	251/252	HindIII	AAGCTT	6	167984060	ss196521458	G/A	G

DNA samples

DNA Sample Name	Genotype
1	CML102
4	B73
7	CML228
10	CML 52
13	P39
22	Oh43

**two of these lines (CML228 and Oh43) were phenotyped in the previous sections

List of Materials Needed for Section 3

0.2 ml PCR tubes
0.2 ml PCR tubes with DNA samples (kept in freezer)
1.5 ml tubes
50xTAE running buffer (kept in room temperature)
50ml purple cap tube
15ml purple cap tube
1L bottle
8-well comb
Blue freezer box (kept in freezer)
CutSmart buffer (kept in freezer)
DMSO (It helps to relieve secondary structures when amplifying high GC templates. **Note: It should be kept in room temperature.**)
Gel marker (kept in freezer)
Gel box
Gel tray
Gloves (The gel contains SYBR safe DNA Gel Stain, which is a less hazardous alternative to ethidium bromide)
Ice bucket (not included)
Lid of gel box
Marker (dCAPs marker/a pair of primers, kept in freezer)
Microwave (**Not for Food**)
Mini Microcentrifuge for 1.5ml tube
Mini Microcentrifuge for PCR tube strip
Nuclease-free water
P-20 micropipette
P-200 micropipette
PCR machine (thermal cycler)
Power supply for gel box
Pre-prepared 4% agarose gel (kept in room temperature)
Promega GoTaq Master Mix (kept in freezer)
Restriction enzyme(s) (kept in freezer)
SYBR safe DNA Gel Stain (kept room temperature)
Tips for micropipette
UV box
Wedges

Part 7: Set up PCRs:

1. Thaw the Promega GoTaq Master mix, Marker, and nuclease-free water (*these will be labeled at each station*). Once they thaw, place them in an ice bucket with ice.
2. Take a 1.5ml tube, label the tube as "Green MasterMix+Marker", put the tube in the ice bucket.

3. Using P-20 and P-200 micropipettes, add the following to the "Green MasterMix+Marker" tube on ice:

   14µl*7 nuclease-free water
   4µl *7 Marker
   2µl *7 DMSO (**kept in room temperature**)
   25µl*7 GoTaq Green Master Mix

   Note: we make "Green MasterMix+ Marker" for 7 reactions but only use 6 reactions in step 6.

4. Gently mix the solution in the "Green MasterMix+ Marker" tube by finger-tapping the bottom of the tube. Quickly spin the solution down for 2-3 seconds in a Mini Microcentrifuge that holds 1.5ml tubes, and place the tube back on ice to keep it cold.
5. Take out the 6, 0.2ml PCR tubes (in a strip) with a DNA sample (5µl) in each tube (*a set of DNA samples that are labeled for each group*).
6. Aliquot 45µl of the "Green MasterMix+ Marker" into each 0.2ml PCR tube for a total volume of 50µl in each tube. Close the cap of 0.2ml PCR tube and your PCR is ready to go.
7. Put the PCR tubes in the PCR machine, or thermal cycler, and start the program. The reaction will proceed as follows (*once programmed, the machine will automate the entire reaction*):

Step	Temperature	Time	Number of Cycles
Initial Denaturation	95°C	2 minutes	1 cycle
Denaturation	95°C	0.5minute	34 cycles
Annealing	60°C	0.5minute	
Extension	72°C	0.2min	
Final Extension	72°C	5 minutes	1 cycle
Soak	4°C	Indefinite	1 cycle

PCR products can be stored in freezer for months.

Part 8: Set up Restriction Endonuclease reactions:

1. Thaw the 10xCutSmart buffer and nuclease-free water, and once they are thawed place them in the ice bucket with ice.
2. Take out the Restriction endonuclease from the freezer and place it in the blue freezer box (included in the trunk). The Freezer box should be precooled in the freezer the day before. **Note: Restriction endonucleases should always stay cold to keep their enzymatic activity.**
3. Take a new 1.5ml tube, label it as "RE mix", and put the tube on ice.
4. Using a P-20 micropipette, add the following to the "RE mix" tube on ice:

   2µl *7 nuclease-free water
   2.5µl*7 10XCutSmart buffer
   0.5µl*7 Restriction endonuclease (*enzyme assigned to each group*)

   Note: we make "RE mix" for 7 reactions but only use 6 reactions in step 6.

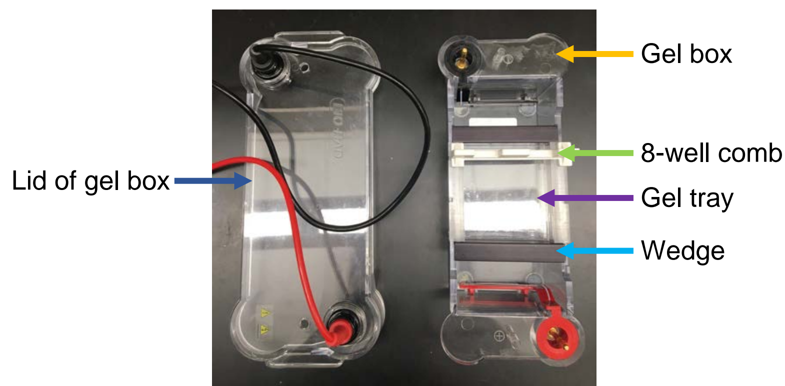
5. Gently mix the solution in the "RE mix" tube by finger-tapping the bottom of the tube. Quickly spin the solution down for 2-3 seconds in a Mini Microcentrifuge that holds 1.5ml tubes and put the tube back on ice to keep it cold.
6. Take 6 new 0.2ml PCR tubes and label them with "RE+DNA sample name" and aliquot 5µl "RE mix" into each 0.2ml PCR tube. The DNA sample name corresponds to the DNA sample name of each PCR reaction (from **Part 7: Set up PCRs**).
7. Pipet 20µl PCR product from the individual PCR reactions (from **Part 7: Set up PCRs**) into the bottom of the corresponding PCR tube with 5µl "RE mix" for a total volume of 25µl. Repeat this step for the other 5 PCR reactions. Close the cap of 0.2ml PCR tube and mix gently by finger-tapping the bottom of the tube. Quickly spin the solution down for 2-3 seconds in a Mini Microcentrifuge that holds PCR tube strips. If a Mini Microcentrifuge that holds PCR tube strips is not available, you can finger-tap the tube gently until the solution settles to the bottom of the PCR tube.
8. Put the Restriction Endonuclease reactions in the "RE+DNA sample name" tubes in the PCR machine to incubate at 37˚C for 3h.

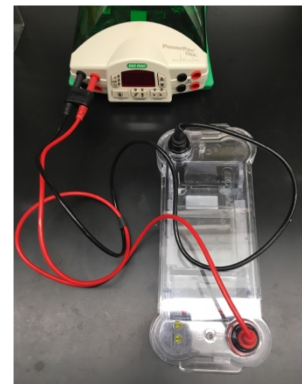
Restriction Endonuclease reactions can be stored in freezer for months.

Note: DO NOT PUT Restriction Endonuclease reactions at room temperature for a long time.

Part 9: Pour a Mini gel for analysis of Restriction Endonuclease reactions.

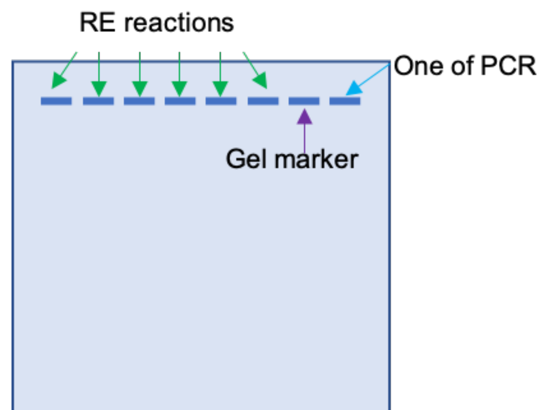
1. Place the gel box on a level surface.
2. Place a gel tray in the gel box, and wedges at either end (See image below).
3. Set an 8-well comb into the gel tray so that the sample wells are near the cathode (black). DNA fragments will migrate toward the anode (red) during electrophoresis.
4. **Note: teacher should perform this step**. Loosen the cap of a gel bottle and completely melt the pre-prepared 4% agarose gel in a microwave for 3 minutes. Open the cap of the gel bottle and use P-20 micropipette to add 10µl SYBR safe DNA Gel Stain to the melted gel (Wear gloves!). Close the cap of bottle and slowly swirl the gel bottle to mix the Gel stain with melted gel thoroughly. Slowly swirl every other 30 second for about 3 minutes to homogenously cool down the gel. **Note: immediately move to the next step to avoid the 4% agarose gel getting solid.**
5. Measure out 30ml of melted 4% agarose gel using a 50ml blue cap tube and pour the gel into the gel tray. Let the gel harden (~10min).



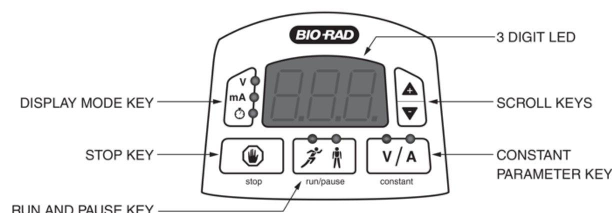


Part 10: Run the Mini gel to analyze Restriction Endonuclease reactions.

1. Carefully remove the 8-well comb from the solidified gel and the wedges from gel box.
2. Pour 1X TAE running buffer into the gel box to submerge the gel beneath 2 to 6 mm of buffer.
3. For the 6 Restriction Endonuclease reactions (RE reactions) (from **Part 8: Set up Restriction Endonuclease reactions**), Pipet 15µl of an individual reaction and load it into individual wells (1-6) using a P-20 micropipette. Using a P-20 micropipette, pipet 5µl of the gel marker and load it into the 7th well. Also, load 15µl of the remaining PCR product from any one of the PCR reactions (from **Part 7: Set up PCRs**) into the 8th well (See diagram below).



4. Place the lid on the gel box carefully without disturbing the samples. The lid should attach to the box in only one orientation. To attach the lid correctly, match the red and black banana jacks on the lid with the red and black banana plugs on the box.
5. Press the power switch located on the right side of the power supply to turn on power.
6. Connect the gel box to the power supply. The power leads are color-coordinated to the output terminals in red and black. **Note: power leads must be inserted perpendicular to the curve of the case.**
7. Press the CONSTANT PARAMETER KEY to select voltage, then use the SCROLL KEYS to set voltage to 120 volts.
8. Use the DISPLAY MODE KEY to select time, and use the SCROLL KEYS to set to 25 minutes.
9. Press the RUN AND PAUSE KEY to start the run. The run LED is lit.



10. When a run is completed, i.e., a timed run has ended or an untimed run is stopped, press the STOP KEY and then turn the power switch to the off position.
11. Remove the gel from the gel tray and place it on a UV box in a darkened room.
12. Record the resulting restriction patterns and corresponding DNA sample IDs, marker used for PCR, and restriction endonuclease used for digestion.

Part 11: Clean up.

1. Once finished with reagents, all remaining reagents should be placed back in their storage temperatures.
2. When you finish your genotyping experiment, the remaining PCR reactions and RE reactions in 0.2ml PCR tubes, as well as the gel, can be trashed. Empty the gel box and rinse gel box, gel tray, 8-well comb, and wedges with clean water. Allow them to air dry and repack them in the way they were received.
3. Clean the surface of the UV box with a dampened paper towel.
4. Repack everything in the way they were received.

DNA sample name	Primers	Restriction Endonuclease	Restriction Pattern