Using "Reverse Genetics" to Learn the Biological Function of a Gene

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Abstract: Reverse genetics begins with the DNA sequence of a gene and works toward ascertaining the mutant phenotype. Because its genome is fully sequenced, the plant *Arabidopsis thaliana* is amenable to reverse genetics. A large collection of T-DNA insertion lines has been generated and sites of insertion within the Arabidopsis genome have been mapped. This workshop describes use of websites to identify T-DNA insertion mutations in a gene of interest and to design PCR primers that identify plants that are homozygous or heterozygous for the insertion. The exercise reinforces concepts in gene segregation through the genotyping of individual plants from insertion lines.

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Introduction

With the completion of the genome sequencing projects of many model genetic organisms, the next major task is to determine the function of all of the genes. Reverse genetics is a powerful approach to achieve this goal. In this approach, functional study of a gene starts with the gene sequence rather than a mutant phenotype. Using techniques such as insertional mutagenesis, a gene's function is altered and the effect on the organism is analyzed. Because its genome has been completely sequenced, the small dicotyledonous plant *Arabidopsis thaliana* is particularly amenable to reverse genetics. Moreover, many Arabidopsis resources are publicly available and plants are easy to grow. The Arabidopsis research community has generated a large collection of T-DNA insertion lines and the Salk Institute Genome Analysis Laboratory (SIGnAL) has used high-throughput sequencing methods to identify the sites of T-DNA insertion within the genome. The T-DNA insertions not only alter the genes into which they insert but serve as tags for identifying the sites of insertion.

As part of a laboratory course in Genetics and Biotechnology, we have developed an exercise in which students use the TAIR and SIGnAL websites to identify T-DNA insertion mutations in a gene of interest. Using the SIGnAL website, they design PCR primers that will distinguish plants that are homozygous for, heterozygous for, or completely lacking the T-DNA insertion. Seed stocks of insertion lines for the gene of interest are obtained from the Arabidopsis Biological Resource Center at Ohio State, grown to seedling stage and DNA is extracted from the leaves of individual plants for genotyping by PCR. This exercise not only introduces students to the power of reverse genetics, it reinforces basic concepts in gene segregation through the genotyping of individual T_3 plants from insertion lines.

The course in which we use this exercise is taken by junior and senior biology majors, and it is assumed that the students already have basic knowledge of genetic principles. The exercise is carried out in two laboratory sessions. The first session is web-based and requires about 1 hour to complete. We overlap this with another exercise that the students are about to complete and do not need the entire lab period for. In this first session, students are given the name of a gene of interest and they use that information to identify several T-DNA insertions in that gene. The second session is where the students genotype the plants. DNA extraction and setting up of the PCR reactions requires 1-1.5 hours and students complete these steps during the scheduled lab time. Students come in on their own time to run agarose gels of the completed PCR reactions. (When these lab sessions are done in back-to-back weeks, there is not adequate time between sessions to order plant seeds with the desired insertion and grow the plants to the appropriate stage for DNA extraction. We choose a gene or two to study before the course begins and order the seeds well in advance of when needed so that we have the Arabidopsis seedlings ready for the students the week after they have done the website searches.)

Advance instructor set-up for this lab includes identification and ordering of seeds with the desired T-DNA insertions and growth of the Arabidopsis plants to the rosette stage (seedlings with multiple true leaves). Seeds require cold treatment for several days before sowing. Seedlings reach the rosette stage in approximately 3 weeks post-germination. Other advance preparations included ordering of custom PCR primers, preparation of buffers, and distribution of reagents into aliquots for student use. The total hands-on preparation time, including identifying T-DNA-containing seed stocks and ordering, is approximately 10-12 hours.

Student Outline

Introduction

The *Arabidopsis thaliana* genome is sequenced and annotated. It contains 26,207 genes. With this information, the goal of understanding the function of each of these genes becomes possible. Once the goal is achieved, we will understand in detail how a plant functions as a biological system. In addition to forward genetics, reverse genetics is another powerful approach to achieve this goal. Reverse genetics is a strategy to determine a particular gene's function by studying the phenotypes of individuals with alterations in this gene. The most effective way to destroy the function of a gene in Arabidopsis is through Agrobacterium-mediated T-DNA insertion into this gene. Because the target of the T-DNA is random, one has to create many T-DNA insertional mutations throughout the genome (usually, but not always, one T-DNA per genome) in order to target a specific gene.

Large scale T-DNA insertional mutations have been created by a few laboratories worldwide, one of which is the Salk Institute (http://signal.salk.edu/tabout.html). Once the insertional mutants have been generated, they are sequenced for genomic regions flanking the T-DNA. The sequence information combined with the annotated genome sequence allows the mapping of each T-DNA insertional site. So far, more than 70% of the genes in Arabidopsis have at least one T-DNA insertion in this collection. A researcher with his/her genes of interest in mind can use the information provided in the above website to identify one or more suitable T-DNA insertional mutants in these genes.

Mutant seeds can be obtained from the Arabidopsis Biological Research Center at Ohio State University. However, the mutant seeds are a mixture of wild type (for the T-DNA inserted gene), homozygous for the mutant allele (with the T-DNA insertion) and heterozygous (one wild type allele and one T-DNA inserted allele) seeds. The SIGnAL website has instructions (and a computer program) for the design of PCR primers that will identify each of the three genotypes among a segregating population of seeds. Two different primer pairs are used for this purpose. One of the primer pairs (LP and RP for each gene) will amplify the wild type allele. The other primer pair (BP and RP) will amplify the insertion-containing allele. The strategy is diagrammed in Figure 1.

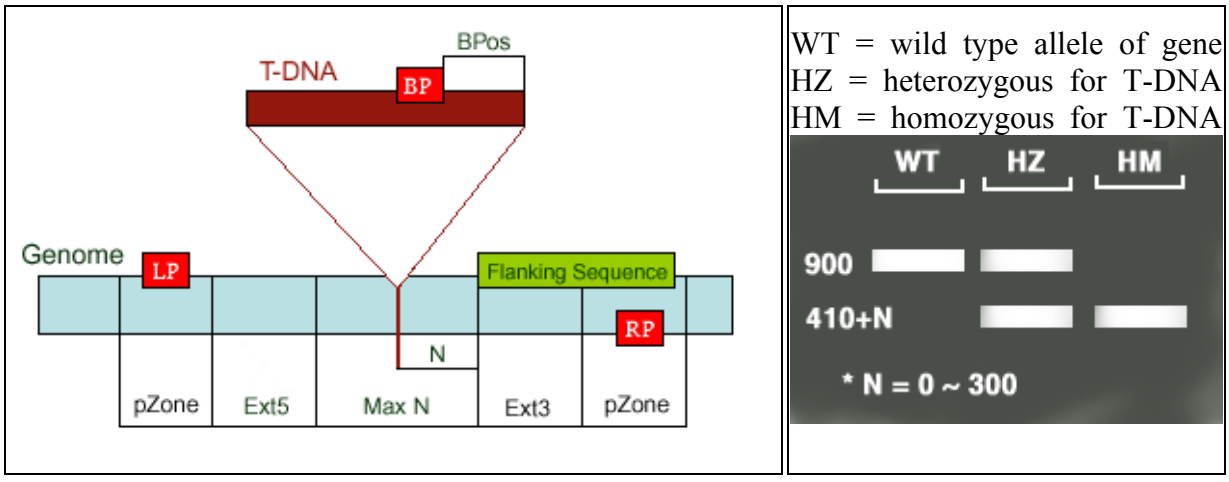

Figure 1. For the LP+RP reaction, there should be a product for WT or HZ lines, but a blank lane for HM lines. For the BP + RP reaction, you should get a band for HM or HZ lines, but a blank lane for WT.

Experimental Procedures

Week 1: Using TAIR and SIGnAL Websites to Identify T-DNA Insertions in a Gene of Interest

1. Go to the TAIR website (http://www.arabidopsis.org/index.jsp). Search for genes At3g05040 and At1g08830. You will find a brief description of the function of these genes. Write down the gene names and a brief description of the product that each encodes.

2. Go to the SIGnAL website (http://signal.salk.edu/tabout.html). Go to **SALK T-DNA** in the main menu and select **T-DNA Express** from the submenu. In the Query box, type in the two gene names (one at a time); click on Search. You will see a display of the genome region that includes the gene of interest. Find the SALK T-DNA insertions for each gene of interest and record the names of these lines below. (NOTE: SALK lines depicted in green have already been made homozygous for the T-DNA insertion whereas those in pink represent a mixture of genotypes.)

   At3g05040: _____

   At1g08830: _____

3. Return to the SIGnAL homepage and select the **iSect Tool**. Next select 1. SIGnAL T-DNA Verification Primer Design. This page will give you an explanation on how to use the appropriate primers to distinguish the three genotypes (the alleles with or without T-DNA insertion) in the population.

4. Scroll further down on the same page to 2. SALK T-DNA primer verification design, and in the box labeled primerL, submit the names of the T-DNA insertions lines identified in Step 2. For each gene, the program will give you the sequence of two primers (based on the flanking Arabidopsis genomic sequence). Record the LP and RP primers and product size for each gene.

   At3g05040:
   LP _____
   RP _____
   Product Size _____

   At1g08830:
   LP _____
   RP _____
   Product Size _____

Week 2: Isolation of Genomic DNA from Plant Seedlings and Genotyping by PCR

You have visited the websites TAIR and SIGnAL and have learned about the two genes *HASTY* (At3g05040) and *CSD1* (At1g08830). *HASTY* encodes a protein in the family of exportins involved in transporting microRNAs from the nucleus to the cytoplasm. *CSD1* encodes a cytosolic copper/zinc superoxide dismutase involved in the response to oxidative stress. In order to use reverse genetics to learn about their biological functions, you identified T-DNA insertional mutants in each gene. The seeds of two lines (SALK_079290 for the *HASTY* gene and SALK_009306 for the *CSD1* gene) were obtained from ABRC and plant seedlings were grown for the purpose of genotyping. When plants homozygous for the T-DNA insertion have been identified, they can be studied to determine the effect of the loss of that particular gene's function.

Materials

Seedlings from T_3 seeds of SALK_079290 and SALK_009306 lines
DNA Extraction Buffer (200 mM Tris-HCl, pH 7.5-8; 250 mM NaCl; 25 mM EDTA, 0.5% SDS)
Isopropanol
TE Buffer (10 mM Tris-HCl, pH 7.5-8; 1 mM EDTA)
TBE Buffer (for gel electrophoresis)
Agarose
250 ml Erlenmeyer flask (for melting agarose)
Balance and weighing supplies
Micropipettors and tips
1.5 ml microcentrifuge tubes
Plastic pestles (that fit into 1.5 ml tubes)
0.2 ml PCR tubes with PCR beads
Sterile ddH_2O water
Primer Mix A (BP + RP for gene At3g05040) at 12.5 µM
Primer Mix B (LP + RP for gene At3g05040) at 12.5 µM
Primer Mix A (BP + RP for gene At1g08830) at 12.5 µM
Primer Mix B (LP + RP for gene At1g08830) at 12.5 µM
6X Cresol Red Loading Dye (0.25% Cresol red, 4 g sucrose in 10 ml ddH_2O)
DNA size standards (1 Kb Plus from Invitrogen)
Microcentrifuge
37°C incubator
Thermocycler
Microwave
Gel electrophoresis equipment (casting tray; buffer chamber and power supply)
Ethidium bromide solution (in sandwich boxes)
Rotating platform
Fotodyne Photodocumentation System
Waste box

Isolation of Plant Genomic DNA

1. You will use the lid of a 1.5 ml microcentrifuge tube to clip off a young leaf from an Arabidopsis seedling. Clip one leaf each from two SALK_079290 plants and one leaf each from two SALK_009306 plants (for a total of four leaves). Use a separate tube for each leaf and be sure to label each tube with the plant number. Also record the plant numbers below for future reference.

   SALK_079290: _____
   SALK_009306_____

2. Use a small plastic pestle to grind the leaf; grind for ~ 15 seconds without buffer. Use a separate pestle for each plant.

3. Add 400 µl of DNA Extraction Buffer to each tube and vortex for ~ 5 seconds. (At this stage, samples can be kept at room temperature for up to 1 hour until all samples have been collected).

4. Centrifuge that tubes at full speed (>10,000 rpm) for 1 minute to pellet cellular debris.

5. Transfer 300 µl of the supernatant from each tube into fresh, labeled 1.5 ml tubes. Avoid taking any of the debris from the first tube.

6. Add 300 µl isopropanol to each tube, mix by inverting the tubes several times, and leave at room temperature for ~ 2 minutes.

7. Centrifuge the tubes at full speed for 5 minutes to pellet the DNA.

8. Remove all the supernatant and dry the pellets by incubating the open tubes at 37^{o}C for several minutes. Do not overdry the pellets or it will be difficult to dissolve the DNA.

9. Add 100 ml of sterile TE or ddH_2O to each pellet and dissolve by gentle flicking of the tube (do not vortex).

10. The DNA is of suitable quality for digestion with restriction enzymes and for PCR. Freeze unused portions until needed.

Set-up of PCR Reactions

For each plant DNA sample, you will set up two different PCR reactions. The first (A) will be carried out the BP primer for the T-DNA and the RP primer for the At3g05040 gene or with the BP primer and the RP primer for the At1g08830 gene. The second set of reactions (B) will be carried out with the LP and RP primers for the At3g05040 gene or with the LP and RP primer for the At1g08830 gene. Reaction tubes 1 and 2 in the table are for the Salk_079290 plants and reaction tubes 3 and 4 are from the SALK_009306 plants.

1. Set up the reactions using sterile micropipet tips. Pipet each reagent, in the order shown in Tables 1 and 2, directly into the tube with the PCR bead. Although it is not shown in the tables, it also is advisable to set up a pair of control reactions without any added DNA.

Table 1. Set-up of PCR reactions with primers BP + RP.

Reagent/Tube	**1A**	**2A**	**Reagent/Tube**	**3A**	**4A**
Sterile water	21 µl	21 µl	Sterile water	21 µl	21 µl
Primers BP + RP At3g05040	2 µl	2 µl	Primers BP + RP At1g08830	2 µl	2 µl
DNA Salk_079290 Plant #	2 µl	2 µl	DNA SALK_009306 Plant #	2 µl	2 µl
Total Volume	25 µl	25 µl	Total Volume	25 µl	25 µl

Table 2. Set-up of PCR reactions with primers LP + RP.

Reagent/Tube	**1B**	**2B**	**Reagent/Tube**	**3B**	**4B**
Sterile water	21 µl	21 µl	Sterile water	21 µl	21 µl
Primers LP + RP At3g05040	2 µl	2 µl	Primers LP + RP At1g08830	2 µl	2 µl
DNA Salk_079290 Plant #	2 µl	2 µl	DNA SALK_009306 Plant #	2 µl	2 µl
Total Volume	25 µl	25 µl	Total Volume	25 µl	25 µl

2. Mix the contents of each tube by gently tapping the tubes on the bench; make sure everything collects in the bottom of the tube when you do this. The contents of the tube should be clear when they are mixed well (and PCR bead has thoroughly dissolved).

3. Place the PCR tubes into one of the thermocyclers. You will use the following program:

1X: 5 min @ 94°C

40X: 30 sec @ 94°C
30 sec @ 60°C
1 min @ 72°C

1X: 5 min @ 72°C

4. Prepare a 1.5% agarose gel with at least 9 wells for electrophoretic analysis of the PCR products.

5. When the PCR reactions are complete, add 5 µl of 6X cresol red loading dye to each and mix.

6. Load 20 µl of each PCR reaction in the gel. Load 10 µl of the DNA size standard.

7. Run the gel at 100 volts until the Cresol Red has run about 2/3rd the length of the gel.

8. Stain the gel with ethidium bromide for 15 minutes and photograph with the Fotodyne photodocumentation system.

9. Send an electronic copy of your results to your TA. The results will be pooled from the whole class.

Evaluation of the PCR Analysis

For each pair of plants that you extracted DNA from, you will need to evaluate the results of both sets of PCR reactions. Recall that the reactions with Primer Mix A only will amplify the alleles that have the T-DNA insertion whereas reactions with Primer Mix B only will amplify the wild type allele of the genes. Record your results and infer the genotype of each plant using the table below.

SALK Line	Plant #	T-DNA Specific Fragment (A:BP + RP)	WT Specific Fragment (B:LP + RP)	Plant Genotype?		
				Homozygote for T-DNA insert	Heterozygote (insert/WT)	Homozygote for WT allele
079290						
009306						

Lab Report

You are required to hand in a lab report for this experiment. The following information should be included. The report should be no more than 5 pages in length.

1. Introduction. What is "reverse genetics"? Why was the Salk T-DNA collection made? What are the genes we chose to analyze in this experiment? What are the genotypes (of the disrupted gene) of the seeds for each line? Your introduction should clearly describe the experimental goal.

2. Materials and Methods. Include a very brief description of the procedure. Include any deviations that may have affected the experimental outcome.

3. Results. Your results including a photograph of the gel with all the lanes labeled.

4. Discussion. Clearly describe your interpretation of the results. If the results are not what you expected, explain why. If the experiment failed completely, also explain why. Finally, if you get what you set out to look for, describe what you plan to use it for in future experiments.

References

http:// www.arabidopsis.org/index.jsp
http://signal.salk.edu/tabout.html

Materials

Computers linked to the internet - ideally one computer per student or pair of students
Seedlings from T_3 seeds of 1 or 2 SALK T-DNA insertion lines - each pair of students needs 4 plants
DNA Extraction Buffer (200 mM Tris-HCl, pH 7.5-8; 250 mM NaCl; 25 mM EDTA, 0.5% SDS) – 2 ml per pair of students
Isopropanol – 1.5-2 ml ml per pair of students
TE Buffer (10 mM Tris-HCl, pH 7.5-8; 1 mM EDTA) – 1 ml per pair of students
TBE Buffer (for gel electrophoresis) – several liters for a class
Agarose (LE) – 1 bottle for a class
250 ml Erlenmeyer flask (for melting agarose) – 1 per pair of students
Balance and weighing supplies – 1 or 2 per classroom
Micropipettors and tips (2-20 µl, 20-200 µl, and 100-1000 µl ranges and boxes of matching tips) – 1 set per pair of students
1.5 ml microcentrifuge tubes – a minimum of 8 tubes per pair of students
Plastic pestles (that fit into 1.5 ml tubes) – 1 per plant that DNA will be extracted from or a minimum of 8 per pair of students
0.2 ml PCR tubes with PCR beads – 8-10 per pair of students plus some extras as back-ups
Sterile ddH_2O water – one small bottle or 10 ml per pair of students
Waste box – 1 per classroom
Primer Mix A (BP + RP for gene At3g05040) at 12.5 µM – 10 µl per pair of students
Primer Mix B (LP + RP for gene At3g05040) at 12.5 µM - 10 µl per pair of students
Primer Mix A (BP + RP for gene At1g08830) at 12.5 µM - 10 µl per pair of students
Primer Mix B (LP + RP for gene At1g08830) at 12.5 µM - 10 µl per pair of students
6X Cresol Red Loading Dye (0.25% Cresol red, 4 g sucrose in 10 ml ddH_2O) – 100 µl per pair of students
DNA size standards (1 Kb Plus from Invitrogen) - 10 µl per pair of students
Microcentrifuge – ideally 1 per pair of students
37°C incubator – 1 per classroom
Thermocycler – number needed depends on size of class and the number of samples that the thermocycler holds
Microwave – 1 or 2 per classroom
Gel electrophoresis equipment (casting tray; buffer chamber and power supply) – 1 apparatus per pair of students with a comb that can make 9-12 wells
Ethidium bromide solution (in sandwich boxes) – 1 per pair of students
Rotating platform – 1 per classroom
Fotodyne Photodocumentation System – 1 per classroom

Instructor Notes

Growing Arabidopsis plants from seeds

If you have never worked with Arabidopsis before, the following are useful websites. The TAIR website also has other useful links.

http://arabidopsis.info/InfoPages?template=newgrow;web_section=arabidopsis
http://arabidopsis.info/protocols/grow.html
http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/handling.htm

Seeds may be ordered, for a reasonable fee, from ABRC. The website is: http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/index.html.

Performing PCR Reactions

If you are not familiar with the Polymerase Chain Reaction, a nice animated tutorial can be found at www.dnalc.org. Go to Resources (top right of homepage) and select Biology Animation Library.

While somewhat pricey, we have found that using PCR beads (*Ready to Go Beads*, GE Healthcare product #27955702) for the reactions are worth the cost. These beads contain the heat-stable DNA polymerase, deoxynucleoside triphosphates, magnesium, and buffer. We have greater consistency and success of PCR reactions from undergraduate students when we have them use the beads. Instructor-prepared or manufacturer Master PCR mixes are another option to consider. The fewer refined measurements there are to make, the fewer the measurement errors by the students.

We have had better results using two paired reactions, rather than a single reaction with three primers (BP + LP + RP), for the genotyping of plants. Primer Mix A (BP + RP) amplifies the T-DNA insertion allele. Primer Mix B (LP + RP) amplifies the wild type allele. The BP primer is common to all T-DNA insertion lines but the LP and RP primers are unique for each gene (determining the sequence of the LP and RP primers is part of the student exercise). For each mix, the concentration of the primer should be 12.5 μM in sterile water. Primers can be obtained from a variety of vendors. Our vendor of choice is Integrated DNA Technologies, Coralville, IA (www.idtdna.com). IDT is a national supplier of synthetic oligonucleotides and their products are affordably priced.

Agarose Gel Electrophoresis

The percentage of agarose to use depends on the expected sizes of the PCR products. Most products range in size from 500 bp – 1200 bp. A 1.5% agarose gel (made up in either TAE or TBE buffer) works well for separating DNA molecules in this size range.

Recipe for 10X TAE (1 L)	Recipe for 5X TBE (1 L)
48.4 g Tris Base	60.5 g Tris Base
11.4 ml Glacial Acetic Acid	30.0 g Boric Acid
20.0 ml 0.5 M EDTA, pH 8	3.7 g EDTA
Bring to 1 L with dH_2O	Bring to 1 L with dH_2O

About the Authors

Brenda G. Leicht is a laboratory coordinator and Adjunct Assistant Professor in the Department of Biology at The University of Iowa. She coordinates the labs for a large non-majors course as well as coordinating two advanced investigative laboratory courses for majors, *Cell Biology Laboratory* and *Genetics and Biotechnology Laboratory*. She received her Ph.D. in Genetics from Indiana University in 1987.

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