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Understanding the Genetic and Molecular Mechanisms of the *SHL3* phenotype in *Arabidopsis thaliana*

A Capstone Project Submitted in Partial Fulfillment of the
Requirements of the Renée Crown University Honors Program at
Syracuse University, and Distinction in Biochemistry

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May 2010

Honors Capstone Project in Biochemistry

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Date: _____

ABSTRACT

The study of plant genetics and the molecular mechanisms regulating plant-pathogen interactions is an intensely studied area of research in molecular genetics. Gaining an understanding of the defense mechanisms of plants has proven highly useful in the construction of transgenic plants for increased crop yield. The focus of my research was on the defense mechanisms of *Arabidopsis thaliana*, a plant in the mustard family. Several features of *Arabidopsis* make it an excellent model plant for molecular genetic studies. A few of these include a short life cycle (approximately 8 weeks), a completely sequenced genome, high fecundity, and the availability of a large mutant pool. My research more specifically focused on the *shl3* (*suppressor of the hrl1 3*) mutant and its suppression of the *hrl1* (*hypersensitive response-like lesions 1*) mutant phenotype. Previous work in the lab has demonstrated that *hrl1* mutant plants up-regulate multiple pathogen defense pathways, resulting in constitutive expression of defense genes and much higher resistance to several pathogens than wild-type plants. Additionally, *hrl1* plants are much smaller in size than wild-type plants and display leaf lesions similar to those on plants undergoing the hypersensitive response. To identify additional components of the defense pathway regulated by *hrl1*, insertional mutagenesis was carried out in *hrl1* plants to identify suppressors of the *hrl1*-associated phenotype. In this screen a mutant, *shl3*, was identified to cause a near-complete reversion of the *hrl1*-associated phenotypes. My research involved investigating the molecular mechanisms involved in regulating this suppression and the role of *shl3* in regulating pathogen defense. In addition, I determined the effects of this insertion on the genes flanking the *shl3* insertion and the relationship of *shl3* to expression levels of other defense-related genes of *Arabidopsis*. These studies should help to gain a better understanding of the role of *shl3* in the defense pathways in *Arabidopsis*.

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completion of this thesis was very much a group effort, and the labor of love of many people.

INTRODUCTION

The study of plant-pathogen interactions is an extremely useful medium for studying the defense pathways of plants. The study of the plant defense system is vital to the understanding of how to combat the critical food famine that affects us as well as our livestock. According to census reports the world population has been rapidly increasing over the past years, in the year 2010 the population is 6,830,586,985, and expected to increase to 7,557,514,266 in 2020 (census.gov) Unfortunately, food production has not been able to increase accordingly because of crop loss caused by pests and diseases. By gaining the knowledge of the plant defense mechanisms and the genes that regulate the pathways, we expect to be able to construct transgenic plants that will be able to defend themselves against bacterial, viral, fungal, and insect attacks, providing a higher crop yield. Though there are many ongoing debates about the safety of consuming transgenic plants as well as feeding them to our livestock, the development of transgenic plants allows for an environmentally friendly and sustainable alternative to the use of chemicals and pesticides.

One of the most widely used model plants in the study of plant-pathogen interactions is *Arabidopsis thaliana* (Fig. 1). It is a small flowering plant in the mustard family and is useful for genetics research for a number of reasons. Its small size allows it to be grown year-round in growth chambers and greenhouses. Additionally, it has a short life cycle (approximately 8 weeks), a completely sequenced genome, high fecundity, and is easy to transform to produce transgenic plants for genetics research.

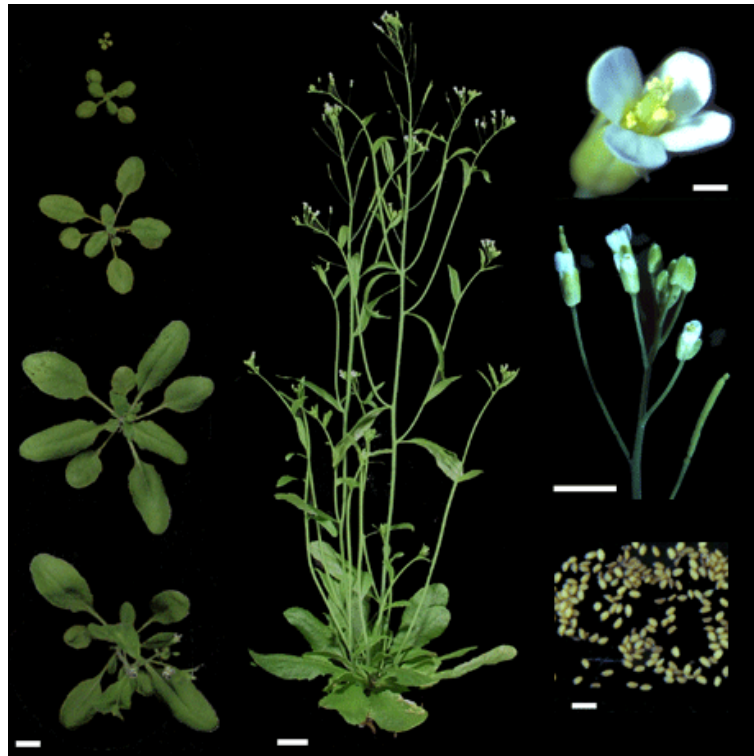


Figure 1. *Arabidopsis thaliana*. This plant is the model organism used for understanding the genetics and molecular mechanisms of plant defense (www-ijpb.versailles.inra.fr/.../Planche.gif).

There are two major pathogen defense systems in plants. The first system is known as the basal defense. This is similar to the innate immune response. The plant recognizes features of the pathogen, more specifically pathogen associated molecular markers (PAMPs), which lead to activation of a defense signaling cascade and ultimately to the accumulation of specific hormones: salicylic acid, jasmonic acid, and ethylene. These hormones play a critical role in activation of defense against pathogens. The second type of defense is the induced defense or more commonly known as the gene-for-gene defense pathway. In this defense pathway there are two possible outcomes for the plant pathogen interactions: compatible and incompatible. Compatible interactions involve a virulent pathogen and a susceptible host plant (Fig. 2a). In this interaction the pathogen enters into the plant and is unrecognized by the plant's defense system and is able to drain the plant of its nutrients, causing the plant to develop water soaked patches, which become necrotic lesions. These lesions then show chlorosis, a condition where the chlorophyll is broken down causing a yellow speckled appearance. This series of events eventually leads to death of the infected tissue and eventually the plant. The second form of plant pathogen interaction is known as an incompatible interaction and it involves an avirulent pathogen and a resistant plant (Fig. 2b). In this type of interaction, pathogen is recognized by the plant and plant activates a rapid defense response, known as hypersensitive response. In this pathway the pathogen produces avirulence factors which are recognized by the resistance gene (*R* gene) products of the plant. This triggers an ion flux which ultimately leads an oxidative burst supplying the plant with

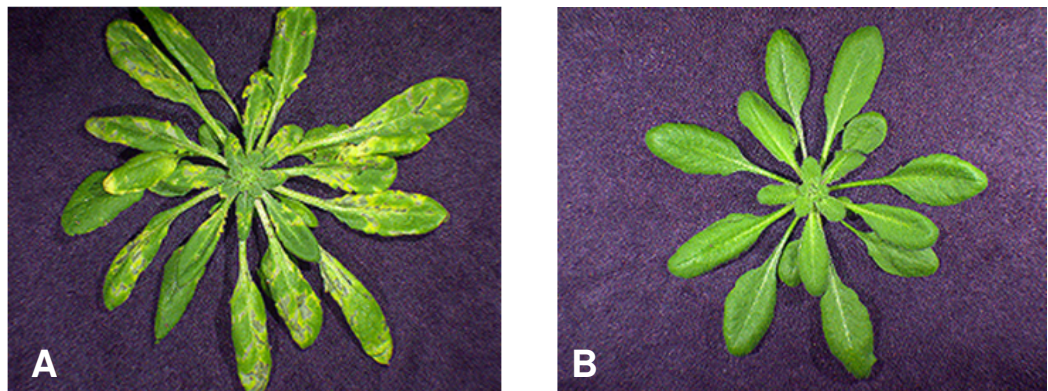


Figure 2. Types of Gene for Gene Interactions

(A) Compatible interaction is when the bacterium is able to successfully infect the plant because the plant is unable to detect the pathogen and protect itself against the attack. The infection leads to water soaked lesions that become necrotic and ultimately lead to cell and plant death.

(B) Incompatible interaction is when the hypersensitive response (HR) is triggered leading to defense associated plant cell death or apoptosis inhibiting further spread of the bacterial infection.

reactive oxygen species (ROS). The ROS induces a signal cascade leading to programmed cell death of the plant cells and the infecting pathogen and thus halting the further spread of the infection.

Previous research in the Raina lab focused on the characterization of the *hrl1* (*hypersensitive response-like lesions 1*) mutant, a lesion mimic mutant of *Arabidopsis* generated by EMS mutagenesis. *hrl1* mutants are much smaller than the wild-type parents and spontaneously develop lesions similar to those seen in plants undergoing the hypersensitive response (Fig. 3). Additionally, *hrl1* plants constitutively express several defense genes and are more resistant to a variety of pathogens. Positional cloning of the *HRL1* gene showed that the observed phenotypes are due to a point mutation in the gene At4g23660, encoding for a 4-hydrobenzoate polyprenyl diphosphate transferase enzyme. This point mutation changed a cytosine to a thymine in a highly conserved region of the gene, causing leucine to change to phenylalanine. This is a key enzyme responsible for the synthesis of ubiquinone and the *hrl1* mutation is predicted to result in a partial loss of function. The resulting decrease in ubiquinone may be causing high levels of reactive oxygen species to accumulate in leaves, which triggers pathogen defense pathways and thus resistance against pathogens.

To identify additional components of the defense pathway regulated by *hrl1*, insertional T-DNA mutagenesis was carried out in the *hrl1* mutant background to identify suppressors of *hrl1*-associated phenotypes. In this screen, the mutant *shl3* (*suppressor of hrl1 3*) was identified to cause a complete

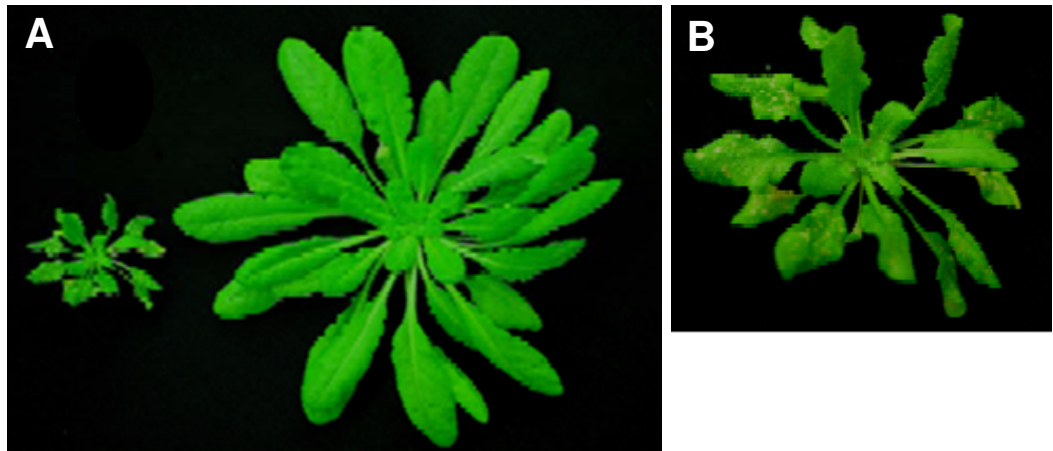
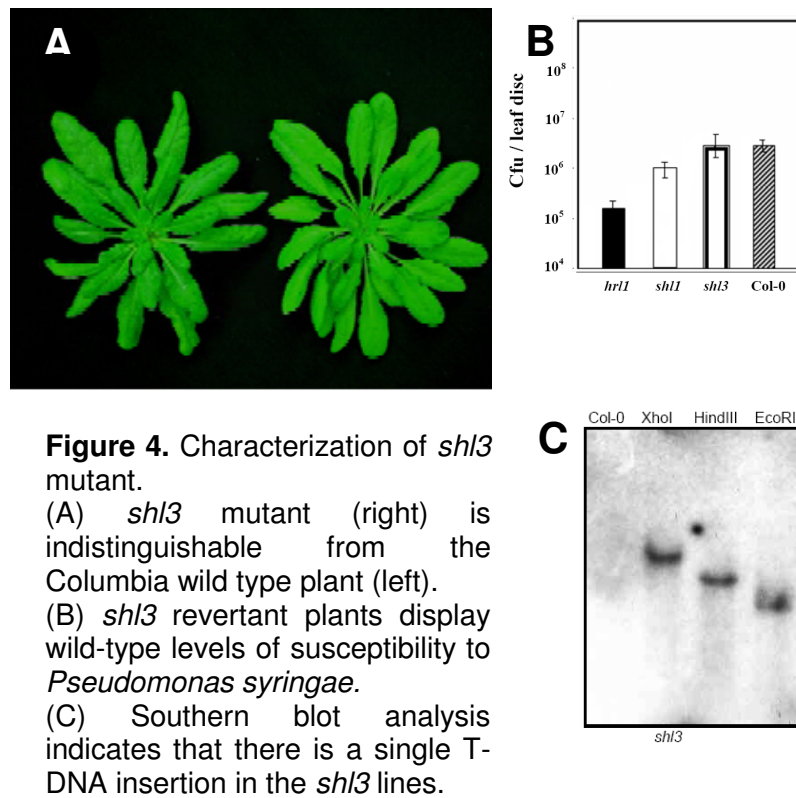


Figure 3. Phenotype of *hrl1* mutant.

(A) On left, the *hrl1* mutant which is approximately 1/5th the size of the Columbia wild type (right).

(B) A close up of the *hrl1* plant (right) shows lesions indicative of the hypersensitive response.



reversion of the *hrl1*-associated phenotype. *shl3* plants are similar to wild-type Columbia plants and show wild-type levels of resistance to *Pseudomonas syringae* (Fig. 4a, b). Using Southern blot analysis, it was determined that the *shl3* reversion is due to a single T-DNA insertion (Fig 4c.). Identification of the *shl3* mutation via plasmid rescue revealed that the insertion is located on the 4th chromosome in the intergenic region between genes At4g12050 and At4g12060 (Fig. 5).

The overall goal of my research was to determine the molecular mechanisms involved in the suppression of the *hrl1* phenotype in the *shl3* mutant. For this, I used reverse transcriptase-PCR (RT-PCR) to investigate whether the insertion of the *shl3* T-DNA affects the expression of either of the flanking genes and to test whether the region of the *shl3* insertion contains an unannotated gene. I also used RT-PCR to study expression levels of defense marker genes in *shl3* to determine their expression levels in the *shl3* mutant. Finally, I attempted to restore the *hrl1* phenotype in *shl3* plants by complementation with a wild-type copy of the genomic region between the genes At4g12050 and At4g12060.

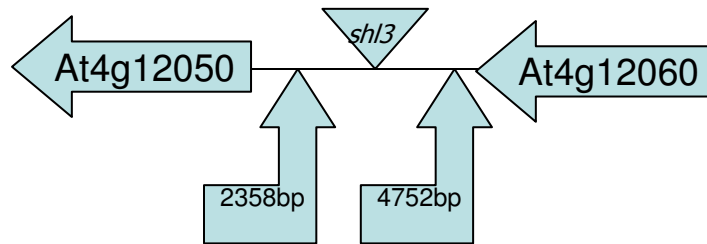


Figure 5. Graphical representation of the location of the T-DNA insertion on the 4th chromosome of *sh13* mutant.

MATERIALS AND METHODS

Polymerase Chain Reaction

PCR was performed in a PTC-225 thermocycler (MJ Research, Waltham, MA, USA). A reaction volume of 15 μ L was typically used, consisting of 10.5 μ L H₂O, 1.5 μ L 10x PCR buffer (10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5 mM MgCl₂), 0.75 μ L of 200 μ M dNTP, 0.75 μ L forward primer, 0.75 μ L reverse primer, 0.2 μ L Jump Start RedtED Taq DNA Polymerase (Sigma, St Louis, MO, USA) and 1 μ L of the appropriate DNA. When colony PCR was performed an additional μ L of H₂O was added and instead of DNA a small amount of the colony was picked from the plate using a sterile pipette tip and mixed into the PCR solution. The PCR program used contained a five minute initial denaturation step at 95°C, followed by 3 cycling steps. They were composed of 30 seconds of denaturing at 94°C, 30 seconds of annealing at 54 to 65°C, and 30-90 seconds of extension at 72°C. These three steps of cycling were performed for 25-40 cycles. Completed reactions were checked on 1% agarose gels run in a 1x SB buffer (made from a 20X stock solution of 8 g NaOH, 45 g boric acid in 1 L water) for 15 minutes at 135V. List of primers used can be found in Table 1.

Table 1. Primers Used

Lab ID	Target Sequence	Sequence	Tm °C
RXR67	PR1coding seq	CCACAAGATTATCTAAGGGTTC	56.5
RXR68	PR1coding seq	GGCTTCTCGTTCACATAATTCC	58.2
RXR317	PDF1.2-f	AATGAGCTCTCATGGCTAAGTTTGCTTCC	55.2
RXR318	PDF1.2-r	AATCCATGGAATACACACGATTTAGCACC	53.7
RXR1640	T7 primer on TDNA	5' TAATACGACTCACTATAGGG 3'	58.2
RXR2448	shl3 f	TTGGTCATCCGATCCGTAATCTTC	61.1
RXR2449	shl3 r	CTTCAGAACTCAAACGCTGGTC	60.2
RXR1822	At4g12050-500bp-f	TGT ATG GCT ACG TTC GCT AGA C	60.2
RXR1847	At4g12050-r	AT GAG CTC AGC AAT CTA ATA ATC AAT ACG GTG GC	60.1
RXR1820	At4g12060-500bp-f	GAA AAA GCC CAA ATG GTC ATG GAG	61
RXR1846	At4g12060-r	AT GGA TCC GAT GTA ATA TAC TAT TCA TCT AAA AAG CCA G	60.2
RXR2391	UBC (ubiquiti conj enz) f	TCAAATGGACCGCTCTTATC	55.3
RXR2392	UBC (ubiquiti conj enz) r	CACAGACTGAAGCGTCCAAG	59.4

Reverse Transcription

Reverse transcription was conducted in 0.2mL strip tubes in a thermalcycler (MJ Research, Waltham, MA, USA). The first step was to add 8.2 μ L DEPC H₂O, 4 μ L dNTPs (2.5mM in DEPC water), 1 μ L oligo(dT)₁₈ (200ng/ μ L), and 1 μ L RNA (previously diluted from a small aliquot of isolated RNA to 1 μ g/ μ L). This solution was heated to 65°C for 5 minutes and incubated at 4°C for 1 minute. Then 4 μ L 5x first strand buffer, 1 μ L 0.1M DTT, 0.4 μ L RNAsin and 0.4 μ L Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) were added to each tube. After gently mixing the reaction it was incubated for 60 minutes at 50°C followed by a heating it to 70°C for 15 minutes. The cDNA was stored in a -75⁰C freezer.

RNA isolation

All work performed for RNA isolation was done with gloves and RNase free material. Leaf samples were collected from plants and placed into individual 2.0mL tubes. The tubes were then submerged in liquid N₂ to freeze the tissue until subsequent steps could be performed. Standard ceramic mortars and pestles were used for grinding. Approximately 30mL of liquid N₂ was added into the mortar and allowed to evaporate, cooling the mortar and pestle. This was repeated once, and then the leaf sample was placed into mortar along with liquid N₂. The leaf was slightly crushed as the N₂ evaporated and fully ground when the N₂ had fully evaporated. Once the liquid N₂ fully evaporated, the tissue was scraped into 2mL centrifuge tubes that had been previously filled with 1mL of Trizol Reagent

(Invitrogen, Carlsbad, CA). The solution was then vortexed vigorously and incubated for 3 minutes at room temperature.

After incubation, 200 μ L of chloroform was added to each tube. The tubes were inverted a few times to mix and incubated for 3 minutes at room temperature. The tubes were centrifuged for 15 minutes at 11,000rpm at 4°C. The supernatant was transferred into new 1.5mL centrifuge tubes and 500 μ L isopropanol was added. The tubes were mixed by inverting them a few times and incubated at room temperature for 45 minutes. The tubes were then centrifuged for 15 minutes at 12,000rpm at 4°C. The supernatant was decanted and the pellet was washed with 1mL of 75% ethanol (diluted in DEPC H₂O). The tubes were then centrifuged for 15 minutes for 12,000rpm at 4°C. The supernatant was decanted and the tubes were placed in 37°C incubator for 8 minutes to dry. Lastly the RNA pellet was resuspended in DEPC-treated H₂O and incubated in 37°C for 30 minutes then on ice for 1 hour. The RNA was stored at -80°C.

RNA Quantification

After RNA isolation it was quantified via obtaining the OD at 260nm using an optical density calculator at a 1:100 dilution. The concentration of the RNA was calculated using the formula below.

$$(\text{OD} \times 40\text{ng}/\mu\text{L} \times \text{dilution})/1000 = [\text{RNA}] \mu\text{g}/\mu\text{L}$$

Once the concentration was calculated, 2-5 μ L RNA was diluted to 1 $\mu\text{g}/\mu\text{L}$.

1 μ L of normalized RNA of each sample was run on a 1% agarose gel in 1X SB buffer (made from 20X stock - of 8 g NaOH, 45 g boric acid in 1 L water) for 15 minutes at 135V to check RNA quality and accuracy of normalization.

DNA isolation

A small piece of a leaf was placed into a 1.5mL eppendorf tube. The leaf was ground with a blue pestle, and 400 μ L of extraction buffer (200 mM Tris HCL pH 7.5, 250 mM NaCl, 25mM EDTA, 0.5% SDS) was added. The tube was centrifuged for 2 minutes at 13,000rpm. The supernatant was transferred into a new 1.5mL eppendorf tube along with 300 μ L of isopropanol. After inverting the tube a few times to mix, the solution was incubated for 2 minutes at room temperature. The solution was centrifuged for 5 minutes at 13,000rpm and the supernatant was decanted. The DNA pellet was placed in a 65°C heat block until it was dry upon which it was resuspended in 100 μ L of H₂O. The DNA was stored in a -20°C freezer.

Seed Sterilization

All *Arabidopsis* seeds were sterilized prior to placing in soil. Approximately 50 seeds were placed in a 1.5mL eppendorf tube along with 1mL of H₂O. The tubes were vortexed and the H₂O was aspirated out. One milliliter of 20% bleach was added to the seeds and vortexed. The tubes were placed on a shaker for 8 minutes then the bleach was aspirated out. The seeds were then washed 3 times with 1mL of sterile H₂O. After the last wash with H₂O the seeds were suspended in 1mL of top agar (0.1% w/v bactoagar in distilled water) and stored at 4°C for 2 days prior to planting in soil.

Plasmid Isolation

A small smear of frozen bacterial culture was inoculated into 3mL of liquid LB media (10g Bacto-Tryptone, 5g Bacto-yeast extraction, and 10g NaCl

in 1L H₂O, autoclaved) with 50µg/mL of kanamycin in a 15mL culture tube. The culture was incubated at 37°C overnight with 250rpm of shaking. Two hundred microliter of the BAC culture was transferred into a 500mL flask (autoclaved) containing 200mL of liquid LB media and 200µL kanamycin (50 mg/mL) and incubated overnight under the same conditions. The cells were harvested into 2 50mL centrifuge tubes by centrifuging at 5,000rpm for 15 minutes. The cells were stored at -70°C overnight prior to isolation. The pellet was thawed and resuspended in 4mL of 10mM of EDTA, pH 8.0 by pipetting up and down with a 10mL pipette to prevent cells from rupturing. After being resuspended the suspension was incubated at room temperature for 5 minutes. The cells were lysed with 8mL of alkaline solution (0.2N NaOH and 1% SDS) by swirling until solution was homogeneous, and then incubated at room temperature for 5 minutes. Immediately 6mL of cold 3M KOAc (50mL of 7.5M KOAc with 23mL of HOAc and 127mL of ddH₂O, stored at 4°C) was added. The tube was inverted a few times before being stored at -80°C overnight. The lysate was cleared from the precipitated SDS proteins, membranes, and chromosomal DNA by centrifuging at 10,000rpm for 15 minutes at 4°C. The supernatant was filtered through miracloth into a new 50mL tube. An equal volume of isopropanol was added and mixed by swirling. The solution was centrifuged at 5000rpm for 15 minutes and the supernatant was decanted. The pellet was resuspended in 1.8mL of TE (10mM Tris-HCl, pH 7.6, 50mM EDTA, pH 8.0) and 0.9mL of 7.5M KOAc. After inverting a few times the solution was stored at -70°C overnight. After thawing the solution was centrifuged at 10,000rpm for 10 minutes and the

supernatant was transferred to a single new 50mL tube. DNase-free RNase A was added to a final concentration of 100 μ g/mL and incubated at in a 37°C H₂O bath for 1 hour. The DNA was precipitated by the addition of 30mL of cold 95% ethanol and incubation for 15 minutes in an ice bath. The DNA was pelleted by centrifuging for 25 minutes at 3000rpm. After removing the supernatant, 30mL of 70% ethanol was added to wash the pellet. The DNA was again pelleted by centrifuging at 3000 rpm for 25 minutes. The DNA pellet was dried and resuspended in 200 μ L of H₂O and stored at 4°C overnight to ensure complete resuspension of the DNA.

Digestion

Two separate double digestions were performed, one for the BAC DNA, and the other for the vector (pDMC99, Curtis and Grossniklaus 2007 This reference is not in the bibliography). For the BAC digestion 200 μ L of the BAC DNA, 30 μ L of NEB 4 buffer, 4 μ L EcoN1 (New England Biolabs, Ipswich, MA) and 65 μ L of H₂O were added to an eppendorf tube and incubated at 37°C overnight. For the vector digestion, 50 μ L of vector DNA, 2 μ L of EcoR1 (New England Biolabs, Ipswich, MA), 10 μ L of NEB 3 buffer, and 38 μ L of H₂O were added to an eppendorf tube and incubated at 37°C overnight. End-filling of EcoN1 and EcoR1 ends was performed using Klenow (New England Biolabs, Ipswich, MA). Four microliters of Klenow and 2 μ L of dNTPs were added to the reaction and incubated at room temperature for 30 minutes, then tubes were incubated for 60 minutes at 75°C to inactivate the Klenow enzyme. Digestion was completed by adding 3 μ L of BSA, and 4 μ L of BamH1 (New England Biolabs,

Ipswich, MA) to the BAC digest and 2 μ L of BamH1, 1.7 μ L of Buffer 3 and 1 μ L of BSA to the vector digest, then incubating at 37°C overnight. After each step, digestions were checked on a 1% agarose gel run in SB buffer for 5 hours at 35V.

Ligation

For the ligation of the BAC DNA into the vector, 6.5 μ L insert DNA (cut from BAC F16J13), 10 μ L of vector DNA (pMCD99), 2 μ L 10x Buffer with ATP, and 1.5 μ L T4 DNA ligase (New England Biolabs, Ipswich, MA) were added to a 1.5mL eppendorf tube and left at 16°C overnight to complete the reaction.

Transformation of Competent *E. coli* Cells

Cells used for bacterial transformation of the ligation mixture were Alpha-Select Silver Efficiency Chemically Competent cells from Bioline (www.bioline.com) and were transformed according to the manufacturer's protocol. Cells were stored in -80°C freezer. The cells were thawed on wet ice and gently mixed by flicking the tube. One hundred microliters of cells were aliquoted into a chilled eppendorf tube and the remaining cells were flash frozen in liquid N₂ before storing in -80°C freezer. Ten microliters of DNA ligation solution was added per 100 μ L of cells, mixed gently and incubated on ice for 30 minutes. The solution was then placed into a 42°C H₂O bath for 45 seconds for heat shock and immediately placed back into the ice bath for 2 minutes. 1mL of liquid LB media was added to the tube and incubated at 37°C for 1 hour while shaking at 200rpm. One hundred microliters of cells were plated on an LB agar plate containing kanamycin. The remaining cells were spun down and resuspended in 100 μ L of

liquid LB and plated on another LB/Kan media plate. The plates were incubated at 37°C overnight. The presence of colonies was determined after 16 hours.

Gel Electrophoresis

DNA and RNA concentrations were tested on a 1% agarose gel run in a SB buffer for 15 minutes. Gels were visualized and photographed using Gel Documentation System (Alpha Innotech, San Leandro CA, USA).

Low Melt Gel Electrophoresis

The BAC and the vector DNA were digested with the restriction enzymes and DNA fragments were fractionated by electrophoresis on low-melting agarose gels. The DNA was run on a 0.8% gel (100mL1X TE Buffer, 0.8g low-melting agarose, 5µL ethidium bromide) for 8 hours at 35V in a 1X TE buffer solution. Desired fragments were cut from the gel using a clean razor blade and placed into 1.5mL centrifuge tubes.

Gel Elution

All work containing buffer-saturated phenol and ether was performed in a hood. Tubes containing the gel fragments were heated for 10 minutes in a 65°C water bath, then mixed by flicking the tubes and heated for a further 5 minutes. 5M NaCl was added at a volume of 1/10th of the melted gel. The tube was mixed and again heated for 5 minutes. An equal volume of buffer-saturated phenol was added to the tube and mixed. The solution was spun at 13,000rpm for 5 minutes and the upper (aqueous) layer was transferred to a new tube. The buffer-saturated phenol step was repeated two times. To extract any remaining phenol, double the volume of ether was added and mixed. The solution was spun at 13,000rpm for 3

minutes. The ether (top layer) was removed with an aspirator. The tube was placed open in a 65°C water bath for several minutes to ensure all ether had evaporated. 1mL of cold absolute ethanol was added and mixed, then tubes were stored in a -70°C freezer overnight. The solution was spun for 10 minutes at 10,000rpm and the ethanol was decanted off. The pellet was washed with 70% ethanol and spun at 10,000rpm for 2 minutes. The ethanol was removed and the pellet was resuspended in 25µL water, then concentration was checked by gel electrophoresis.

BASTA Spraying

To select for the insertion of the *shl3* T-DNA, the plants were sprayed with BASTA herbicide. Those that lived contained the insert since the T-DNA conferred BASTA resistance. The BASTA solution contained 100mL of H₂O, 135µL of BASTA solution, and 20µL of silwet. Plants were sprayed once every three days once the seedlings were two weeks old.

RESULTS

Confirmation of *shl3* insertion

Because *shl3* plants are visually indistinguishable from wild-type Columbia plants, PCR was performed on plant genomic DNA to confirm the presence of the *shl3* T-DNA insertion. Two different PCR primer pairs were designed (Fig. 6). The first set had the forward and reverse primer each located 500 base pairs away from the *shl3* insertion site. The second set of primers had the reverse primer located on the T-DNA insert and the forward primer used was the same as the first set (Fig. 6). The selected primers ensured that the *shl3* plants would only show amplification with the primers RXR1640 and RXR2448 but not the primers RXR2448 and RXR2449 vice versa for the Columbia plant. Amplification showed the expected results (Fig. 7).

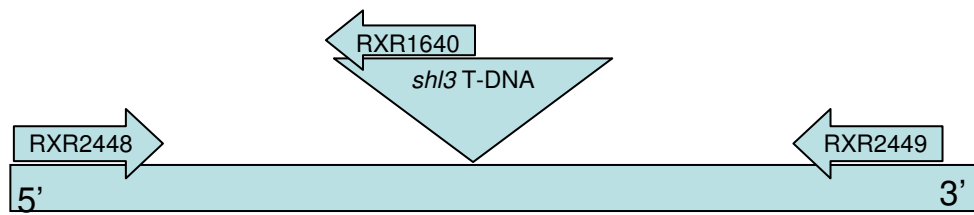


Figure 6. The location of the primers in relation to the *shI3* T-DNA insert. If amplification occurs with the forward and reverse primer, either no T-DNA insert is present or it is heterozygous for the insert. If amplification occurs with the forward and *shI3* primer, T-DNA is present.

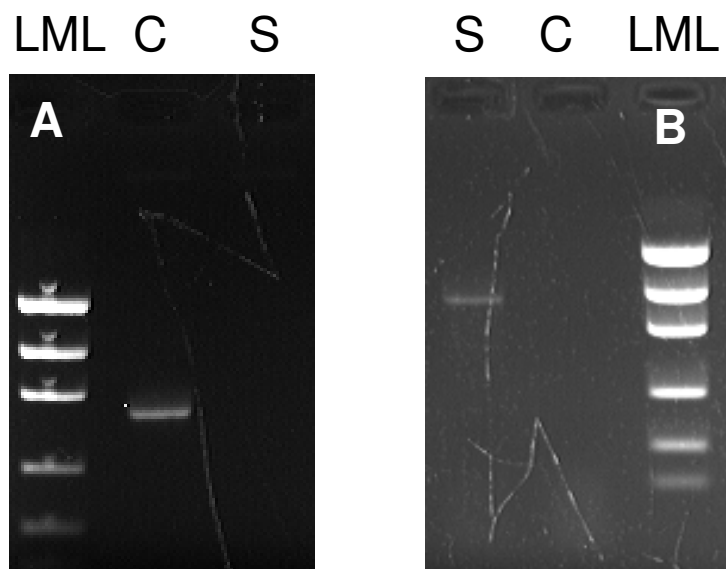


Figure 7. Analysis of *sh13* plants by PCR.

(A) Amplification with the flanking forward and reverse primers showed amplification of Columbia wild type but not *sh13* indicating the lack of an insert in the Columbia plants, and the presence of an insert in *sh13*.

(B) Amplification with the forward primer and the SHL3 primer (on the insert) showed amplification of the *sh13* plants but not the Columbia plants. This indicates that there is an insert in *sh13* but not the Columbia plants. For location of primers see Figure 6. (S – *sh13*, C – Columbia, LML – Low Mass Ladder)

Defense Gene Regulation

To determine whether defense gene expression reverts to wild-type levels in *shl3* mutant plants, RT-PCR was conducted on two standard defense marker genes. *PDF1.2* and *PR1* are not expressed to detectable levels in wild-type plants, but are highly induced upon pathogen infection and have been shown to be constitutively expressed in *hrl1* plants. Tissue was collected from four-week-old wild-type Columbia, *hrl1*, and *shl3* plants and RNA was isolated, then reverse-transcribed to cDNA and amplified. *shl3* plants often begin to show *hrl1*-like lesions as they age, therefore tissue was also processed from 5-week-old *shl3* plants displaying lesions. Amplification at a relatively low number of cycles (24 cycles) showed that constitutive expression of *PDF1.2* and *PR1* was suppressed in the *shl3* plants (Fig. 8). *shl3* plants develop very few lesions very late in the development. RT-PCR analysis revealed that 5-week-old *shl3* plants displaying lesions also express *PDF1.2* and *PR1* defense genes to levels similar to *hrl1*. A housekeeping gene (ubiquitin-conjugating enzyme, UBC) was used as an internal control and amplified to equal levels in all samples, indicating that differences among samples in defense gene expression are not due to differences in amount of RNA among various tissue samples. These results suggest that T-DNA insertion in *shl3* mutant partially complemented spontaneous lesions and defense gene expression of *hrl1*.

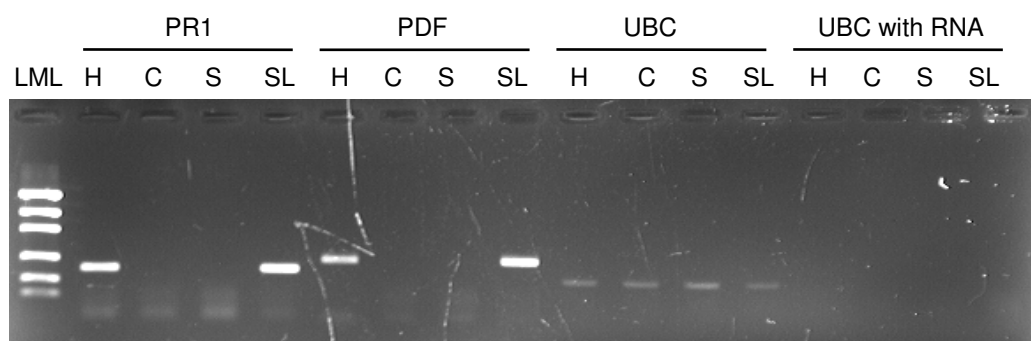


Figure 8. Analysis of expression of defense genes *PR1* and *PDF1.2* in *hrl1* and *shl3* plants.

Gene-specific primers were used for RT-PCR. UBC was used as an internal control to show relative amount of the cDNA. RNA amplified using UBC primers to show that there was no contaminating genomic DNA in the RNA. H – *HRL1*, C – Columbia, S – *shl3*, SL – *shl3* with lesions, LML – Low Mass Ladder.

Expression of Genes Flanking *shl3*

Since the *shl3* insertion is located in an intergenic region between two genes on the fourth chromosome, it is possible that expression of one of the genes flanking the insertion is effected in *shl3* mutant, resulting in suppression of the *hrl1* phenotype. To test this, RT-PCR was conducted on the two genes that flanked the insertion, At4g12050 and At4g12060. Based on the data collected there does not appear to be a significant effect on the expression of either gene caused by the *shl3* insertion (Fig. 9).

T-DNA Insertion in *shl3* is not in an Unannotated Gene

Because the *shl3* insertion is located between two genes and does not appear to have a significant effect on the expression of either of the flanking genes, it is difficult to explain why it is able to cause suppression of the *hrl1* phenotype. One possible reason is that the region in which the insertion is located actually contains a previously unannotated gene. To determine whether any transcript is being produced from that region, RT-PCR was performed on Columbia plants using primers flanking the T-DNA insertion, primers RXR2448 and RXR2449 (Fig. 7). No amplification was detected from cDNA, suggesting that no transcript is produced from that region (Fig. 10).

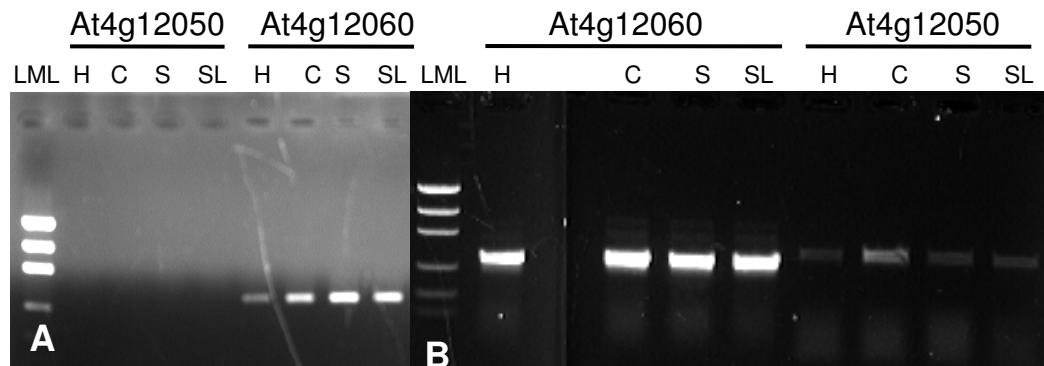


Fig 9. Amplification of genes flanking the *shI3* T-DNA insertion .

(A) Amplification at 25 cycles.

(B) Amplification at 35 cycles.

At 25 cycles amplification of gene *At4g12060* showed its suppression in *hrl1* and slight up-regulation in *shI3*. Amplification at 35 cycles shows saturation of gene *At4g12060*, and indicates that gene *At4g12050* is suppressed in both *hrl1* and *shI3*. H – *hrl1*, C – Columbia, S – *shI3*, SL – *shI3* with lesions, LML – Low Mass Ladder.

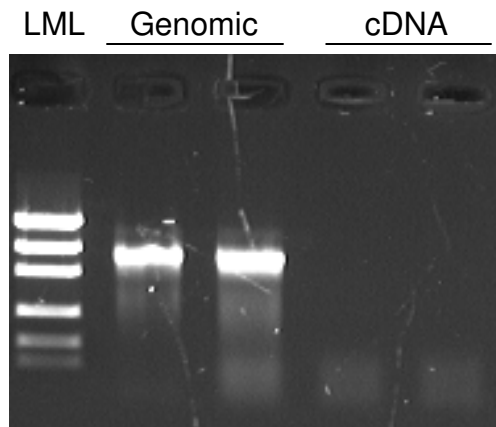


Figure 10. Amplification of Columbia genomic DNA and cDNA at 37 cycles with the forward and reverse primers that flank the *sh13* insert showed that no transcript is generated from that region.

Complementation of shl3

Previous work in the lab had identified the position of the *shl3* insertion to be located in the intergenic region between gene At4g12050 and gene At4g12060. To confirm that the insertion is responsible for the suppression of the *hrl1* phenotype, I attempted to complement the *shl3* plants with a wild-type copy of the genomic region between At4g12050 and At4g12060. The complementation was carried out using the bacterial artificial chromosome (BAC) F16J13, which contained the appropriate chromosomal region. The BAC was cut with the restriction enzyme EcoN1, treated with Klenow and dNTPs to fill in the 3' overhang to create a blunt end and then cut with the restriction enzyme BamHI leaving a sticky end. This created a 8603bp fragment of DNA that contained the appropriate region (Fig. 11a). The pMDC99 vector that was used to clone this piece (Curtis and Grossniklaus 2007) (Fig. 11b). The vector was cut with EcoRI, filled in with Klenow and dNTPs to create a blunt end, and cut with BamHI to leave a matching sticky end to the BAC. The appropriate fragments of the vector and BAC were isolated using low-melting agarose gel electrophoresis (Fig. 11c) and phenol extraction. Ligation of the BAC DNA and the vector was performed using T4 DNA ligase as described in methods. The ligation mixture was transformed into *E. coli*. Unfortunately even after 2 attempts, I did not get any colonies.

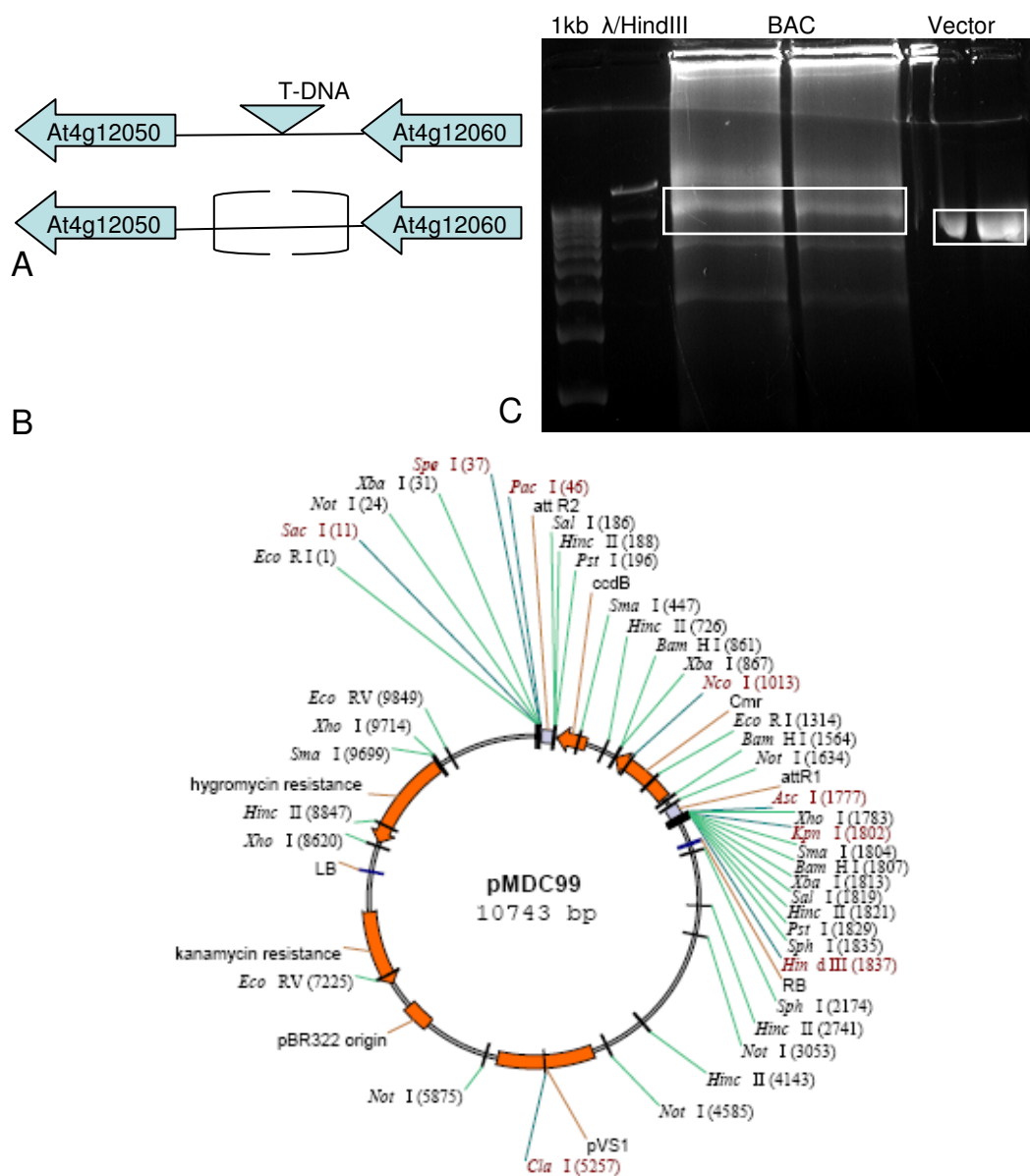


Figure 11. BAC and vector used for complementation.

(A) Site of T-DNA insertion in *shl3* mutant (upper) and region from wild-type Columbia used for complementation (lower).

(B) The vector used for complementation pMDC99.

(C) BAC DNA digested with *Eco*N1 and *Bam*HI. The region highlighted is the region that contains the region flanking the T-DNA in *shl3* and was eluted from low melting agarose for cloning in pMDC99 vector.

DISCUSSION

The *shl3* mutation in *Arabidopsis thaliana* suppresses the phenotypes of the *hrl1* mutant, reverting the phenotype back to that resembling the Columbia wild type strain. *hrl1* plants constitutively express defense genes, show spontaneous hypersensitive response-like lesions, display heightened resistance to pathogens, and are significantly smaller than wild-type plants. The *shl3* T-DNA insertion causes near-complete reversion back to a phenotype resembling the Columbia wild type plant, both visually and on the molecular level. The defense marker genes *PDF1.2* and *PR1* are suppressed in *shl3* to a level similar to that of the Columbia wild type. However, when the *shl3* mutant began to show lesions resembling those of *hrl1*, the expression of the defense genes is up regulated similar to the levels of *hrl1*. This indicates that the *shl3* reversion is not complete and as the plant ages it becomes more like *hrl1*. Through Southern blot analysis, prior studies show that there is only one T-DNA insertion in the *shl3* plants, which is localized in the intergenic region between genes At4g12050 and At4g12060. RT-PCR showed suppression of At4g12050 in *hrl1* as well as in *shl3*. Based on expression levels of the two genes flanking the *shl3* insertion, the insert does not appear to be affecting their expression in a way that would explain reversion of the *hrl1*-associated phenotypes in *shl3*. In addition, possibility of an unannotated gene being disrupted by the T-DNA in *shl3* does not appear to be the case because no transcript originating from this region could be detected by RT-PCR. However, this possibility needs to be further tested using techniques such as RACE PCR.

Unfortunately, the complementation experiments using the wild type chromosomal region flanking the T-DNA in *shl3* plants could not be completed. This would have helped to definitively show if the disruption of the intergenic region by the T-DNA is responsible for the reversion of *hrll*-associated phenotypes in *shl3*. This work will be continued in the Raina Lab by a new undergraduate researcher.

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Understanding the Genetic and
Molecular Mechanisms of the *SHL3*
phenotype in *Arabidopsis thaliana* in
Layman's Terms

A Capstone Project Submitted in Partial Fulfillment of the
Requirements of the Renée Crown University Honors Program at
Syracuse University, and Distinction in Biochemistry

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and Renée Crown University Honors

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The study of plant-pathogen interactions is an extremely useful medium for studying the defense pathways of plants. The study of the plant defense system is vital to the understanding of how to combat the critical food famine that affects us as well as our livestock. According to Census reports the world population has been rapidly increasing over the past years. Unfortunately, food production has not been able to increase accordingly because of crop loss caused by pests and diseases. By gaining the knowledge of the plant defense mechanisms and the genes that regulate the pathways involved, we are able to construct transgenic plants that will be able to defend themselves against bacterial, viral, fungal, and insect attacks, providing a higher crop yield.

There are two different variations of transgenic plants. In the first type a foreign gene is isolated from a different organism and inserted into the plant to express a new function in the plant. The second type of transgenic plant is where a native plant gene's function is disrupted by either up-regulating or suppressing it. Though there are many ongoing debates about the safety of consuming transgenic plants as well as feeding them to our livestock, the development of transgenic plants allows us to develop an environmentally friendly and sustainable alternative to the use of chemicals and pesticides.

One of the most widely used model plants in the study of plant-pathogen interactions is *Arabidopsis thaliana*. It is a small flowering plant in the mustard family and is useful for genetics research for a number of reasons. Its small size allows it to be grown year-round in growth chambers and greenhouses. Additionally, it has a short life cycle (approximately 8 weeks), a completely

sequenced genome, high reproductive rate, and is easy to transform to produce transgenic plants for genetics research.

There are two defense recognition systems apparent in plants. The first system is known as the basal defense. This is similar to the innate immune response in humans. The plant recognizes universal features of the pathogen, more specifically known as pathogen associated molecular markers (PAMPs) and leads to a signal cascade in the plant and ultimately to the accumulation of specific hormones that initiate broad range plant defense. The second type of defense is the induced defense or more commonly known as the gene for gene defense. In the gene for gene defense there are two possible outcomes for the plant pathogen interactions: virulent and avirulent. The first, virulent, is also known as a compatible interaction. In this interaction the pathogen enters into the plant cells and is unrecognized by the plants defense system and is able to drain the plant of its nutrients, eventually leading to plant death. The second form of plant pathogen interaction, avirulent, is also known as an incompatible interaction. Here the pathogen is still able to enter into the plant cells but is recognized by the plant defense system triggering hypersensitive response. A signal cascade leads to apoptosis of the plant cells in contact with the pathogen allowing the plant to reabsorb the nutrients from these cells and halt further spread of the bacterial infection. This resembles the humeral or antibody immune response in humans.

Previous research in the Raina lab focused on the characterization of the *hrl1* (*hypersensitive response-like lesions 1*) mutant, a lesion mimic mutant of

Arabidopsis generated by a single DNA point base pair mutation. *hrl1* mutants are much smaller than wild-type *Arabidopsis* plants and spontaneously develop lesions similar to those seen in plants undergoing the hypersensitive response. Additionally, *hrl1* plants continuously express several defense marker genes and are more resistant to a variety of pathogens than wild-type plants.

To identify additional components of the defense pathway regulated by *hrl1*, insertional T-DNA mutagenesis was carried out in the *hrl1* mutant background to identify suppressors of *hrl1*-associated phenotypes. This means that small identifiable tagged segments of DNA were inserted randomly into the *hrl1* plants and the progeny was screened for plants that displayed the wild-type phenotype. In this screen, the mutant *shl3* (*suppressor of hrl1 3*) was identified to cause a near complete reversion of the *hrl1*-associated phenotype. *shl3* plants are identical in size to wild-type Columbia plants and show wild-type levels of resistance to bacteria. Using southern blot analysis (locates the presence and quantifies sequences of DNA via a probe) it was determined that the *shl3* reversion is due to a single T-DNA insertion. Identification of the *shl3* mutation via plasmid rescue (used to locate unknown region of an insertion) revealed that the insertion is located in the intergenic region of the 4th chromosome between genes At4g12050 and At4g12060. My overall goal was to determine the molecular mechanisms involved in the suppression of the *hrl1* phenotype by *shl3*.

To do this I analyzed the concentration of the defense marker genes *PDF1.2* and *PR1*. The levels were shown to be lower in *shl3* in comparison to the *hrl1* mutant, and at the same level as Columbia wild type. However, when the

shl3 mutant began to show lesions resembling those of *hrl1* the concentration of the defense genes are increased to levels resembling *hrl1*. This indicates that the *shl3* reversion from *hrl1* to wild type Columbia is not complete and as the *shl3* plant ages it becomes more like *hrl1*.

Since the *shl3* insertion is located between two genes on the fourth chromosome, it is possible that expression of either of the genes flanking the insertion is disrupted, resulting in suppression of the *hrl1* phenotype. To test this, I quantified the concentration of the two genes that flanked the insertion, At4g12050 and At4g12060. Analysis of At4g12050 concentration showed a decrease in *hrl1* as well as in *shl3* in comparison to the wild type Columbia. Based on the concentration of the At4g12060 there is no difference across wild type Columbia, *hrl1*, and *shl3*. Based on the data collected there does not appear to be a drastic effect on the expression of either gene caused by the *shl3* insertion, therefore the reversion of the phenotype is not due to the disruption of these two genes.

The prediction that the *shl3* T-DNA is located in an un-annotated gene has not been conclusively tested at this time. Further research could be done using a variety of molecular biological techniques.

Previous work in the lab had identified the position of the *shl3* insertion to be located in the intergenic region between gene At4g12050 and gene At4g12060. To confirm that the insertion is responsible for the suppression of the *hrl1* phenotype, I attempted to insert the wild-type copy of the genomic region between At4g12050 and At4g12060 into *shl3* plants. Unfortunately a successful

transformation of the plants was never able to be completed and no data was able to be collected thus future work in the lab will be to continue to work with this aspect of the *shl3* project. This would have helped to definitively show if the region of the insertion is directly causing the reversion of the plants back to Columbia wild type. This like the un-annotated gene research needs more work to be done and will be continued in the Raina Lab by a new undergraduate researcher.