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Molecular Mechanisms Regulating Pathogen Defense: The Role of JMJ3 in *Arabidopsis thaliana*

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Molecular Mechanisms Regulating Pathogen Defense: The Role of *JMJ3* in
Arabidopsis thaliana

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

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May 2016

Honors Capstone Project in Biology

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Abstract

The Raina lab studies plant-pathogen interactions through genetics and molecular biology by using the model organism, *Arabidopsis thaliana*. A group of genes in *A. thaliana*, the Jumonji gene family, regulates gene expression by epigenetic mechanisms. Jumonji genes influence histone demethylation. Previous research in the Raina lab has shown that some Jumonji (*JMJ*) genes play a role in immune signaling in *A. thaliana* in response to pathogens. My project is to characterize *JMJ3* gene, and see if there was any role that *JMJ3* played in pathogen defense in *A. thaliana*.

The goal of my study was to investigate the possible role of *JMJ3* in pathogen defense. To assess this, *jmj3-1* and *jmj3-3* mutant lines were challenged with the bacterial pathogen, *Pseudomonas syringae*, and the fungal pathogen, *Botrytis cinerea*, and pathogen growth in these mutants was determined. These mutant plants were compared to wild type plants (WT) to determine if there was any change in disease susceptibility due to loss of function of this gene. My results indicate that *JMJ3* negatively regulates defense against *Pseudomonas syringae*.

Executive Summary

I joined the Raina research laboratory at Syracuse in Fall 2014. The laboratory is focused on molecular biology: biology on the molecular level. Molecular biology focuses on cellular pathways, genetics, and proteins. It can be used to explain physical observations that we make about organisms, on the molecular level (what we don't see with the eye). Specifically, the Raina lab applies this to plant biology, studying the molecular mechanisms involved in a plant's immune response to a pathogen. When a plant becomes infected, its immune system responds to the invading pathogen, just like how your body responds when you are sick. What we investigate are the cellular pathways and genes involved in a plant's immune response to a disease.

What are the practical implications of this research? Each year, millions of dollars of crop loss occurs due to pathogens. This is not only bad for business, but also bad for farmers, as this results in lower yields and lost product. The human population is currently growing at an exponential rate; the world population is currently projected to reach 9.5 billion by 2050 (the current world population is around 7.3 billion). This population growth has led to expanded development and urbanization to accommodate for the influx of people, which has resulted in a gradual decrease in farmland. Currently, hunger is a problem around the world. So, our population is growing at an exponential rate, we are losing farmland, crop yields are being hurt by disease, and we are already having trouble feeding people. With all of these factors in consideration, we are going to face an incredible challenge in feeding everyone in the not too distant future.

This is where molecular biology research on plants can help. If we can study and learn the pathways and genes that are involved in resistance to disease in plants, then we can use this knowledge to establish crops that are more resistant to disease. This would make the most of available farmland and result in higher crop yields, which would result in more food to feed the population.

My work involves investigating a group of genes in the Jumonji gene family. The Jumonji genes are important in regulating expression of genes. I am investigating to see if *JMJ3* is involved in immune system defense. The plant that I work with, *Arabidopsis thaliana*, expresses these genes. *Arabidopsis* is easy to grow and its genetic makeup has had a lot of prior research done, making it a good organism to work with. In order to see if *JMJ3* plays a role in pathogen defense, I compared two mutant lines, *jmj3-1* and *jmj3-3*, which did not express *JMJ3* to wild type (WT) plants to see if there was any difference in immune response. I exposed the two lines of *Arabidopsis* to the bacterial pathogen, *Pseudomonas syringae*, and the fungal pathogen, *Botrytis cinerea*, to see if there were any differences in resistance.

Overall, I have seen that there is no difference in resistance to the fungal pathogen, *Botrytis cinerea*, between the *jmj3-1*, *jmj3-3*, and wild type plants. However, I have observed that the *jmj3-1* and *jmj3-3* lines are more resistant to the bacterial pathogen, *Pseudomonas syringae*, than the wild type plants are. This would suggest that *JMJ3* negatively regulates *Arabidopsis*' defense against *Pseudomonas syringae*.

The next step in molecular analysis of *JMJ3* is to analyze RNA. DNA, genetic material, codes for RNA, which influences the expression of genes. *PR1* gene has been associated with immune response in *Arabidopsis*, in previous studies. When *Arabidopsis* is subject to a pathogen, its cells stimulate RNA to express *PR1* in the immune response. Therefore, if *JMJ3* plays a role in pathogen defense, there will be a difference in *PR1* expression between plants that express *JMJ3* and plants that do not express *JMJ3*. In order to test this, mutant lines expressing *JMJ3* and wild type plants will be tested. Tissue samples from non-diseased plants will be collected, along with tissue samples of diseased plants that were treated with *Pseudomonas syringae*. These samples will then be subject to RNA analysis. If *JMJ3* plays a role in pathogen defense, different levels of *PR1* expression will be seen between the two lines that do not express *JMJ3* and the WT plants that do express *JMJ3*. This would reinforce that *JMJ3* plays a role in pathogen defense in *Arabidopsis*.

My results indicate that *Arabidopsis* plants that do not express *JMJ3* are more resistant to *Pseudomonas syringae*, but show no difference in resistance to *Botrytis cinerea*, than their WT counterparts. Based on these results, I expect the RNA analysis to show elevated levels of *PR1* in the *jmj3-1* and *jmj3-3* lines, compared to the WT line. This means that *JMJ3* negatively regulates immune defense against *Pseudomonas syringae* in *Arabidopsis thaliana*.

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Acknowledgements

It has been a long road to get to this point. Before I begin, I would like to take a moment to give thanks where it is due. First off, I would like to thank Dr. Ramesh Raina for giving me this research opportunity and opening the door to me as a junior when many others were deterred from the fact that I was an upperclassman with no prior research experience. Over the last two years, Dr. Raina has given me confidence in working in a research environment and has helped me prepare for the next part of my life, after my time at Syracuse University. He has never once doubted my ability and has offered me support each step of the way through this experience, especially when I felt that I had hit walls. Dr. Raina not only welcomed me as a lab member, but as a friend too, inviting me to family events and dinners. I am incredibly fortunate to have a mentor such as him.

I would also like to thank my good friend, Luke Strauskulage, for turning my attention to the Raina lab when I was looking for labs, and for taking me under his wing to teach me a plethora of lab techniques when he was at a very busy point in his life. Luke has not been just a great person to work with in the lab; he has also been someone that I look up to and am proud to call my friend.

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going on. Thank you for being there for me through all of my success and failure in all aspects of my life. Thank you for listening to me when I have something going on and for offering insight and encouragement when I felt like throwing in the towel. Thank you for telling me to not settle for less than I could achieve. I am so lucky to be able to call you two my mom and my dad. I am so grateful for you two supporting me all the way both in and out of the classroom and when I decided to completely shift direction in my life. I know that I work hard and have a good head on my shoulders, but I do not think that I would have gotten to where I am now without your love, wisdom, and support.

I have learned a lot during this experience and believe that what I have learned in this lab will carry into other aspects of my life down the road, both inside and outside of a work environment. I hope that what I have worked on will help do some good in the world.

Introduction

Our current food system faces numerous challenges that threaten the well being of the human race. Every year, millions of dollars and a variety of crops are lost due to crop damage. This does not just hurt business; it affects everyone. It hurts the farmer who attempted to grow the crop, and it hurts the hungry consumer. These crop losses are not just because of inclement weather or droughts, pests and diseases contribute significantly to crop loss too.

In addition to this, our population is growing at an exponential rate. The current global population is around 7.3 billion people. By 2050, the world population is expected to be around 9.5 billion people (Figure 1). That is roughly a 30% increase in just thirty-four years. This is not helped by the fact that population growth and expansion is also leading to a decrease in the amount of available farmland. The 2012 National Resources Inventory shows that agrarian land is being converted over to developed land. In 1982, there were over 970 million acres of agricultural land in the U.S., in 2007, there was over 918 million acres of agricultural land (Table 1). Nearly 24 and a half million acres of agricultural land have been converted to developed land between 1982 and 2012 (Table 1). Society is progressively becoming more urbanized. Over 43 million acres of rural land have transformed into developed land since 1982 (Table 1). Land that was once used to grow crops is now being turned into land for humans to reside on. This means that if the human population grows, as predicted, more farmland will have to be turned into habitable land for humans. If this is to happen, the agricultural sector is going to have to find ways to be much more efficient with the farmland that is left.

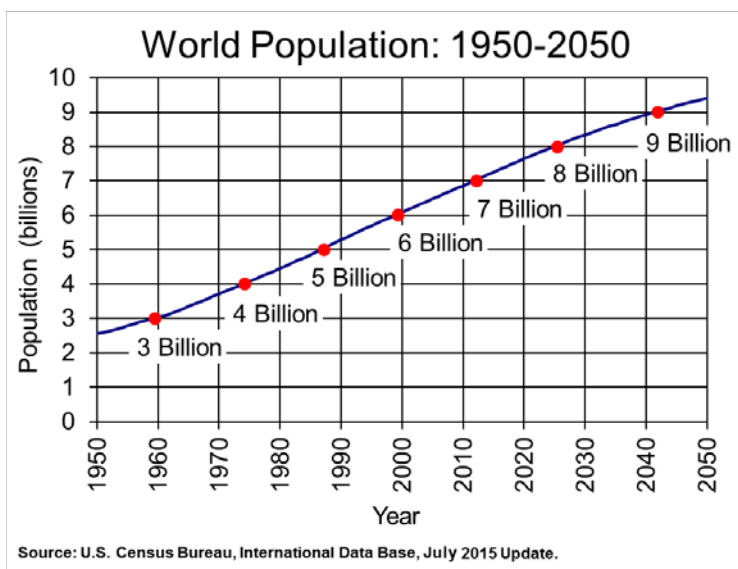


Figure 1: The estimated world population growth curve from 1950-2050, as reported by the U.S. Census Bureau.

2012 National Resources Inventory

	1997-2002	2002-2007	2007-2012	1982-2012
Agricultural land converted to developed land (acres)	4,867,400	3,539,100	1,824,500	24,498,500
Agricultural land at the beginning of the reporting period (acres)	937,815,600	926,494,400	918,305,300	971,232,700
Prime agricultural land at the beginning of the reporting period (acres)	271,816,400	267,502,300	264,112,000	281,483,300
Rural land converted to developed land (acres)	9,183,500	6,473,200	3,368,500	43,380,800
Rural land at the beginning of the reporting period (acres)	1,394,623,100	1,383,608,000	1,376,363,300	1,424,297,300
Prime rural land available at the beginning of the reporting period (acres)	322,336,800	319,284,200	316,818,800	329,978,700
Total surface area (acres)	1,944,142,600	1,944,142,600	1,944,142,600	1,944,142,600

Table 1: The National Resources Inventory from 2012, obtained from the Farmland Information Center. The table shows the amount of farmland that has been lost or converted for development over various time periods.

Furthermore, hunger is already an issue in the present day. A study done by the World Food Programme in 2015 shows that hunger is prevalent in many third world countries. Much of Africa, Asia, and areas in South America are critically affected by hunger (Figure 2). According to the study, the United States is not severely affected by hunger (Figure 2). However, think about this: there are many homeless people across the United States. People also rely on federal systems such

as food stamps in order to obtain food. The country also has food deserts spread across it. Food deserts are areas where access to food is severely restricted. An example of this would be an area that people reside in that does not have a grocery store, forcing the people to depend on a gas station or convenience store as a source of food. If these issues exist in the United States, and the study shows that hunger in the U.S. is not that bad when compared to other areas in the World, think about how many people are starving in those countries. The bottom line is that our current crop yields will not be able to satisfy the needs of a drastically larger future population, especially if agrarian land is disappearing. The system is not sustainable.

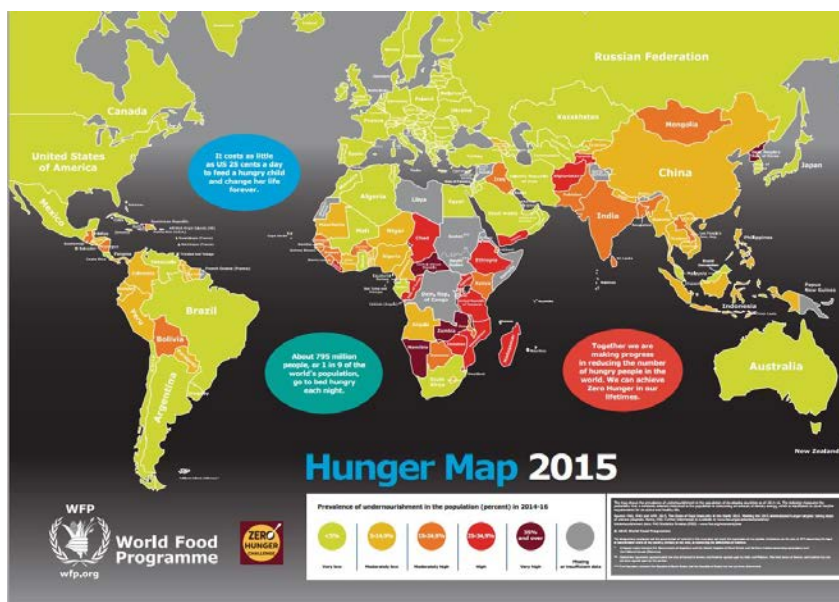


Figure 2: A map showing the distribution of hunger across the World in 2015. The World Food Programme performed the study. Areas in green are where hunger is least prevalent. Yellow, orange, red, and maroon show areas of increasing hunger, respectively. Areas in grey are countries where not enough information could be collected for an assessment on hunger.

What if there was a way to study how plants react to these pests and diseases on the molecular level? What if it was possible to figure out how to fight these pests and diseases? This would save millions of dollars and result in fewer crops lost, which in turn would help meet the dietary needs of the present and future populations. Studying pathogen resistance will help make the most of available

farmland and maximize its utilization to the fullest potential. These are the implications of molecular research in plants.

I joined Dr. Raina's molecular Biology research team at the end of September 2014. The model organism of choice in the laboratory is the plant *Arabidopsis thaliana*. *Arabidopsis* is a model organism because it has a small, completely sequenced genome. This makes it a great organism for molecular and genetic studies; it is easy to work with. *Arabidopsis* also grows relatively quickly, is easy to maintain, and has a wide variety of mutant genotypes to work with. I hope to see what genes are involved in the immune signaling of *Arabidopsis* against pathogens.

My main focus is the Jumonji gene family. The family is comprised of 21 different genes. Jumonji genes play an important role in gene regulation, especially chromatin regulation and development. One class of Jumonji genes, the JmjC domain, regulates histone demethylase activity (Takeuchi, 2006). Jumonji genes also influence flowering in *Arabidopsis*, a condition that would be affected by pathogens. I investigated to see if a group of Jumonji genes, *JMJ3*, are involved in immune system signaling. These genes can hopefully serve as genetic tools to provide insight on gene regulation and immune response; they are also conserved across many organisms, including humans. What we learn from the Jumonji genes in *Arabidopsis* can potentially be extrapolated to other areas of research, notably human diseases. The results of this research could shed some new light not just on how pathogen defense works in plants at the molecular level, but also on many other areas of the scientific world.

The goal of my research has been to investigate if *Arabidopsis* plants that express *JMJ3* are more resistant to bacterial and fungal pathogens than their common wild type counterparts are. A link between *JMJ3* and increased resistance to bacterial and fungal pathogens could be beneficial to establishing crops that are less susceptible to disease, thus raising crop yields.

Methods

Preparing Materials: LB media was made for developing bacterial cultures. Batches were made in 500mL bottles. 4g of tryptone, 2g of yeast extract, 10g of sodium chloride, and 320mL of de-ionized water were mixed and the remaining water was added after a pH of around 7 was confirmed for the media. If agar needed to be added to the LB media, to solidify it, 3.2g of agar were added per 400mL of LB.

A stock solution of 0.5M MgSO₄ was made in 100mL batches by combining 6.0185g of MgSO₄ in 100mL of de-ionized water. This stock solution served as an initial concentration for MgSO₄ in various experiments. The MgSO₄ was diluted to other concentrations with water. Mathematical calculations were performed to determine the right amounts to mix for proper concentration. All of my materials were sterilized before use by being autoclaved on a liq 15 cycle in an autoclave machine in the Life Sciences building.

DNA Isolation: Upon starting my research, I was presented with two separate growth lines for *JMJ3*, *jmj3-1* and *jmj3-3*. These lines had to be homozygous, meaning that they were not expressing *JMJ3*. The lines used for the study had to be homozygous, so that I would know that the observed results are due to the gene, *JMJ3*. Plants that were heterozygous possessed a copy of the gene for *JMJ3* and a copy of another gene; these were called wild type (WT) plants. In order to confirm that the lines were homozygous and that no WT plants were present in the lines, DNA had to be isolated and analyzed. Tissue samples were collected from *jmj3-1*, *jmj3-3*, and WT plants. Two leaf discs were collected from several plants of each line,

using a hole punch, and transferred into sterile microfuge tubes for each individual plant. The tissue was then macerated using small plastic pestles. Then, 400 μ L of DNA extraction buffer was added to each microfuge tube and the samples were vortexed for five seconds. The DNA extraction buffer was made from 200mM Tris HCl at pH 7.5, 250mM NaCl, 2.5 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% SDS. The DNA extraction buffer was made in 50mL batches (10mL Tris HCL, 12.5mL NaCl, 2.5mL EDTA, 5mL SDS, 20mL water). After being vortexed, the samples sat at room temperature for half an hour, to allow DNA to be extracted from the sample. Afterwards, the samples were put in a microfuge and centrifuged for one minute at 13,000rpm. Next, 300 μ L of the supernatant from each sample was transferred to new microfuge tubes. 300 μ L of isopropanol was added to each tube and the samples sat at room temperature for five minutes, in order to precipitate DNA from the solution. Then, the samples were centrifuged in a microfuge for five minutes at 13,000rpm in order to form a pellet of DNA at the bottom of each tube. The supernatant was discarded from each sample and residual salts were then washed away by adding 500 μ L of 70% ethanol to each tube. The samples were then centrifuged in a microfuge for five minutes at 13,000rpm, and the supernatant was discarded, leaving a purified DNA sample in each microfuge tube. The DNA pellets were then dissolved by adding 50 μ L of water to each sample and then stored in a freezer at -20°C for preservation.

DNA Analyses: PCR was performed to analyze the DNA samples. Both gene specific and T-DNA PCRs were performed. The master mix for the gene specific PCR was made with 6.3 μ L of water, 1.5 μ L of 2.5mM DNTP₃, 1.5 μ L of 10X buffer, 1.5 μ L of 20mM MgSO₄, 1.5 μ L of the appropriate forward primer, 1.5 μ L of the appropriate reverse primer, and 0.1 μ L of Taq. Each of the values of the ingredients were multiplied by the number of samples, plus 2, for pipetting error, and then 1.5 μ L of DNA was added to each PCR tube after the master mix was evenly distributed (13.9 μ L per tube). The T-DNA PCR master mix followed the same protocol, except the reverse primer was substituted with 1.5 μ L of 948 primer. The samples were then run in a PCR machine and then loaded into agarose gels along with 2 μ L of loading dye for each sample. A DNA mass ladder was also loaded as a reference marker to help identify expression of *JMJ3* in the samples. The gels underwent gel electrophoresis for 15 minutes and were then visualized using an Alpha Innotech Multi Image Light Cabinet and a computer. Agarose gels were prepared by mixing 0.4g of agarose powder and 50mL of 1X buffer into a flask. The flask was then heated in a microwave, to dissolve the powder, and then cooled in a hood. 2.5 μ L of ethidium bromide was added to the solution, which was then poured into a casting tray and hardened to form a gel. I had to wear lab gloves whenever I was working with ethidium bromide, as it was carcinogenic. The plants that were confirmed to be homozygous in the *jmj3-1* and *jmj3-3* lines were kept, while the heterozygous plants were discarded. The remaining plants were kept to mature and put in the greenhouse in the Life Sciences Complex in order to dry out to collect seeds.

Collecting Seeds: Seeds were collected from plants, to be used for my experiments. Once the plants in the greenhouse had dried up, they were individually grinded up by being rubbed between papers to release the seeds. The ground up plant was then put through a strainer, to separate the seeds from excess plant material. The seeds were stored in microfuge tubes and the leftover plant material and soil was autoclaved and then disposed of.

Sewing Seeds: Seeds that were to be used for experiments had to be washed and sterilized. The seeds for an experiment were allocated into 1.5 μ L centrifuge tubes and washed with 1mL of 20% bleach (bleach that was diluted with water) and then vortexed. The bleach was then removed via pipet and 1mL of water was added to the tubes, which were vortexed and drained by pipet again. The process was repeated three times, and then 1mL of water was added to each tube. The tubes were then put in a box that was wrapped in foil, to prevent light from causing the seeds to germinate prematurely, and the box was put in a refrigerator, overnight, to allow the seeds to stratify. Seeds were then sewn the next day. The tubes were drained of water and had top agar added to them. Top agar is more viscous than water, so it allows for more control over the seeds than water does while pipetting them into the soil. The seeds were pipetted into trays that had been potted and filled with autoclaved soil. Plastic domes were then misted and put over the trays, which then sat on racks, under light, in the growth room. The dome remained on, tight, until there were signs of germination. Then, the domes were put ajar and water was

added to the bottom of the trays. Once the *Arabidopsis* plants began to grow, the domes were removed from the trays.

Plant Maintenance: The *Arabidopsis* plants were carefully monitored in a growth chamber that had a constant temperature of 72°F and around 48% humidity. The plants were exposed to 12-hour cycles of light and dark. They were watered as needed, usually every 2-3 days. Forceps were also used to remove additional young plants that had germinated in the pots, so that one plant remained in each pot. Peter's fertilizer was mixed with water and given to the plants every two weeks to help with growth, as needed. Plants generally matured for experimental purposes within 4-5 weeks. When seeds had to be collected, the plants grew for longer, reaching the flowering stage. Wooden sticks and thin rope were used to tie the flowers, so that they would not tangle with each other and cross-pollinate. Plants that were to be used for seed collection were kept in the greenhouse, where they needed water every 2 days due to the increased sunlight.

Fungal Assays: The *Arabidopsis* plants were infected with the fungus, *Botrytis cinerea*, to see if the plants not expressing *JMJ3* were more or less susceptible to the fungus than the WT plants were. Large petri dishes were used for the fungal assays (Figure 3). They contained Peter's solution (mixture of Peter's fertilizer, water, and agar), which served to keep the leaves used in the assay intact for the duration of the assay. The plants used in the fungal assays grew for about four weeks. The plants were large enough to work with, but not large enough to make infiltration

difficult. Equal numbers of leaves from the *jmj3-3* and WT lines were collected from the plants grown for the assay and set in the Peter's solution in the petri dishes (Figure 3). Leaves from another line of *Arabidopsis*, 603, were collected and set in the dishes too. The 603 line was highly susceptible to fungal pathogens, so it was used as a control group to make sure that assay worked. If the 603 leaves appeared to be infected with the fungus, then the assay worked. A marker was used to divide up the dishes for each of the genotypes (Figure 3). The same process was done for fungal assays that also contained samples from *jmj3-1* plants (Figure 4).

The fungus was grown beforehand by sub culturing a small sample of fungus into a culture dish with melted PDA (PDB with agar). The dish was sealed with parafilm and wrapped in cloth to insulate it. The culture was then stored in a drawer and grown over the span of a month leading up to the fungal assay. When it was time to perform the fungal assay, a scoop of the fungus from the culture was mixed with 10mL of PDB in a 50mL tube, in a hood. The tube was then vortexed to break up the clump of fungus and release the spores. The mixture was then filtered into another 50mL tube using Miracloth. The filtered mixture was then vortexed again. A dilution had to be performed in order to have a proper spore concentration for the fungal assay. A hemocytometer was used to calculate the average number of fungal cells in a sample. Ten microliters of the mixture was loaded into each side of a counting slide, which was then put into the hemocytometer. The hemocytometer then counted the number of cells in the two samples, which were then averaged. A concentration of 2.5×10^5 cells was desired; the desired concentration value was divided by the average concentration that was obtained, and then multiplied by

10mL (the final solution would be 10mL). The resulting value was the volume of the fungal mixture that was to be used in making the solution for the assay. This value was subtracted from 10 to obtain the amount of PDB that needed to be added to dilute to mixture to the desired concentration. The obtained values of the fungal mixture and PDB were combined in a 15mL tube and vortexed, to ensure that fungal spores were distributed throughout the solution.

The leaves in the petri dishes were then each infected with 10 μ L of the final solution. The solution was pipetted onto the center of the topside of each leaf. The petri dishes were then sealed with parafilm and sat on a rack, under a light, for three days, until lesions from the fungus had developed. At that time, the diameter of each lesion was measured in cm, using a ruler, and recorded. The lesion diameters for each line were averaged and compared.



Figure 3: The setup for a fungal assay. 603 leaves in the middle served as the control group to test if the fungus was successful in infection. WT and *jmj3-3* leaves are arranged around the dish. Genotypes were divided by marking the plate. A small drop of the fungal solution was pipetted onto the center of each leaf. The dishes were sealed with parafilm and sat on a rack, under light, for three days to allow lesions from the fungus to develop.



Figure 4: A fungal assay setup containing leaves from *jmj3-1*, *jmj3-3*, WT, and 603 plants.

Preparing Bacteria for Disease Assays: *Pseudomonas syringae* (DC3000) was the bacteria used for the disease assays. The DC3000 was prepared prior to the disease assays. Two days before infiltrating the plants, several primary cultures were made. Cultures were by combining 5mL of LB Media, 5 μ L of rifampicin, 5 μ L of kanamycin, and a small clump of bacteria, from a glycerol stock that was kept frozen at -80°C, into a 15mL tube. Glycerol stocks were prepared with 0.7mL of 50% glycerol and 1.5mL of a prior primary culture in a small tube. The tube was snap frozen with liquid nitrogen and transported to the -80°C freezer, where it was stored. The primary cultures were sealed with parafilm and put into a shaker at 28°C to sit overnight. Secondary cultures were made during the evening of the day before infiltrating the plants. Three secondary cultures were made, containing 2 μ L, 3 μ L, and 4 μ L of primary culture, respectively. The given amounts of primary culture were added to 5mL of LB media, 5 μ L of kanamycin, and 5 μ L of rifampicin in the three tubes. The secondary cultures were sealed with parafilm and stored in a shaker at 28°C for overnight. I had to wear gloves while preparing the primary and secondary cultures, since rifampicin was carcinogenic.

Bacterial Infiltration of Arabidopsis: Infiltration of *Arabidopsis* had to be done in the morning, as that was when the stomata of the plants were most open to absorb the bacteria. The secondary cultures were taken out of the shaker and put in an Allegra 25R Centrifuge at 6000rpm for 10 minutes to form bacteria pellets. The volume of LB media in each tube was marked, and then the LB media was discarded and replaced with 10mM MgSO₄. Dilutions of the secondary cultures were then prepared

in 1.5µL micro centrifuge tubes; 500µL of the secondary cultures were mixed with 500µL of 10mM MgSO₄. The dilutions were made to analyze the bacteria using a Smartspec Plus Spectrophotometer. The spectrophotometer was calibrated with 1mL of 10mM MgSO₄. The bacteria dilutions were analyzed using the OD 600 setting on the spectrophotometer. An OD value for each of the dilutions was given and recorded. I wanted to use a secondary culture that had an OD value between 0.2 and 0.5, as that bacteria was at an ideal stage for infiltration. Once I chose the proper secondary culture, I diluted the bacteria down to a concentration of 5×10^5 , with 10mM MgSO₄. In the winter, I diluted the bacteria down to 1×10^6 , because infiltration was more difficult to perform on plants, so I wanted a higher bacteria concentration to ensure success. The final solution of bacteria was made in a 50mL tube and was constantly shaken by hand, to ensure that the bacteria did not settle down on the bottom of the tube.

The *Arabidopsis* leaves that were subject to infiltration were marked with a marker. Three leaves were infiltrated for each plant. Trays containing *jmj3-3* plants, *jmj3-1* plants, WT plants, and *gdg1-1* plants were subject to infiltration. I used *gdg1-1* as a control group for the disease assay, since it is highly susceptible to DC3000. If the *gdg1-1* plants showed signs of infection, then I know that the infiltration was a success. A syringe was used to force the bacteria into the leaf through the stomata on the underside of the leaf. After infiltration of the plants was complete, excess bacteria solution was cleaned off and the trays of plants sat for three days, to allow the bacteria to spread through the leaves before performing the disease assay.

Bacterial Disease Assay: Square culture plates were used for the disease assays, each plate could hold samples from four plants in a genotype. The plates were marked in a 4 by 4 grid pattern, with the rows representing the different plants and columns representing the different dilutions of bacteria collected from the plants. Eight of the nine plants for each genotype were used; so two plates were used for each genotype, per assay. LB media with agar was used to develop the bacteria samples obtained from the assay. The LB media with agar was melted down in a microwave and then cooled off in a hood. Once the media had cooled down enough, rifampicin and kanamycin were added to it. The antibiotics were added in a 1mL:1 μ L ratio, LB media to antibiotics. Each plate required 50mL of media so 50 μ L of each antibiotic were added, per plate. The media was poured into the plates and then sat to solidify. Once solidified, the plates were covered, to prevent the media from drying out while performing the assay.

Micro centrifuge tubes were also prepared before collecting tissue samples from the plants. Each of the tubes were labeled for a distinct plant, eight tubes were used for each genotype. The tubes were each filled with 800 μ L of a 10mM MgSO₄ & silwet solution. The solution was prepared by combining 1mL of 0.5M MgSO₄, 10 μ L of silwet, and 49mL of water in a 50mL tube. I used forceps and a hole punch to collect tissue samples from the plants. Discs of tissue were punched from the leaves that I had marked while infiltrating. I infiltrated three leaves per plant, so a total of three leaf discs were collected from each plant and stored in their appropriate centrifuge tube. Samples were collected from *jmj3-3* plants, *jmj3-1* plants, and WT plants. I did not collect samples from the *gdg1-1* plants because they were only used

to observe if the infiltration worked. I tried to take samples from one side of the midrib, to ensure that as much tissue could be collected as possible. Once all of the samples were collected, the centrifuge were placed in a beaker and put in a shaker at 28°C for one hour, to allow the solution to extract the bacteria from the tissue samples.

While the samples were in the shaker, I set up the next part of the assay. I took a 96-well plate and filled each of the wells with 180µL of 10mM MgSO₄, using an 8-pronged pipet. I divided the wells into four columns of eight wells, per genotype. Once an hour had passed, the samples were removed from the shaker and briefly vortexed to suspend the bacteria in solution. I pipetted 20µL of bacteria into the first well of each row in the marked columns. The first well of each row corresponded to a different plant. Once all of the samples were loaded, I performed a dilution series. I used the 8-pronged pipet to transfer 20µL of sample from the first column into the second column, then 20µL from the second column to the third column, and finally, 20µL from the third column to the fourth column. The dilution series allowed for bacteria colonies to be easily counted once they were plated.

After the dilution series was finished, the samples were plated on the culture plates that were prepared earlier. Each column marked on the plate represented a different plant, while each row represented a different dilution (1st, 2nd, 3rd, 4th). Each square on the marked grid received 20µL of the corresponding dilution via pipet and the plates were gently swirled by hand to spread the bacteria out. Once all of the dilutions were plated, the plates were set to dry for a few minutes before

being sealed and stored upside down in an incubator at 28°C for 24-26 hours, to allow for bacterial colonies to develop.

After the time had passed, the plates were removed from the incubator and the bacterial colonies were counted. I used the 4th dilution for counting, since the colonies were easier to identify in that set than in the 1st, 2nd, and 3rd dilutions (Figure 5). The plates were held up to a light and I counted the number of colonies for each plant.

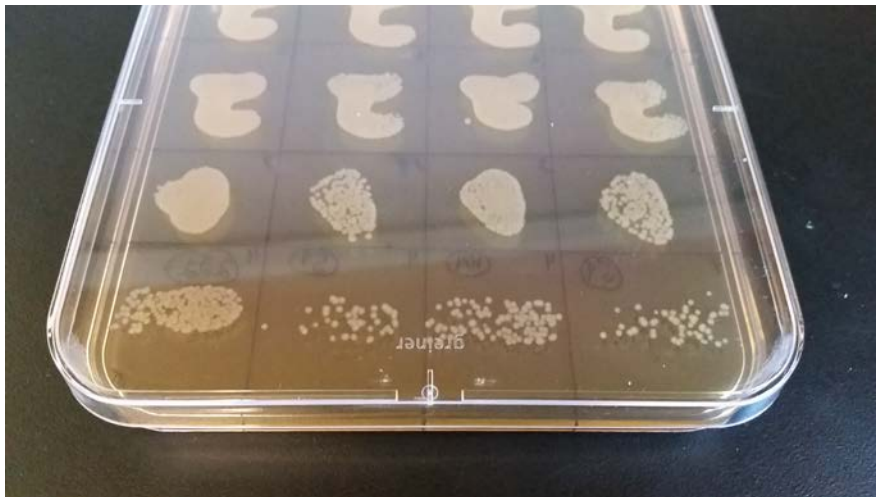


Figure 5: A bacterial disease assay plate. Each column represents a different plant. Each row designates a different dilution, from the 1st at the top, to the 4th on the bottom.

RNA Isolation: RNA will also be analyzed in order to see if expression of *JMJ3* is linked to pathogen response. *PR1* is a gene transcript that has been shown to be associated with immune response. It is stimulated by an invading pathogen. When a pathogen invades a cell, it stimulates the RNA in a cell to express *PR1*. If *JMJ3* has a role in pathogen defense, it will influence RNA synthesis and *PR1* expression. So, if RNA analyses of *jmj3-3* and *jmj3-1* show different levels of *PR1*, compared to levels in WT plants, then I will know that *JMJ3* influences expression of *PR1*, playing a role in the immune response of *Arabidopsis*.

Plants subject to RNA infiltration were infiltrated with DC3000 in the same manner as for the bacterial disease assays. Four plants were subject to infiltration for each line (*jmj3-1*, *jmj3-3*, and WT). Samples were collected for analysis over the course of 24 hours, at 6-hour intervals: Upon infiltration, six hours post infiltration, twelve hours post infiltration, and twenty-four hours post infiltration. Leaves from non-infiltrated plants for each line were also collected for comparison, upon infiltration. One leaf from each infiltrated plant was collected at the proper time interval, grouped together according to line, and stored in a 2 μ L RNA tube. I had to wear gloves when initially obtaining the RNA tubes from packaging so as not to contaminate the stock with my own RNA from my hands. Once samples were collected, they were snap-froze via liquid nitrogen and stored in a freezer at -80°C until I was ready to work with them.

Throughout the entire RNA analyses process, I had to wear gloves. This was because my own RNA, on my hands, could contaminate the samples and cause the RNA from the plants to degrade. Before RNA was extracted from the samples. I had

to clean down my workbench and pipets with diluted ethanol to minimize the chance of degrading the RNA. I also had to use a special type of water for RNA isolation, DEPC (diethylpyrocarbonate) water. DEPC was added to water in order to inhibit RNase that would be present in the water and cause RNA samples to degrade. The samples had to be grinded down using a mortar and pestle, which were also cleaned prior using water and bleach. The samples were stored in liquid nitrogen during the process, to keep them frozen. Liquid nitrogen was also used to chill down the mortar and pestle and to keep the samples frozen while working with them. Everything was kept cold to minimize the chance of RNA degradation. When the liquid nitrogen in the mortar evaporated, I ground the samples into a fine powder, and then transferred the powder back into the respective RNA capsule, which was then held in liquid nitrogen. Once all of the samples were ground, 1mL of Trizol was added to each of the samples. The Trizol is used to extract RNA from the powder. The mixture of Trizol and powder was briefly vortexed, and then stored at -80°C until I was ready to continue the RNA extraction process.

I added 200 μL of chloroform to each sample after letting them thaw from -80°C . Each tube was vortexed and then sat for three minutes to allow the compounds of the mixture to separate. Afterwards, the samples were centrifuged at 10,000rpm for fifteen minutes at 4°C , using an Eppendorf 5403 refrigerated centrifuge. The centrifuged samples contained a solid layer on the bottom of the tube and an aqueous layer on the top. The aqueous layer contained the RNA. I extracted 600 μL from the aqueous layer of each sample and transferred the extracted samples to fresh tubes. I also added 600 μL of isopropanol to each of the

extracted samples and vortexed them. After the samples sat for ten minutes, they were centrifuged under the same conditions as before, except for ten minutes. A small white pellet of RNA formed in the bottom of the tube. Next, the isopropanol was removed from the tubes and 1mL of 70% ethanol (ethanol diluted with DEPC water) was added to each tube. The samples were vortexed and then centrifuged again at 7,500rpm at 4°C for five minutes. Then, the ethanol was removed from the tubes and the samples were centrifuged again under the same conditions, except for one minute. Excess ethanol was removed and the samples sat until they were dry. The RNA samples were then dissolved with 35µL of DEPC water and stored at -20°C until I was ready to proceed with quantifying the RNA.

RNA Quantification: RNA samples had to be at equal concentrations to assess expression of *PR1*. Gel electrophoresis was first used to make sure that RNA was present in the isolated samples. Then, concentrations were analyzed using the RNA OD mode on a spectrometer. Readings were taken by adding 1µL of sample to 99µL of DEPC water. Once all of the values were recorded, samples were diluted accordingly with DEPC water to achieve a target concentration of 0.5µg/µL for all samples. Gel electrophoresis was used to check and see if sample concentrations were equal.

cDNA Synthesis: Once the RNA samples were quantified, they could be used to create cDNA, which will be used to assess expression levels of *PR1*. RT-PCR was used to synthesize the cDNA from the RNA samples. The master mix was created using the

following formula: 1 μ L of oligo dT (500 μ g/ μ L), 8 μ L of dNTPs (2.5mM), and 1.1 μ L of DEPC water. This formula was multiplied by the number of samples used, to obtain the proper amount of mixture; 10.1 μ L of the master mix was added to each PCR tube. RNA samples were then added to the tubes, 5 μ L of RNA was added per tube. The tubes were then placed in the PCR machine for five minutes at 65°C. Then, the tubes were removed and placed on ice for one minute. A separate mix was made and added to the original mixture. This mixture was made using the following formula: 4 μ L of 5X Buffer, 0.2 μ L of RNase inhibitor, 0.3 μ L of DEPC water, and 0.4 μ L of RT enzyme. This formula was also multiplied by the number of samples; 4.9 μ L was added to each PCR tube. The samples were then subject to RT-PCR to generate the cDNA. After RT-PCR, 20 μ L of DEPC water was added to each sample and the samples were stored at -20°C.

Results

DNA Analyses:

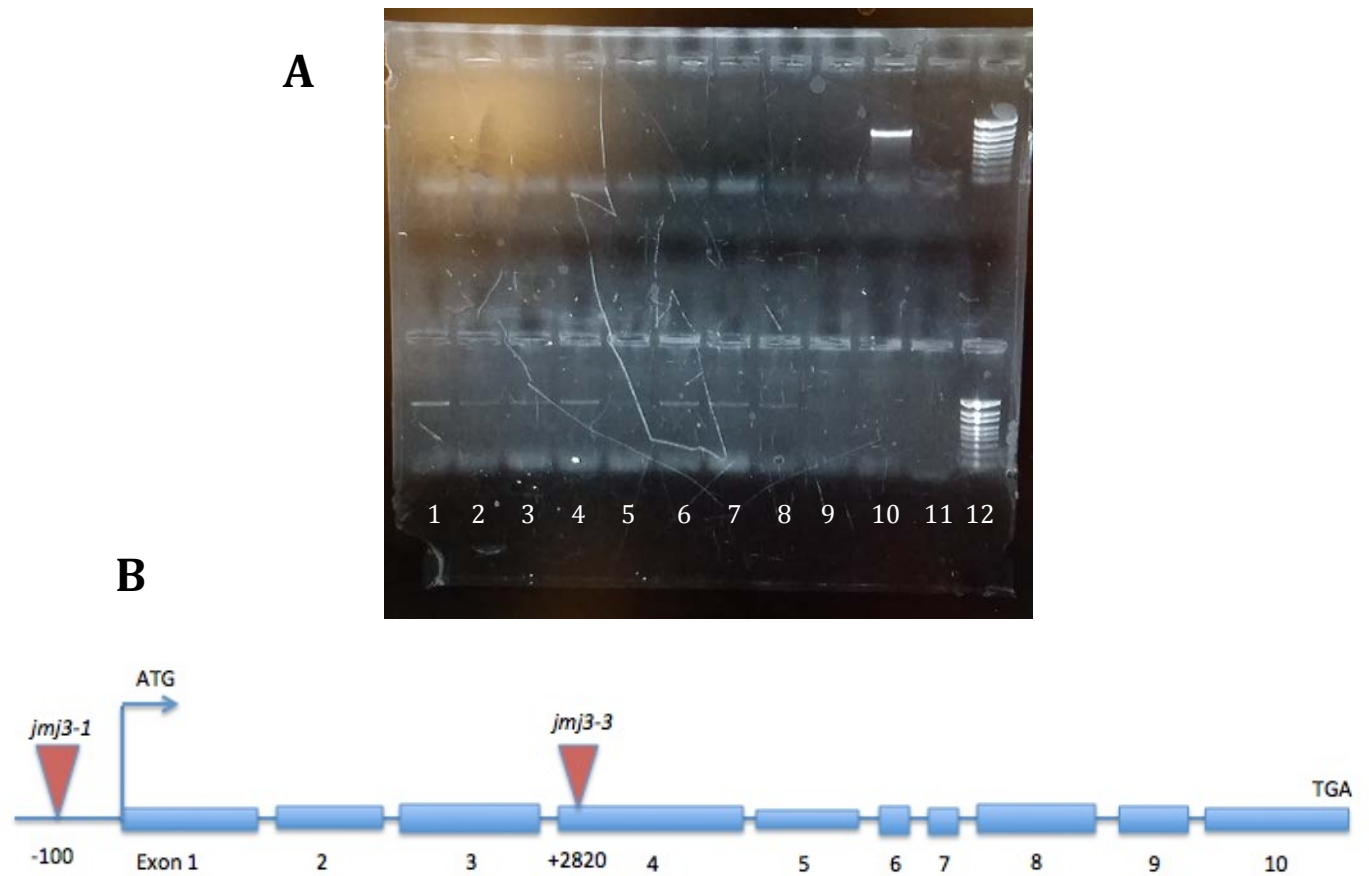


Figure 6: (A) Analysis of DNA for *jmj3-3* via PCR and gel electrophoresis. Lanes 1-9 correspond to DNA taken from *jmj3-3* plants, lane 10 contained DNA from a WT plant. Lane 11 contained blank PCR mixture, to ensure that no contamination was in the PCR. Lane 12 contained a DNA ladder that was used to identify the expression of *JMJ3*.

(B) A diagram showing the T-DNA insertion lines of the mutant lines in *JMJ3*. *Jmj3-1* was located in the promoter region, 100 base pairs away from ATG; *jmj3-3* was located on the 4th exon, 2820 base pairs away from ATG. This diagram is not to scale.

DNA was analyzed to confirm that the observed results were due to expression of the *JMJ3* gene. Samples were taken from *jmj3-1* and *jmj3-3* plants and compared to DNA from a WT sample. The top row shows a gene specific PCR analysis and the bottom row shows a T-DNA PCR analysis (Figure 6A). Lanes 1-9 contain samples from *jmj3-3* plants, and lane 10 contains samples from a WT plant. In order to make sure that my PCRs had no DNA contamination, I also analyzed a sample containing only PCR mixture (lane 11). Lane 12 contained a DNA ladder that was used to help identify bands in the PCRs. The absence of any bands in lane 11 confirms that there was no contamination in my PCR analyses. The band in the WT sample in the top row, and absence of bands in lanes 1-9 confirm that the *jmj3-3* plants I used were homozygous (Figure 6A). This is further supported by the presence of bands in lanes 1-9 (some are faint), and absence of a band in lane 10, on the bottom row; this confirms that the *jmj3-3* line is homozygous. This means that the difference between WT and *jmj3-3* lines in the bacterial and fungal assays was due to expression of the gene, *JMJ3*. The WT plants were expressing *JMJ3*, while the mutant plants were not expressing *JMJ3*.

Mutant Phenotype Characterization: While I was letting my plants grow, I noticed a phenotype difference between the mutant lines not expressing *JMJ3* and WT plants. There was a difference in the saliks (pods that stored seeds) once the plants reached the flowering stage. I noticed that plants not expressing *JMJ3* had a large number of saliks that looked slightly shriveled. The WT plants had a few abnormal saliks, but much fewer than plants not expressing *JMJ3* did. Almost 76% of the saliks observed

on *jmj3-3* plants were abnormal, while over 42% of saliks on *jmj3-1* plants were abnormal compared to around 25% of the saliks on WT plants were abnormal (Table 2).

Genotype	Percentage of Abnormal Saliks
WT	24.64%
<i>jmj3-1</i>	42.46%
<i>jmj3-3</i>	75.98%

Table 2: A table showing the percentage of abnormal saliks on plants sampled for each of the lines.

Fungal Assays:

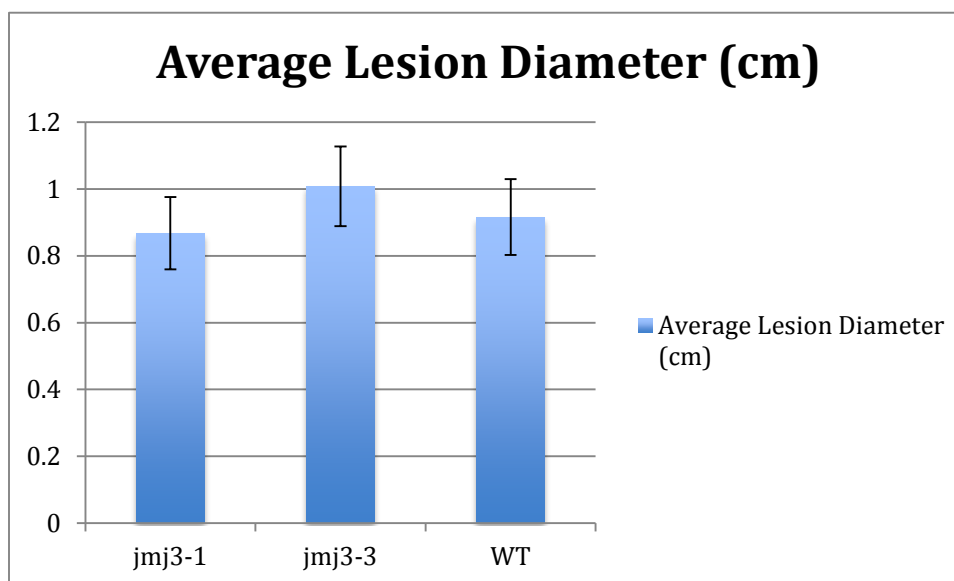


Figure 7: The fungal assays showed that there was not a significant difference between *jmj3-1*, *jmj3-3*, and WT in susceptibility to *Botrytis cinerea*. P-values of 0.761 for *jmj3-1* and 0.57 for *jmj3-3* confirmed that the results were not statistically significant. This experiment was done five times and had similar results each time.

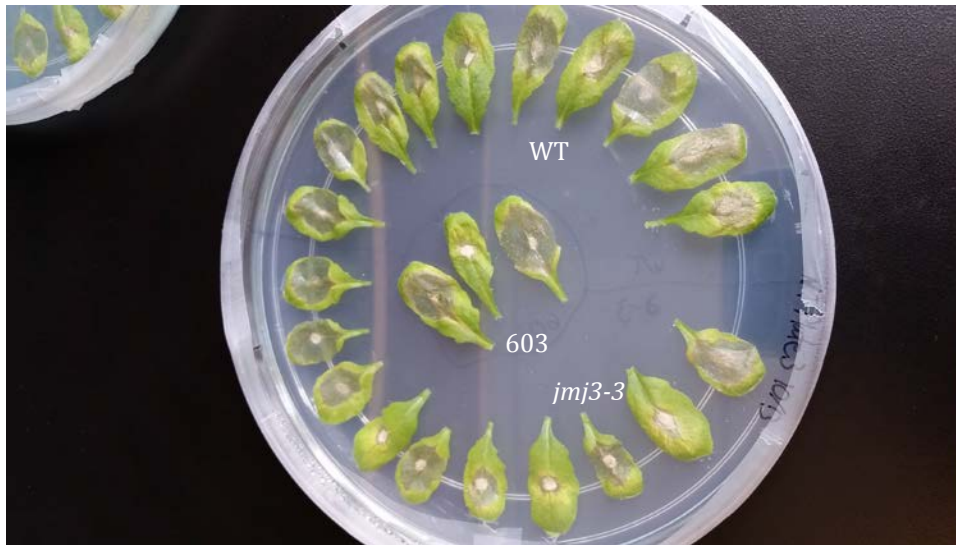


Figure 8: A fungal assay tray, containing *jmj3-3*, WT, and 603. Calices and lesions from *Botrytis cinerea* can be seen on the leaves. The 603 “control group” can be seen in the middle, while the WT and *jmj3-3* leaves are divided around the outer portion of the dish.

The fungal assays that tested susceptibility to *B. cinerea* showed that there was no significant difference between *jmj3-1*, *jmj3-3*, and WT plants (Figure 7). The average lesion length of *jmj3-1* leaves was 0.868cm, 1.008cm for *jmj3-3* leaves, and 0.916cm for WT leaves. The mutant *jmj3-1* and *jmj3-3* lines appeared to have slightly smaller lesions than WT had; however, p-values of 0.761 for *jmj3-1* and 0.577 for *jmj3-3* confirmed that the results were not statistically significant, as both values were well over the 0.05 threshold (Figure 7). Overall, observations showed no significant visible difference between *jmj3-3* and WT (Figure 8). *JMJ3* appears to have no affect on immune response to *B. cinerea*.

Bacterial Disease Assays:

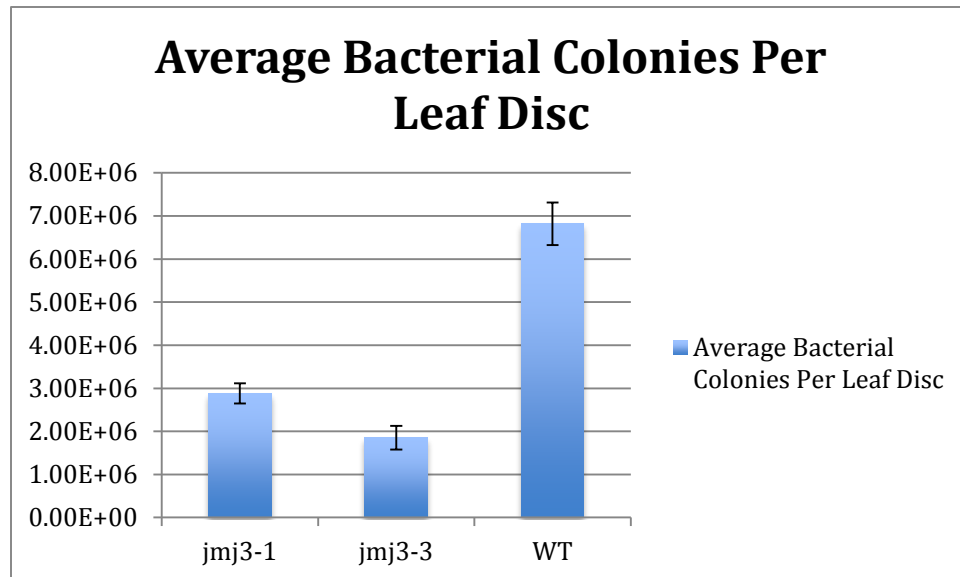


Figure 9: The bacterial disease assays showed a significant difference between *jmj3-1*, *jmj3-3*, and WT in susceptibility to the bacterial pathogen, *Pseudomonas syringae*. *Jmj3-1* was 2.37 times more resistant to *P. syringae* than WT was. *Jmj3-3* was 3.69 times more resistant to *P. syringae* than WT was. P-values of 4.53×10^{-6} for *jmj3-1* and 4.48×10^{-7} for *jmj3-3* confirmed that the results were statistically significant.



Figure 10: Leaves taken from a bacterial disease assay consisting of *gdg1-1*, *jmj3-1*, *jmj3-3*, and WT plants. The leaves from *jmj3-1* and *jmj3-3* plants are visibly less infected than the leaves of WT plants.

The bacterial disease assays performed on *jmj3-1*, *jmj3-3*, and WT showed that *jmj3-1* and *jmj3-3* were more resistant to *P. syringae* than WT was (Figure 9). *Jmj3-1* was 2.37 times more resistant to *P. syringae* than WT was. *Jmj3-3* was 3.69 times more resistant to *P. syringae* than WT was. The leaves of *jmj3-3* and *jmj3-3* plants looked healthier than the leaves of WT plants, in both size and color (Figure 10). Statistical analyses gave p-values of 4.53×10^{-6} for *jmj3-1* and 4.48×10^{-7} for *jmj3-3*, confirming that the results were statistically significant. This experiment was done three times with *jmj3-1* and seven times with *jmj3-3*, with similar results each time.

Discussion

DNA Analyses: The DNA analyses performed show that the mutant lines used in the study *jmj3-1* and *jmj3-3*, were homozygous, they were not expressing *JMJ3*. The PCR confirmed that the WT sample was expressing *JMJ3*, while the mutant was not (Figure 6A). This means that the differences observed between the mutant lines and the WT line were due to expression of *JMJ3*.

Fungal Pathogen Susceptibility: My results indicate that *JMJ3* plays no role in the resistance of *Arabidopsis thaliana* to *Botrytis cinerea*. The average lesion length of *jmj3-1* leaves was 0.868cm, 1.008cm for *jmj3-3* leaves, and 0.916cm for WT leaves (Figure 7). These average values are all very close to each other, and statistical analyses yielded p-values of 0.761 for *jmj3-1* and 0.57 for *jmj3-3* when compared to WT. These p-values show that the results are not statistically significant; there is no difference in susceptibility to fungal pathogens between the *jmj3-1* and *jmj3-3* lines and WT. It can also be seen visually that there is no significant difference between *jmj3-1* and *jmj3-3* leaves and WT leaves when exposed to *Botrytis cinerea* (Figure 8). This experiment was repeated four times, and once again with the *jmj3-1* line, and very similar results were seen each time.

Bacterial Pathogen Susceptibility: My results show that *A. thaliana* plants not expressing *JMJ3* are more resistant to the bacterial pathogen, *Pseudomonas syringae*, than WT plants are. *Jmj3-3* was 3.69 times more resistant to *P. syringae* than WT was, and *jmj3-1* was 2.37 times more resistant than WT was (Figure 9). P-values of

4.53×10^{-6} for *jmj3-1* and 4.48×10^{-7} for *jmj3-3* confirm that these results are statistically significant. The leaves of plants in the *jmj3-1* and *jmj3-3* lines also looked visible healthier than the leaves of WT plants (Figure 10). This experiment has been repeated seven times with *jmj3-3* and three times with *jmj3-1* and similar results have been seen each time. These results show that *A. thaliana* plants that do not express *JMJ3* are more resistant to the bacterial pathogen, *Pseudomonas syringae*.

Overall Implications: My results indicate that *JMJ3* mutants were more resistant to *Pseudomonas syringae* than WT plants were. The bacterial disease assays with *jmj3-1* and *jmj3-3* and WT showed more bacterial colonies for WT than for *jmj3-1* and *jmj3-3*. Leaves of *jmj3-1* and *jmj3-3* plants that were infected also appeared to be healthier than the leaves of infected WT plants. However, *JMJ3* mutants appear to be no different than WT plants in susceptibility to *Botrytis cinerea*. The fungal assays performed showed very similar susceptibility between *jmj3-1*, *jmj3-3*, and WT.

Future Work

My results indicate that *JMJ3* does play a role in defense against *Pseudomonas syringae*. To further analyze this relationship, the cDNA from the RNA analysis will be analyzed to examine levels of *PR1* expression in *jmj3-1*, *jmj3-3*, and WT plants. The cDNA samples will have to be normalized, like the RNA samples, to obtain proper expression levels of *PR1*. Previous studies have shown elevated levels of *PR1* to be associated with increased pathogen resistance. Since *jmj3-1* and *jmj3-3* were more resistant to *P. syringae* than WT was, I would expect to see elevated levels of *PR1* in the mutant lines, when compared to the WT line. This would further support the idea that *JMJ3* plays a role in pathogen defense (Figure 11).

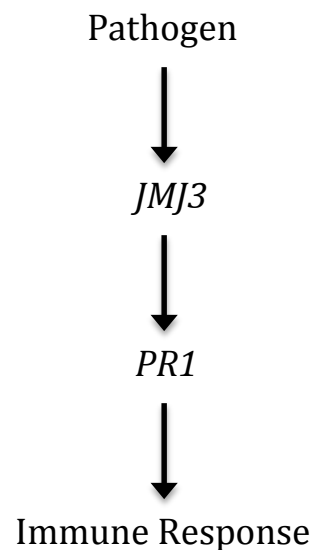


Figure 11: The proposed model of the role of *JMJ3* in pathogen defense. A pathogen influences *JMJ3*, which is expected to influence *PR1*, which results in an observable immune response.

Conclusion

My results show that *JMJ3* does not play a role in defense against the fungal pathogen, *Botrytis cinerea*, but *JMJ3* does play a role in defense against the bacterial pathogen *Pseudomonas syringae*. *JMJ3* negatively regulates defense against *P. syringae*. The mutant lines that were not expressing *JMJ3* showed increased resistance to *P. syringae*. Further work will be done to investigate this relationship; I expect to see increased expression of *PR1* in plants not expressing *JMJ3*, when compared to WT plants.

References

Hunger Map 2015. Digital image. *World Food Programme*. World Food Programme, 2015. Web. 7 Sept. 2015. <https://www.wfp.org/content/hunger-map-2015>.

Takeuchi, T., Y. Watanabe, T. Takano-Shimizu, and S. Kondo. "Roles of Jumonji and Jumonji Family Genes in Chromatin Regulation and Development." *National Center for Biotechnology Information*. U.S. National Library of Medicine, Sept. 2006. Web. 04 Nov. 2014. <http://www.ncbi.nlm.nih.gov/pubmed/16715513>.

World Population: 1950-2050. Digital image. *United States Census Bureau*. U.S. Census Bureau, July 2015. Web. 7 Sept. 2015. <https://www.census.gov/population/international/data/idb/images/worldpop.png>.

2012 National Resources Inventory. Digital image. *Farmland Information Center*. National Resources Inventory, 2015. Web. 12 Jan. 2016. <http://www.farmlandinfo.org/statistics>.