5-2015

Developing double mutant Arabidopsis plants deficient in fatty acid desaturase 7 and a salicylic acid pathway regulator, NPR1-1

Alexys Marrufo
University of Arkansas, Fayetteville

Developing Double Mutant Arabidopsis Plants Deficient in Fatty Acid Desaturase 7 and a Salicylic Acid Pathway Regulator, NPR1-1

An Honors Thesis submitted in partial fulfillment of the requirements for Honors Studies in Biology

By

Alexys Marrufo

2015

Biology
J. William Fulbright College of Arts and Sciences
The University of Arkansas

Acknowledgements

I would like to thank my mentor Dr. Fiona Goggin for her guidance and teaching throughout this project and my thesis writing. Her investment in me has not only allowed me to succeed in the lab, but also to learn valuable practical skills. I would also like to thank Jiamei Li for her willingness to constantly teach me and for her thorough responses to all of my questions. Jiamei's talent and passion for teaching were evident to me every time she helped me. Additionally, I would like to thank the University of Arkansas Honors College for providing me with the opportunity to pursue research as an undergraduate. Research has challenged me intellectually in a way the classroom could not, making it an experience that I am thankful to have had. I would also like to thank each of my committee members for investing the time and energy required as a member of my thesis committee. Finally, I would like to thank the Arkansas Department of Higher Education for its part in funding this project through both the SURF and ASSET grants.

TABLE OF CONTENTS

Abstract

Salicylic acid (SA) is one of several molecules whose accumulation is known to stimulate the synthesis of defense genes in *Arabidopsis thaliana* as well as in other green plants. Fatty acid desaturase 7 is a cytoplasmic desaturase that introduces double bonds into fatty acids. In previous experiments on both *Arabidopsis thaliana* and tomato *(Solanum lycopersicum)*, it was observed that a loss of function mutation in *FAD7* coincided with an increase in SA accumulation in plant cells. Additionally, in those experiments, plants displaying the *fad7-1* mutation displayed increased resistance when challenged with aphids as compared to WT Arabidopsis possessing functional *FAD7*. However, the mechanism whereby increased resistance was mediated was not concluded from the studies. It has been hypothesized that a SA-dependent mechanism was responsible for the displayed increased resistance due to the high SA levels observed. If this is the case, then a regulator of SA pathways, NPR1, is also important in mediating increased resistance. However, plants have not yet been synthesized to specifically test whether *fad7-1* mediates increased resistance via a SA pathway and NPR1 or not. Therefore, in this project, double mutant Arabidopsis plants were generated from single mutant Arabidopsis parental plants. After three generations of pollination, double mutant plants were obtained and plentiful seed was collected from them. Future experiments will utilize the seed collected from this project to test the potential role that *fad7*-1 has in mediating increased plant resistance via a SA-dependent mechanism and NPR1, an important SA pathway regulator.

Introduction.

Despite their size, small piercing-sucking insects can cause significant injury to plants. These insects inject their small mouthparts into a plant and extract fluids that are necessary for the survival of the plant. One such insect is the aphid. Specifically, an aphid extricates sap from the phloem of plants, such as roses or green plants like lettuce and alfalfa. Aphids can cause physically apparent changes to a host as well as changes at the molecular level through the enzymes in their saliva. As is the case with many predators, these modifications are usually harmful and cause damage to the host, but are beneficial to the aphid.

FAD7 is an ω-3 fatty acid desaturase (FAD) located in the chloroplast (Browse, 1986). Fatty acid desaturases are enzymes that remove hydrogen atoms from a fatty acid, thereby introducing double bonds into the fatty acid. Specifically, FAD7 is known to introduce double bonds into C16:2 as well as C18:2 fatty acids to synthesize C16:3 and C18:3. FADs are known to influence plant susceptibility to a wide range of stresses such as drought tolerance and plant adaptation to temperature extremes (Upchurch, 2008). Additionally, FADs play an important role in the synthesis of a defense hormone known as jasmonate, which is an active form of jasmonic acid (JA) (Avila et. al., 2012).

In general, plants have several known defense pathways that may be activated upon physical damage by predators. Jasmonic acid (JA) (Howe and Jander, 2008; Fonseca et al., 2009; Sheard et al., 2010) and salicylic acid (SA) (Vlot et al., 2009) are two of the known molecules whose accumulation stimulates the synthesis of defense genes in plants. JA accumulation has been observed in plants combating fungal pathogens (Kunkel et. al., 2002), combating chewing insects, and in plants responding to being wounded (Avila et. al., 2012). In contrast, SA accumulation has been shown to be useful in protecting plants against piercing-sucking insects (Avila et al., 2012**)** and in establishing systemic acquired resistance (SAR), a state of heightened defense in plants (Kunkel et al., 2002)**.** In rice, it has been observed that when the *FAD7* gene is overexpressed, JA levels are high (McConn et al., 1997). However, in Arabidopsis plants in which *FAD7* and other *FAD* genes have been suppressed (*fad3 fad7 fad8)*, JA levels are depleted (Song et al., 2004 and Martin et al., 1999**)** and SA levels are high. Therefore, evidence supports the idea that particular FADs play a role in mediating JA and/or SA accumulation and, as a result, may play a role in mediating the defense mechanisms that plants use against predators.

A previous study in tomato (*Solanum lycopersicum)* showed that plants displaying a loss-of- function in *FAD7,* noted as *fad7-1,* had 42% fewer aphids on them when compared to wild type plants (Avila et al., 2012). Similarly, increased aphid resistance has also been observed in *Arabidopsis thaliana* mutants with defects in *FAD7* expression (Avila et al., 2012). Based on these two studies, *fad7* appears to mediate plant resistance in both tomato and Arabidopsis. Additionally, in each of the two studies, intracellular SA levels were observed to be higher than they normally are found to be in wild type (WT) Arabidopsis plants with normally expressed *FAD7* (Avila et al., 2012). However, in tomato and Arabidopsis, it is unknown whether *fad7-1/gl1* mediated the increased resistance via a SA accumulation or via some other cascade of signals or via a combination of the two. Therefore, it is necessary to conduct experiments in which either a direct connection between the *fad7-1* mutation, the presence of increased SA levels in the plant, and increased plant resistance can be made or not to clearly define the role of *fad7-1* in stimulating defense signaling in *Arabidopsis thaliana* via SA and NPR1-1.

NPR1-1 is a cytoplasmic protein and a positive transcriptional regulator known to regulate and promote many SA-dependent pathways (Zhang et al., 1999; Dong, 2004). As SA accumulates in a plant cell, NPR1-1 is transported into the nucleus. In the

nucleus, SA binds to NPR1-1 via transition metal ions, such as S-nitrosothiol (SNO) and thioredoxins (TRXs). Binding of SA to NPR1-1 causes a conformational change in NPR1-1 that exposes the NPR1-1 transcription-activating domain (TA) and stimulates the transcription of defense genes (Pajerwoska-Mukhtar, 2013). A previous study in tomato (*Solanum lycopersicum)* showed that silencing of the *NPR1-1* gene coincided with an increase in aphids of about 70% when compared to wild type plants (Avila et al, 2012). Therefore*,* it seems that NPR1-1 plays a necessary role in stimulating increased plant resistance when they are challenged with aphids, though it is not the only regulator that increases plant resistance. Because NPR1-1 regulates SA pathways, it seems possible that the SA pathway may be a mechanism by which plants gain increased resistance to aphids when *FAD7* expression is suppressed.

Glabra 1 (Gl1) is a gene that regulates the development of trichomes in *Arabidopsis thaliana*. Trichomes are small hairs that grow from the epidermis of a plant and can have various functions such as protecting the plant against predators or acting as secretor organs. The *glabra 1* mutation in Arabidopsis causes leaves to be glabrous, or in other words, to lack trichomes. Whether the absence of trichomes has any detrimental affects on Arabidopsis is unknown. However, the phenotypic manifestation of the mutation can be used as an indictor of successful pollination. For instance, suppose one parental plant possesses trichomes and the other does not. Cross-pollination is then performed between these two plants. If cross-pollination was successful, then progeny plants will display trichomes (only 1 copy of Gl1 is necessary for manifestation). By merely observing a phenotypic trait, the plants that are successfully pollinated are easily identifiable by the presence of trichomes, and the absence of trichomes helps to quickly eliminate plants that are not properly pollinated.

As previously mentioned, it has been observed that the *fad7-1* gene coincided with an increase in intracellular SA levels in Arabidopsis. In the *fad7-1* mutants, increased plant resistance to aphids was observed. It is not known whether the increased resistance was directly due to increased SA accumulation/SA-dependent pathway or other factors. Additionally, NPR1-1 is essential for optimal function of SA-dependent plant defense mechanisms because NPR1-1 acts as a receptor for SA. The binding of SA to NPR1-1 mediates the transcription of defense genes (Pajerwoska-Mukhtar et. al., 2013). It is hypothesized that SA is indeed playing a role in increased resistance in *fad7-1* mutant Arabidopsis. If this is so, *NPR1* expression will be necessary to mediate that SA-dependent defense response optimally.

fad7-1/gl1 (decreased synthesis of JA)
↓
increased SA levels
↓
NPR1
↓(**?**)
Increased resistance

Figure 1. Proposed cascade of events causing increased Arabidopsis resistance to aphids.

One way to study the role of *FAD7*, SA, and NPR1 in increased resistance is to generate hybrid double mutant plants with a *fad7-1 x npr1-1* genotype*.* The *fad7-1* mutation, when present with WT expression of *NPR1*, should cause increased plant resistance (Avila et. al., 2012). The *npr1-1* mutation, when present with WT expression of *FAD7*, should cause decreased resistance to aphids when compared with *fad7-1* mutants. Theoretically, when both mutations are present, as in the double mutant plants,

the plants should display decreased resistance when challenged with aphids. The reason for this is that though SA levels may be increased due to the *fad7-1* mutation, the *npr1-1* mutation will decrease the levels of the NPR1 protein present in the cell. The decreased NPR1 levels will leave the elevated amount of SA little NPR1 to bind to, and transcriptional activation of defense genes should not occur to a significant degree. Essentially, if it is observed that abnormal functioning of a critical step in the SA pathway affects plant resistance, then it can be demonstrated that resistance is mediated, at least in part, through a SA-dependent mechanism. Additionally, it will be confirmed that the reduced expression of FAD7, at least in part, mediates the increased resistance through SA and NPR1-1 if *fad7-1* gene expression is unable to mediate increased defense despite the alleles present for NPR1-1. However, double mutant plants have not yet been developed to perform this experiment.

Therefore, the primary goal of this project was to develop *fad7-1 x npr1-1* double mutant Arabidopsis plants and collect plentiful seed from those plants. In each generation, seed was collected only from plants with trichomes due to the fact that cross-pollination with *npr1-1* plants should cause the development of trichomes even though *gl1* was present in parental *fad7-1* plants. The seed collected from this project will be used by other lab members to test the contribution of NPR1 to aphid resistance in Arabidopsis plants with impaired *FAD7* activity. The results of such studies could be an important step towards the ability to create lines of plants exhibiting increased resistance to aphids and other small piercing-sucking insects as well, which are so often devastating to plants. Creating such resistant plants could potentially aid in increasing the yield of green plants, which are often used for food as well as aid in reducing plant loss for gardeners/farmers and the costs associated with such losses.

Materials and Methods.

Plant Materials. All plants were planted in 65mm pots of a peat, vermiculite, perlite (4:3:2 ratio) soil mixture. Pots, trays, forceps, and other utensils used to plant seeds were sprayed with milk one day prior to planting as a preventative measure against tobacco mosaic virus (TMV). In all generations except for F2, soil components were mixed immediately before planting and the soil mixture was moistened before being placed into individual pots for planting. During the planting process, an average of three plants were planted in each pot to reduce the risk of obtaining no viable plants in a pot. All plants were grown under stable conditions (~25°C-28°C; 16:8 L:D photoperiod) in enclosed plant racks in the lab (Figure 2A). All plants were watered twice weekly with tap water, at the beginning and end of the week. The plants were fertilized once weekly with MiracleGro. Plants were given treatments of 1mL/500g Gnatrol once weekly as a preventative measure against fungus gnats. Fertilization and Gnatrol treatments were not given at the same time.

Plant Pollination. In the parental generation (P), *Arabidopsis thaliana (Columbia CS7000) fad7-1/gl1* mutants were used as the maternal parent and were hand-pollinated (crossed) with *npr1-1* mutants. Hand-pollination was performed according to *Protocol for Crossing Arabidopsis Plants* (Goggin and Avila, unpublished data). Only Arabidopsis plants whose buds were still green and closed were emasculated and pollinated with pollen from an *npr1-1* flower. After hand-pollination of the parental plants, F1, F2, and F3 plants were allowed to self-pollinate. To ensure F1, F2, and F3 self-pollination, each plant was placed in a sleeve before it began producing flower buds (Figure 2B).

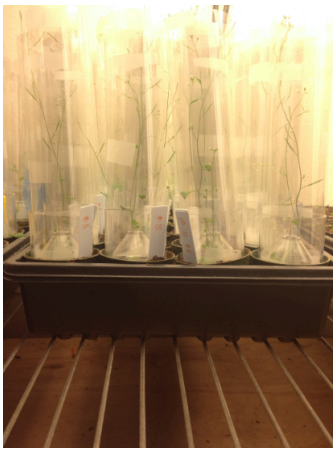
A.  B. 

Figure 2. Plants at various stages of growth; A. Plants growing on plant racks in the lab 1 day after seeds planted; B. Plants 1 week after placed in sleeves.

P

↓ Hand-pollination

F1 (heterozygous hybrids)

↓ Self-pollination

F2 (homozygous *Atfad7-1,* heterozygous *npr1-1)*

↓ Self-pollination

F3 (homozygous *Atfad7-1,* homozygous *npr1-1)*

Figure 3. Overview of the project from P to F3 including the pollination method for each generation.

Tissue collection. DNA was extracted from generations P, F1, F2, and F3 using the one-step DNA extraction method**.** According to *One-Step DNA Extraction from Arabidopsis* (Kasajima et. al., 2004) (Edwards et. al., 1999) small glass beads were placed into a 1.5 mL centrifuge tube along with 200 μl of extraction buffer. A hole punch was then used to extract a circular piece of the plant leaf DNA (average DNA sample weight ≈ 3.6 mg). The leaf sample was placed into the centrifuge tube and was completely submerged in the extraction buffer. The GenoGrinder homogenizer was then used to grind the extracted tissue at 1750 rpm for one minute. After homogenizing, DNA was stored at -20°C until needed for the PCR process.

Genotype screening. To determine the genotypes obtained in each generation variants of TD-PCR scaled for a 25μl reaction volume were used. Plants were first screened for the *fad7-1/gl1* mutation in the F2 generation. TD57- PCR was used when testing with WT FAD7 primers and mutant *fad7-1/gl1* primers. Different forward primers were used for WT and mutant NPR1-1, but they shared a reverse primer. The following primers were used:

AtFAD7 WT(F) 5’ – TTTCAGTGGGCTCGAAGTCC – 3’
Atfad7-1 Pro-leu2 (F) 5’ – TTTCAGTGGGCTCTCGAAGACT – 3’
AtFAD7 WT (R) (shared reverse primer) 5’ – ATCTGCGGGAAAAGATGATG – 3’

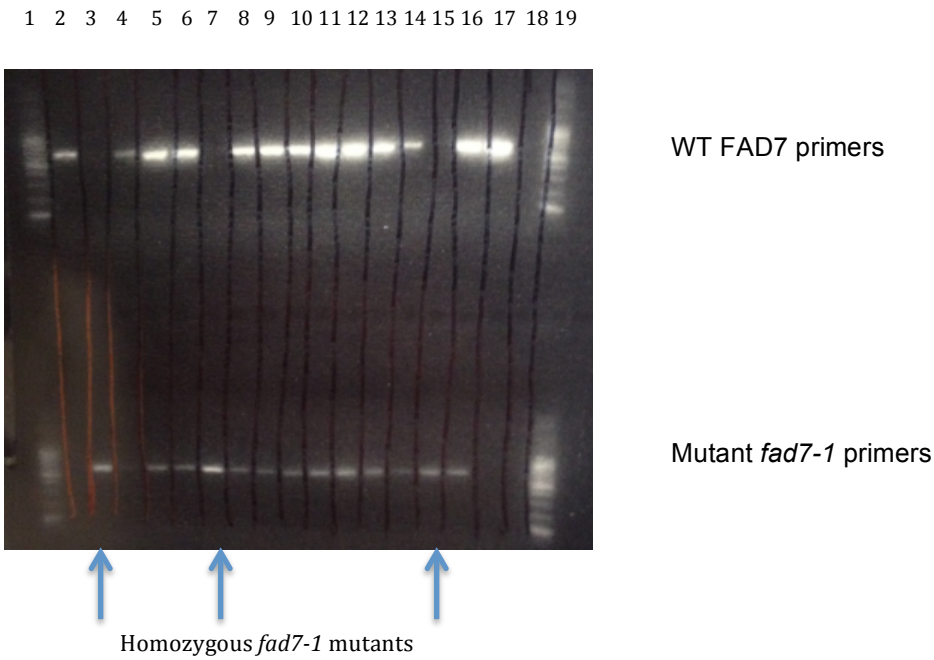


Figure 4, Lanes 1 and 19 contain a 100 base pair DNA size marker. The WT *FAD7* amplicon length is and mutant *fad7-1* amplicon length is 446 base pairs. Lanes 2-17 represent various *fad7-1* plants in the F2 generation. Lane 18 is the negative control, containing only PCR reagents and no DNA. Amplification of DNA (indicated by bands) by the WT primers indicates the presence of at least 1 WT FAD7 allele while amplification by mutant primers indicates presence of at least one *fad7-1* allele. Plants showing bands for both primer sets represent heterozygous plants. In this photo, lanes 3, 7, and 15 represent *fad7-1* homozygotes. Homozygous *fad7-1* plants will be screened for homozygosity at the *npr1-1* locus.

Once homozygous mutant *fad7-1/*gl1 plants were obtained in F2, only those mutant plants were tested for the presence of *npr1-1.* No homozygous mutant *npr1-1* plants were identified in F2. Therefore, seeds produced from the homozygous mutant *fad7-1/gl1* plants were used to plant the subsequent F3 generation. The F3 generation was then tested for the presence of *npr1-1* expression. TD-PCR 56 was used with both WT and mutant *npr1-1* primers. Different forward primers were used for WT and mutant NPR1-1, but they shared a reverse primer. The *npr1-1* primers used were:
At_NPR1-1_WT1 (F) 5’ – CGAGGGGATATACGGTGCTTC – 3’
At_NPR1-1_MU1 (F) 5’ – CGAGGGGATATACGGTGCTTT – 3’
At_NPR1-1_1 (R) (shared reverse primer) 5’ – GCGGTTCTACCTTCCAAAGTT – 3’

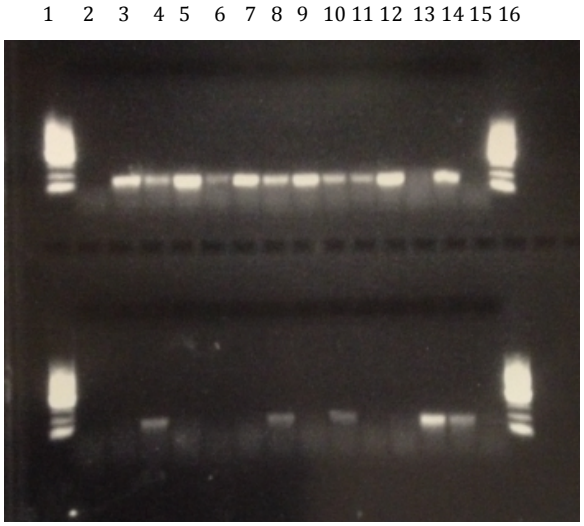


Figure 5. Lanes 1 and 16 contain 100 base pair DNA size marker. The WT NPR1-1 amplicon length is 115 base pairs while the mutant *npr1-1* gene amplicon length is 208 base pairs. Lanes 2-11 represent various NPR1 plants in the F3 generation. Lanes 12-15 represent both positive and negative controls of *fad7-1/gl1, npr1-1, fad7-1 x npr1-1,* and a negative control containing PCR reagents with no DNA, respectively. Amplification of DNA by the WT primers (indicated by bands) indicates the presence of at least 1 WT NPR1-1 allele while amplification by mutant primers indicates presence of an *npr1-1* allele. Plants showing bands for both primer sets represent heterozygous plants.

Gel electrophoresis. Electrophoresis was run in a scooter box on 1% RA agarose gel at 250V for 16 minutes. 20μl of reaction product was loaded into gel wells. 4μl of 100 base pair DNA size marker was loaded into the first and last wells in each row. The UVP Biodoc. was used to read and take photos of the gel.

Seed collection. Seeds were collected from F1, F2, and F3 plants. Seeds were collected in Arabidopsis siliques (the fruit of the Arabidopsis plant) after siliques were a golden brown color. To aid in collecting the small siliques, flat-headed forceps were used to pluck the stalk of the silique off of the live plant. Once collected, seeds were placed in 1.5 ml tubes and labeled with the identity of the seeds. The seeds were then stored at room temperature until needed for cleaning or sterilization.

Seed cleaning. Before seeds were sterilized, plated on agar, and afterwards planted, it was necessary to remove them from their siliques (cleaning the seeds). To clean, 1.5 ml tube contents were emptied onto a piece of white paper. Forceps were then used to remove siliques and any other debris from among the pile of seeds. After removing all debris, seeds were placed back into their 1.5 ml storage tubes and stored at room temperature until needed for sterilization.

Seed sterilization. Before seeds were plated and then planted, they were sterilized. This process involved washing the seeds with 70% ethanol and then with a 6% bleach solution. Next, seeds were rinsed 6-7 times with ddH_2O to remove any ethanol or bleach residue as leftover residue can hinder seed germination. Sterilization was performed according to *Germinating Arabidopsis thaliana seeds on MS sterile media* (Lorence, 2008). Sterilization was important to ensure that bacteria and other pathogens that may

have attached to seeds at any point were removed. Often, pathogens can affect seed germination or harm germinating seeds, making them unusable for planting.

Results.

Double mutants. A total of 14 *fad7-1 x npr1-1* double mutant plants were obtained in the F3 generation. First, homozygous *fad7-1* single mutants (homozygous at the FAD7 locus / heterozygous at the NPR1-1 locus) were obtained in F2. Theoretically, it was expected that homozygous *npr1-1* mutants would be obtained in F2 as well if we screened a large enough number of F2 plants. However, they were not obtained until F3. The proportions of observed *fad7-1* and *npr1*-1 plants quickly diverged from each other in F2, exemplifying Mendel's *law of segregation*. Due to the fact that pairs of alleles can segregate independently, after obtaining single mutant plants, it was necessary to grow another generation in order to obtain double mutant plants.

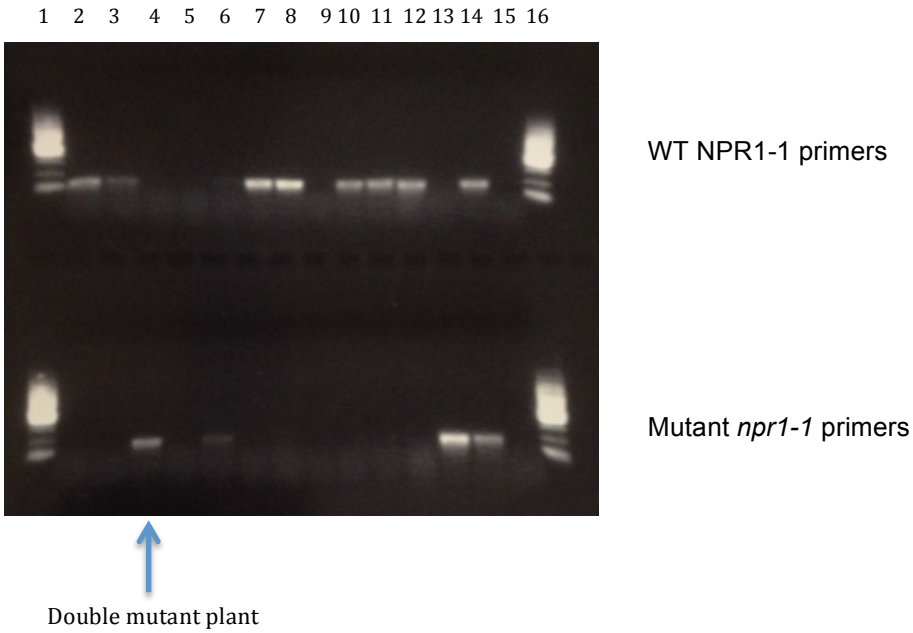


Figure 6. Lanes 1 and 16 contain 100 base pair DNA size marker. The WT NPR1-1 amplicon length is 115 base pairs while the mutant *npr1-1* amplicon length is 208 base pairs. Lanes 2-11 represent various F3 *npr1-1* gene mutant plants. The plant in lane 4 shows amplification (a band) with mutant primers but no amplification with WT primers. This verifies that it is a homozygous *npr1-1* mutant. Each of the plants represented on this gel were produced from parents who were homozygous for the *fad7-1* gene mutation. Therefore, plant 3 is homozygous for both the *fad7-1* mutation and the *npr1-1* mutation. It is a double mutant plant. Additionally, lanes 12-15 represent both positive and negative controls of *fad7-1/gl1, npr1-1, fad7-1 x npr1-1,* and a negative control containing PCR reagents with no DNA, respectively.

The χ^2 test was used to determine whether the observed number of plants in each generation significantly deviated from the expected number. For F1 plants, there was no statistically significant deviation in observed versus expected numbers of plants that were homozygous WT, heterozygous, or homozygous mutant plants at the FAD7 locus. For F2 plants, there was a significant deviation in observed versus expected numbers of homozygous WT FAD7 plants, leading to a greater number of homozygous WT plants than expected. But, no significant deviation was observed in heterozygous or homozygous mutant plant numbers.

For F1 *npr1-1* plants, there was no significant deviation in observed versus expected numbers of homozygous WT, heterozygous, or homozygous mutant plants. For segregation of *npr1-1* in the F2 generation, there was a significant deviation observed in homozygous WT and homozygous mutant plants, yielding higher numbers of plants than expected. There was no significant deviation in heterozygous plants. In F3 *npr1-1* plants, there was no significant deviation in homozygous WT, heterozygous, or homozygous mutant plant numbers.

For F1 plants, there was a significant deviation in the trichomes present category. Fewer plants with trichomes were observed than were expected. For F2 plants, at the *gl1* locus, there was no significant deviation observed in either trichomes present or trichomes absent. For F3 plants, there was a significant deviation only in the trichomes absent category yielding fewer plants with trichomes than expected.

Seed totals. Estimation was used to determine the total number of seeds obtained. To estimate, one 1.5 ml centrifuge tube containing seeds collected from one plant was used as a reference. The number of seeds in the reference tube was counted. Then, the seed numbers in remaining tubes were estimated based on visual comparison to the reference tube. In total, about 2700 seeds were obtained from the 14 double mutant plants. Based on these numbers, the average number of seeds collected from each plant was 193 seeds, although some plants produced considerably more seed than others.

Figure 7. The reference tube used for estimating seed number in all tubes. This tube contains ~200 double mutant Arabidopsis seeds.

Discussion. The ultimate goal of this project was accomplished upon obtaining double mutant *fad7-1 x npr1-1* plants. However, there were various issues encountered throughout the project. For instance, in the *fad7-1/gl1, npr1-1,* and *gl1* genes in various generations, significant deviation was seen in observed versus expected numbers of specific plant genotypes. Any F1 deviations may have been due to error in hand-pollinating technique. Because this process requires work with tiny flower buds and contains many steps, there are many possibilities for error, such as damaging buds or unsuccessful pollination. In the F2 generation, a number of plants desiccated and died. Because only living plants were used and taken into account, any F2 deviations in observed versus expected numbers may have been caused by unintended desiccation. The observed desiccation may have possibly been due to the fact that the soil was not

mixed immediately before planting as it was done in other generations. Instead, soil that had been made the day before was used. However, a cause and effect relationship between the day-old soil and desiccation was not confirmed. Any significant F3 deviations in observed versus expected numbers may have been due to the shorter sleeve length used, which may have possibly led to the rare cross-pollination rather than self-pollination. In all generations, the sample size may have affected the observed versus expected differences, as the expected numbers are expected to be observed in a sufficiently large sample of plants.

In addition to occasional technical mistakes and problems, gel/gel picture problems were also an issue throughout the project. Various times, after performing PCR and then running gel electrophoresis, no DNA amplification was observed at all upon placing the gel under UV light. Other times, slight amplification was observed, but no sharp or clear bands were seen by which a definitive conclusion could be reached. To fix the issue, various solutions such as using new agarose to make the gel, and/or replacing old PCR reagents with new ones, and/or using different PCR machines were tested. Each time a gel problem was encountered, one of these solutions was successful in fixing the problem.

Additionally, an issue was encountered in determining a successful method by which to identify the presence of the mutant *npr1-1* genotype. The first attempt was to identify the presence of *npr1-1* using the NlaIII restriction enzyme. The only genetic difference between WT and mutant NPR1 is a point mutation. Therefore, the restriction enzyme, which was supposed to cleave the WT NPR1 but not recognize or cleave the mutant *npr1-1* due to the point mutation, was experimented with for about three months. However, proper cleavage was not observed and differentiation of WT versus mutant NPR1-1 was not possible. This could have been due to using too low of a purified DNA concentration or incubating the reaction mixture for too short of a time. Additionally, at the time I used the restriction enzyme, I was encountering gel clarity issues. Consequently, any results that I may have obtained from the restriction may have appeared smudged or unclear. Therefore, no conclusions could be reached. Hence, an alternative method of identifying *npr1-1* was required. Next, experimentation with WT specific NPR1 and mutant specific *npr1-1* primers began. Four different sets of WT and mutant primers were designed. After testing three of the four sets, one WT primer set and a separate mutant primer set were found to provide sufficiently clear results in identifying the presence of *npr1-1*. Therefore, using allele-specific primers proved to be the most efficient method of identifying *npr1-1* in F2 and F3.

In essence, although the various problems encountered occupied substantial time, the goal of this project was completed. The plentiful number of seeds obtained from double mutant plants will allow for growing and studying Arabidopsis plants to clarify the role of FADs, specifically FAD7, in regulating SA dependent defenses against aphids via NPR1. Specifically, newly synthesized double mutant plants may show that *fad7-1/gl1* up-regulates Arabidopsis defenses against aphids (Avila et. al., 2012) via SA signaling by showing that optimal increased resistance cannot be attained without a necessary component of the SA pathway, NPR1 (Pajerwoska-Mukhtar et. al., 2013). In other words, when newly synthesized plants are challenged with aphids, they should display decreased resistance if NPR1 (silenced) and the SA pathway (silenced in part) are crucial to causing increased resistance. If these results are seen, then the proposed mechanism (Figure 1, pg. 4) of increased resistance in Arabidopsis will be confirmed. With this understanding, additional studies, which challenge double mutant plants with aphids, should be conducted with a greater number of plants. If results are consistent with previous lab studies, then, in the future, it may be possible to introduce the *fad7-/gl1* mutation into crops that are used for human consumption and observe whether *fad7-*

1/gl1 could mediate increased resistance to aphids in those plants. However, it is important to remember that mutant FAD7 causes an increase in SA while mediating a decrease in JA (Avila et. al., 2012) which is another defense molecule that is important in plant responses to other insects and pathogens (Kunkel et. al., 2002). Therefore, the potentially negative consequences of silencing one mechanism to reap the benefits associated with another mechanism must be considered and experimented with before the *fad7-1/gl1* mutation is introduced into crops.

Contrastingly, it may be observed that the proposed hypothesis is incorrect and that increased Arabidopsis resistance to aphids can occur independently of the SA pathway. If plants deficient in a crucial SA pathway regulator are still able to elicit significant defenses against aphids, then this may show that SA is relatively unimportant in increased resistance or at least does not play a crucial role in increased resistance. If this is the case, then at least one method thought to be responsible for increased resistance may be eliminated from consideration and future studies can shift their focus to the study other pathways that may possibly be responsible for increased Arabidopsis resistance to aphids.

Conclusion.

In conclusion, Arabidopsis *fad7-1 x npr1-1* double mutant plants were produced in this project. The seed collected from those plants will be used as a basis for future experimentation in studying the role that *fad7-1* plays in stimulating plants defenses against aphids via SA accumulation in the cell and NPR1-1, a SA pathway regulator. Future experimentation on these double mutant plants may allow researchers to make direct connections between *fad7-1,* increased SA accumulation, and increased Arabidopsis resistance. Otherwise, researchers will be able to rule out SA-dependent pathways as a mechanism for increased resistance. At any rate, future experiments will provide insight into the mechanism/s behind increased plant resistance against aphids. If these mechanisms are better understood, future agricultural practices could include generating plants, such as lettuce or alfalfa, with altered fatty acid desaturase function in order to produce crops that demonstrate increased resistance to insect pests, like aphids and/or other piercing-sucking pests. This could lower the amount of crops lost to piercing-sucking insect pests and reduce the unexpected costs associated with such losses.

Tables

fad7-1 gl/1	49
npr1-1	23
Hybrids	0

Table 1. Number of parental plants representing each respective genotype. 72 total plants were identified as possessing trichomes in the parental (P) generation and were used for collecting seed.

	O	E	$(O-E)^2$	$(O-E)^2 / E = X^2$
Homozygous WT	12	0	144	NA
Heterozygous	46	58	144	2.48
Homozygous mutant	0	0	0	0.00

Table 2. Observed and expected genotype distribution of all used (living and possessing trichomes) F1 plants at the *fad7-1* locus. 58 total plants possessed trichomes and were used for collecting seed. Additionally, X^2 calculations and values are shown. For X^2 test, df = 2; critical value = 5.991; P = 0.05.
*Highlighted sections indicate plants that were used for planting the subsequent generation.

	O	E	(O-E)	$(O-E)^2 / E = X^2$
Homozygous WT	40	23	289	12.6
Heterozygous	35	47	144	3.06
Homozygous mutant	18	23	36	1.50

Table 3. Observed and expected genotype distribution of all used (living and possessing trichomes) F2 plants at the *fad7-1 l*ocus. 93 total plants possessed trichomes and were used for collecting seed. Additionally, X^2 calculations and values are shown. For X^2 test, df = 2; critical value = 5.991; P = 0.05.
*Highlighted sections indicate plants that were used for planting the subsequent generation.

fad7-1 gl/1	49
npr1-1	23
Hybrids	0

Table 5. Number of parental plants representing each respective genotype. 72 total plants possessed trichomes in the parental (P) generation and were used for collecting seed.

	O	E	$(O-E)^2$	$(O-E)^2 / E = X^2$
Homozygous WT	12	0	144	NA
Heterozygous	46	58	144	2.48
Homozygous mutant	0	0	0	0.00

Table 6. Observed and expected genotype distribution of all used (living and possessing trichomes) F1 plants at the *npr1-1* locus. 93 total plants possessed trichomes and were used for collecting seed. Additionally, X^2 calculations and values are shown. For X^2 test, df = 2; critical value = 5.991; P = 0.05.
*Highlighted sections indicate plants that were used for planting the subsequent generation.

	O	E	$(O-E)^2$	$(O-E)^2 / E = X^2$
Homozygous WT	49	23	625	26.04
Heterozygous	44	48	16	0.33
Homozygous mutant	0	24	576	24.0

Table 7. Observed and expected genotype distribution of all used (living and possessing trichomes) F2 plants at the *npr1-1* locus. 93 total plants possessed trichomes and were used for collecting seed. Additionally, X^2 calculations and values are shown. For X^2 test, df= 2; critical value = 5.991; P = 0.05.
*Highlighted sections indicate plants that were used for planting the subsequent generation.

	O	E	$(O-E)^2$	$(O-E)^2 / E = X^2$
Homozygous WT	23	14	81	5.79
Heterozygous	24	28	16	0.57
Homozygous mutant	14	14	4	0.25

Table 8. Observed and expected genotype distribution of all used (living and possessing trichomes) F3 plants at the *npr1-1* locus. 57 total plants possessed trichomes and were used for collecting seed. Additionally, X^2 calculations and values are shown. For X^2 test, df= 2; critical value = 5.991; P = 0.05

fad7-1 gl/1	48
npr1-1	22
Hybrids	0

Table 9. Number of parental plants representing each respective genotype. 72 total plants possessed trichomes in the parental (P) generation and were used for collecting seed.

	O	E	$(O-E)^2$	$(O-E)^2 / E = X^2$
Trichomes present	58	70	400	5.13
Trichomes absent	12	0	144	NA

Table 10. Trichome characteristics of all living F1 plants. Additionally, X^2 calculations and values are shown. For X^2 test, df = 1; critical value = 3.841; P = 0.05
*Highlighted sections indicate plants that were used for planting the subsequent generation.

	O	E	$(O-E)^2$	$(O-E)^2 / E = X^2$
Trichomes present	93	92	1	0.01
Trichomes absent	29	23	36	1.57

Table 11. Trichome characteristics of all living F2 plants. 93 plants were used for collecting seed because they possessed trichomes. However, a total of 122 plants were planted and lived. Additionally, X^2 calculations and values are shown. For X^2 test, df =;1 critical value = 3.841; P = 0.05
*Highlighted sections indicate plants that were used for planting the subsequent generation.

F3: 57 total plants	O	E	$(O-E)^2$	$(O-E)^2 / E = X^2$
Trichomes present	57	48	81	1.68
Trichomes absent	7	16	81	5.06

Table 12. Trichome characteristics of all living F3 plants. 57 plants were used for collecting seed because they possessed trichomes. However, a total of 64 plants were planted and lived. Additionally, X^2 calculations and values are shown. For X^2 test, df= 1; critical value = 3.841; P = 0.05
*Highlighted sections indicate plants that were used for planting the subsequent generation.

Punnett Squares

	fad7-1/gl1	*fad7-1/gl1*
FAD7	*fad7-1/gl1* FAD7	*fad7-1/gl1* FAD7
FAD7	*fad7-1/gl/1* FAD7	*fad7-1/gl1* FAD7

Punnett Square 1. The Mendelian genetics of the F1 generation at *fad7-1* locus.

	fad7-1/gl1	FAD7
fad7-1	*fad7-1/gl1* / *fad7-1/gl1*	FAD7 / *fad7-1/ gl1*
FAD7	*fad7-1gl1* / FAD7	FAD7 / FAD7

Punnett Square 2. The Mendelian genetics of the F2 generation at *fad7-1* locus.
*Highlighted box represents the desired genotype, homozygous mutant.

	NPR1-1	NPR1-1
npr1-1	NPR1-1 / *npr1-1*	NPR1-1 / *npr1-1*
npr1-1	NPR1-1 / *npr1-1*	NPR1-1 / *npr1-1*

Punnett Square 3. The Mendelian genetics of the F1 generation at *npr1-1* locus.

	NPR1-1	*npr1-1*
NPR1-1	NPR1-1 / NPR1-1	NPR1-1 / *npr1-1*
npr1-1	NPR1-1 / *npr1-1*	*npr1-1* / *npr1-1*

Punnett Square 4. The Mendelian genetics of the F2 generation at *npr1-1* locus.

.

	NPR1-1	*npr1-1*
NPR1-1	NPR1-1 / NPR1-1	*npr1-1* / NPR1-1
npr1-1	*npr1-1* / NPR1-1	*npr1-1* / *npr1-1*

Punnett Square 5. The Mendelian genetics of the F3 generation at *npr1-1* locus.
* Highlighted box represents the desired genotype, homozygous mutant.

	gl1	*gl1*
Gl1	*gl1* / Gl1	*gl1* / Gl1
Gl1	*gl1* / Gl1	*gl1* / Gl1

Punnett Square 6. The Mendelian genetics of the F1 generation at *gl1* locus.
Only 1 copy of mutant *gl1* is necessary for the presence of trichomes.
*Highlighted box represents the *gl1* genotype used for planting the next generation of plants.

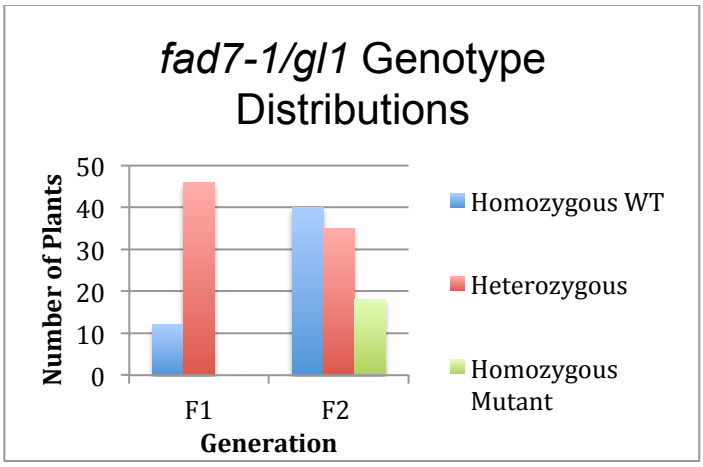
	gl1	GL1
gl1	*gl1* / *gl1*	*gl1* / Gl1
Gl1	*gl1* / Gl1	Gl1 / Gl1

Punnett Square 7. The Mendelian genetics of the F2 generation at *gl1* locus.
*Highlighted box represents the *gl1* genotype used for planting the next generation of plants.

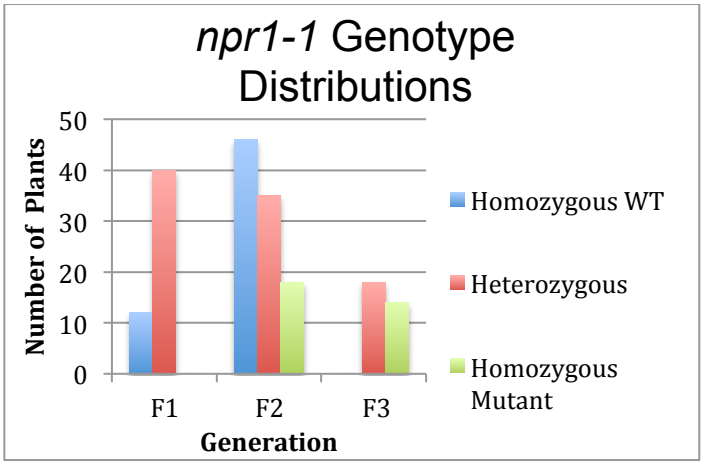
	gl1	*gl1*
gl1	*gl1* / *gl1*	*gl1* / Gl1
Gl1	*gl1* / Gl1	*gl1* / Gl1

Punnett Square 8. The Mendelian genetics of the F3 generation at *gl1* locus.

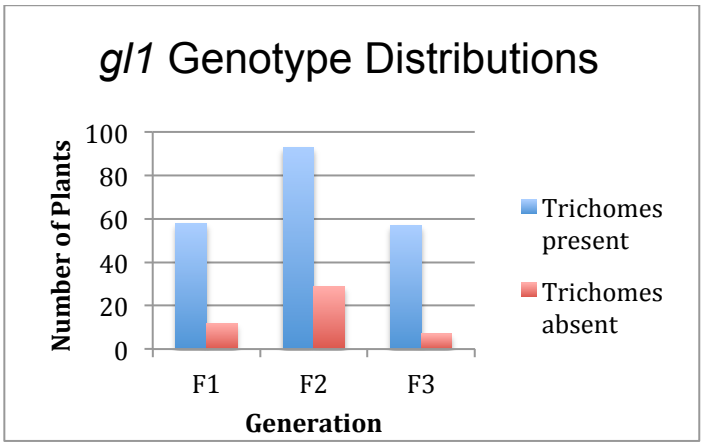
Graphs



Graph 1. Distribution of genotypes at the *fad7-1* locus in F1 and F2.



Graph 2. Distribution of genotypes at the *npr1-1* locus in F1, F2, and F3.



Graph 3. Distribution of genotypes at the *gl1* locus in F1, F2, and F3.

Literature Cited:

Avila C, Arévalo-Soliz L, Jia L, Navarre D, Chen Z, Howe G, Meng Q, Smith J, Goggin F (2012) Loss of Function of FATTY ACID DESATURASE7 in Tomato Enhances Basal Aphid Resistance in a Salicylate-Dependent Manner. Plant Physiol 158:2028-2041.

Browse J, McCourt P, Somerville C (1986) A mutant of Arabidopsis deficient in c(18:3) and c(16:3) leaf lipids. Plant Physiol 81: 859-864.

Edwards K., Johnstone C., Thompson C. (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucl. Acid. Res.* 19: 1349

Goggin F, Avila C (2014) Unpublished data.

Heidel AJ, Baldwin IT (2004) Microarray analysis of salicylic acid- and jasmonic acid-signaling in responses of Nicotiana attenuata to attack by insects from multiple feeding guilds. Plant Cell Environ 27: 1362–137.

Howe GA, Jander G (2008) Plant immunity to insect herbivores. Annu Rev Plant Biol 59: 41-66.

Jasmonate-Mediated and Salicylate-Mediated Signals in Plant Defense. The Plant Cell 16 no 2: 319-331.

Kasajima I., Ide Y., Ohkama-Ohtsu N., Yoneyama T., Fujiwara T. (2004) A protocol for rapid DNA extraction from Arabidopsis thaliana for PCR analysis. *Plant Mol. Biol. Rep.* 22, 49-52

Kunkel BN, Brooks DM (2002) Cross talk between signaling pathways in pathogen defense. Current Opinion in Plant Biol 5: 325-331.

Li J, Brader G, Palva E (2004) TheWRKY70 Transcription Factor: A Node of Convergence for Jasmonate-Mediated and Salicylate-Mediated Signals in Plant Defense. The Plant Cell 16 no. 2: 319-331.

Lorence A (2008) Germinating *Arabidopsis thaliana* seeds on MS sterile media.

Nishiuchi T, Hamada T, Kodama H, Iba K (1997) Wounding changes the spatial expression pattern of the Arabidopsis plastid ω-3 fatty acid desaturase gene (FAD7) through different signal transduction pathways. Plant Cell 9: 1701–1712.

Martín M, León J, Dammann C, Albar JP, Griffiths G, Sánchez-Serrano JJ (1999) Antisense-mediated depletion of potato leaf v3 fatty acid desaturase lowers linolenic acid content and reduces gene activation in response to wounding. Eur J Biochem 262: 283–290.

McConn M, Creelman RA, Bell E, Mullet JE, Browse J (1997) Jasmonate is essential for insect defense in Arabidopsis. Proc Natl Acad Sci USA 94: 5473–5477.

Pajerwoska-Mukhtar KM, Emerine DK, Mukhtar S (2013) Tell me more: roles of NPRs in plant immunity. Trends in Plant Science. Vol.18 No. 7: 1360-1385.

Song J, Lee DE, Jung S, Kim H, Han O, Cho BH, Lee IJ, Back K (2004) Characterization of transgenic rice plants expressing an Arabidopsis FAD7. Biol Plant 48: 361–366.

Vlot AC, Dempsey DA, Klessig DF (2009) Salicylic acid, a multifaceted hormone to combat disease. Annu Rev Phytopathol 47:177-206.

Upchurch RG (2008) Fatty acid unsaturation, mobilization, and regulation in the response of

plants to stress. Biotechnol Lett 30: 967–977.

Van der Does D, Leon-Rayes A, Koornneef A, Van Verk MC, Rodenburg N, Pauwels L, Goossens A, Korbes AP, Memelink J, Ritsema T, Van Wees SCM, Pieterse CMJ (2013) Salicylic Acid Suppresses Jasmonic Acid Signaling Downstream of SCFCOI1-JAZ by Targeting GCC Promoter Motifs via Transcription Factor ORA59. Plant Cell 25: 744-761.

Walling LL (2008) Avoiding effective defenses: strategies employed by phloem-feeding insects. Plant Physiol 146: 859-856.

Zarate SI, Kempema LA, Walling LL (2007) Silverleaf whitefly induces salicylic acid defenses and suppresses effectual jasmonic acid defenses. Plant Physiol 143: 866–875.

Zhang YL, Fan WH, Kinkema M, Li X, Dong XN (1999) Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. Proc Natl Acad Sci USA 96: 6523-6528.