Differences in Defense Response to Herbivory across Arabidopsis Mutants

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Introduction

Arabidopsis thaliana is a model plant, commonly used in research. Despite the extensive use of this plant, the exact genetic pathways of its defense responses are not clear. According to recent studies, the Jasmonic Acid defense pathway is the most commonly activated pathway against insect attack. Defensive compounds are not constantly synthesized because the plant has to maintain a balance between growth and defense (Rehrig et. al., 2014). If the balance shifts too far in one direction, the plant may not be successful. However, a slightly shift towards defense may aide plants in the fight against herbivores. If a specific mutation can be isolated that strengthens the defense response of a plant, it could be introduced to crops so that farmers may reduce pesticide usage.

Once a defense response is triggered by insect feeding, downstream genes activate a positive feedback loop, spurring the defense response on. In order to moderate the immune response, suppressor genes combat the defense signal. To determine the balance of these competing forces, two genes were compared in a gene expression assay: LOX2 and JAZ1(Chung et. al., 2008). (see figure 1.)

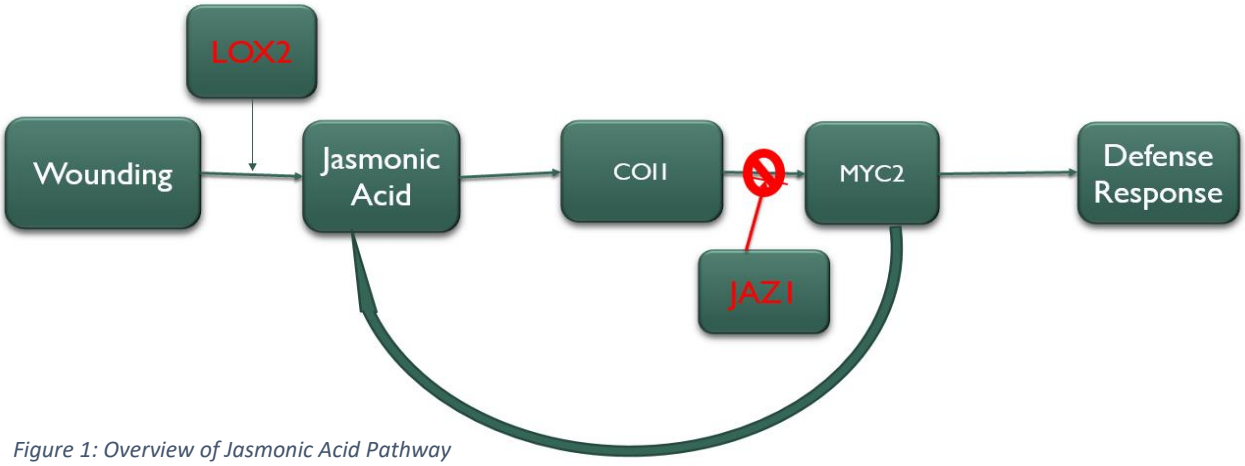

Figure 1: Overview of Jasmonic Acid Pathway

Figure 2: Beet Armyworm, 1st instars

Several *Arabidopsis* mutants already exist that have mutations along the pathway above. This experiment tests which mutations are the most effective against insect attack, using the model insect *Spodoptera exigua* (beet armyworm). Eight *Arabidopsis* genotypes were chosen. Colombia is the wild-type of this plant and served as the control. RLD is another wild type that has a defect which makes it slightly more susceptible to attack (Nguyen et. al., 2015). The remaining six genotypes were chosen because their mutations were all along the Jasmonic pathway above, yet were in distinct enough places that the results would help elucidate the pathway. The most important mutant was named SRFR 1-1, because it had a mutation in the suppressor regulator of RLD wild type, meaning its defense response should be less attenuated than RLD. The remaining mutants used were EDS 1-2 (enchanced disease susceptibility), EDM 1-1 (enhanced mold resistance), TCP Triple (three mutations in the TCP protein along the Jasmonic acid pathway), and two double mutants, EDS 1-2 SRFR 1-4 and EDM 1-1 SRFR 1-4. SRFR 1-1 and SRFR 1-4 have mutations in the same suppressor regulator, but at different places so the resulting protein has slightly different functionality (Nguyen et. al., 2015; Rehrig et. al., 2014).

Methods

All of the *Arabidopsis* plants were germinated on ½ MS petri plates for 10 days, then transferred to soil. The plants grew for approximately 3 weeks before experimentation began.

Figure 3: Experiments one and three running concurrently

Experiments one and three were carried out concurrently with experiment two immediately following. The first day of experiments one and three was October 4th, and experiment two concluded on October 12th.

Experiment 1: Plant Performance and insect growth

Figure 4: Insect Growth Experiment

Experiment one is the most straightforward test of the plant mutations. 1st instar *Spodoptera exigua* were placed on 4 week old *Arabidopsis thaliana* of each genotype. Two insects were placed on each plant, with two plants for each genotype. The plants were sealed with aerated cups to keep the insects inside. The caterpillars were left on the plants for 1 week (until one of the plants was entirely eaten). Condensation from the cup kept the soil moist, so the plants were only watered once (Nguyen et. al., 2015). To water the plants, water was placed inside the tray and was absorbed through the bottom of the cup for 1 hour. Once the week had elapsed, the insects were removed from the plants and weighed together. The plants were photographed.

Experiment 2: Cannibalism

Experiment two focused on the plants' effect on the insects. Two types were conducted. The first assay, infected, took the insects from the experiment above and placed them in a petri dish immediately after weighing. The insects were taken out and weighed together after 3 days.

Figure 5: Example of Petri Plate in Infected Cannibalism Assay

The second cannibalism experiment was with "fresh" insects. Caterpillars were taken directly from the food substrate and placed in large plastic cups, with a Parafilm seal on the top. Four insects were placed in each cup, with four leaves from control plants for each genotype (Castells, 2017). Since these caterpillars had been kept in the 37 °C incubator, they had advanced to the 4th and 5th instar stages, while the "infected" caterpillars were 2nd and 3rd stage instars.

Figure 6: Example of cup for Fresh Cannibalism Assay

Experiment 3: Gene expression

The objective of experiment three was to compare the relative gene expression across the genotypes. Foam cages were placed on single leaves from each plant, four cages to each plant. Each cage held one 1st instar caterpillar. Each genotype had at least one plant for control, and one experimental plant. The insects were left on the plants for 12 hours (until significant damage was seen in the leaf). After the cages and insects were removed, the damaged leaf samples were collected over time (Nguyen et. al., 2015).

Figure 7: Cages for Gene Expression Assay

In order to find the 'peak' defense response, genotypes with extra plants provided different time points. The total number of samples collected are shown in Table 1. Not all samples made it to final qPCR values due to intermediate process issues. Additionally, due to supply issues, the final genotypes were not completed prior to the deadline. However, the work will continue next semester, with nine more primers, and the final genotype will be completed at that time.

Table 1: Number of samples collected

Number of 100 mg samples taken for each genotype					
	Control	6 HAI	8 HAI	10 HAI	24 HAI
Col	1	0	0	3	0
RLD	4	0	0	5	0
srfr1-1	2	0	0	4	3
eds1-2	2	0	0	3	0
edm1-1	2	0	0	2	1
tcptrip	2	0	0	4	0
eds1-2,srfr1-4	2	2	0	3	2
edm1-1,srfr1-4	2	2	3	3	1

To collect samples, the damaged leaves were removed and weighed out to 100 mg of leaf material as quickly as possible. The leaves were immediately placed in a pre-labelled 15 mL conical tube. Liquid nitrogen was added to the tube to freeze the sample. After the liquid nitrogen boiled away, the tube was sealed and placed in a -20 °C freezer until all samples were collected for that time period. Then, the samples were placed in boxes according to their time of collection and placed in a -80°C freezer (Nguyen et. al., 2015). The first step for gene expression is RNA extraction. Initially, a Viogene kit was used for approximately 30 practice samples. The results were highly varied, sadly resulting in the loss of many samples. An Invitrogen kit was attempted for the next 20 samples and the results worked well, with only three exceptions. A third RNA extraction kit, Abbott plant extraction, finished the rest of the samples with a perfect record. The concentration and quality of each sample was checked via Nanodrop. All samples with a 260/280 and 260/230 measurement over 1.8 were used. All samples that fit within these parameters had an acceptable concentration.

Once a genotype was completed, cDNA was performed. The first half of the samples used an NEB kit, and the second half used an Invitrogen kit. Both gave a final volume of 20 uL of cDNA. The kits were switched to allow samples with lower concentration to be used. The

concentrations of the RNA were balanced to 1000 ng for the cDNA, to make the concentration equal across the samples.

cDNA from each genotype was then used for qPCR to find the relative quantification of each sample. The same amount of cDNA was added to each well across all samples. The first kit used was NEB luna, and when the cDNA switched to an Invitrogen kit, the qPCR switched as well. However, the Invitrogen kit had different temperatures during the elongation cycle, and thus was not effective for the JAZ1 primer. The qPCR kit was moved back to NEB luna. Three primers were used in qPCR: SAN, LOX2, and JAZ1. SAN is the housekeeping gene for *Arabidopsis thaliana*. The purpose of LOX2 and JAZ1 is noted above. The Ct (cycle time) values of each sample type (experimental and control) were averaged. Each sample had three technical replicates, and up to three biological replicates (Biolabs). The relative folds of gene expression were calculated using the delta delta method, as shown in the table below (Nathanial Torres, personal communication, 2017).

Tcp trip 10 hai vs control	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value Experimental (Primer-Control)	ΔCt Value Control (Primer- Control)	Delta Delta Ct Value (Exp. - Cont)	Expression Fold Change
	Target Gene: Experimental Well	Housekeeping Gene: Experimental Well	Target Gene: Control Well	Housekeeping Gene: Control Well	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene San	-	21.86	-	21.71	-7.29	-5.73	-1.56	2.948538435
Lox2	14.57	-	15.98	-				
JAZ	37.85		35.60		15.99	13.90	2.10	0.234068062

Table 2: qPCR analysis method

Results

Experiment one results were two-fold: insect weight and qualitative plant performance. The caterpillars were weighed collectively after removal from the plants. The insects were weighed together, and divided by the number of insects. Thus, standard deviation and other such statistical tests could not be performed. The average weight per insect is shown below (the beginning weight is negligible).

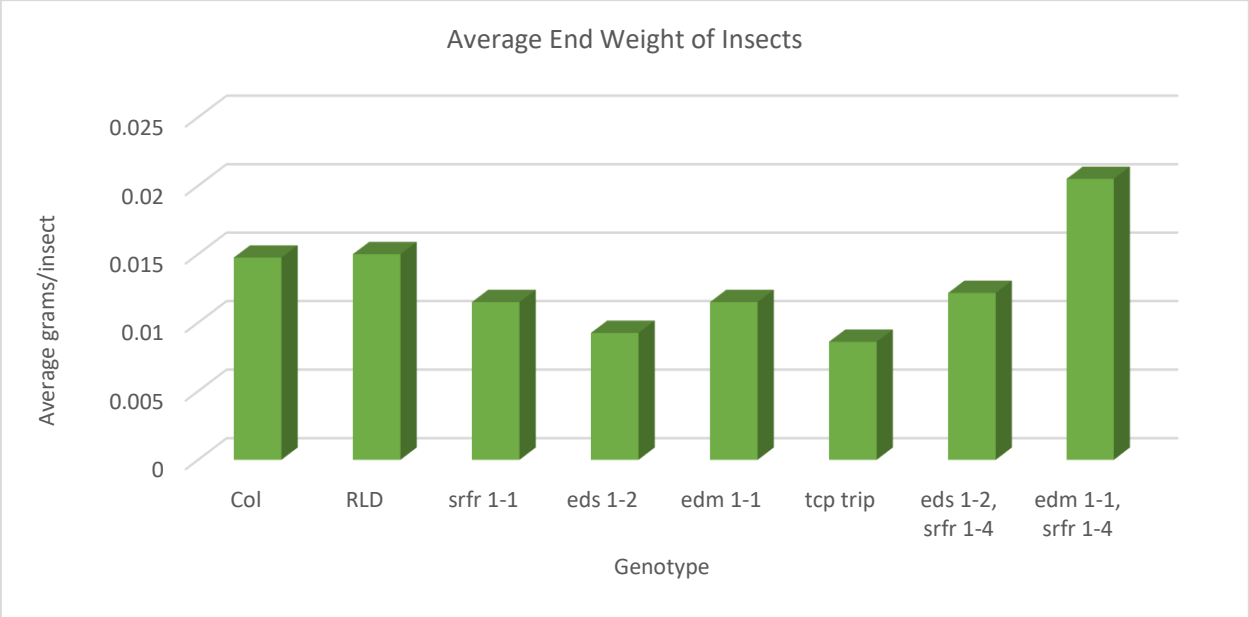

Table 3: Mean end weight per insect

More resistant genotypes should have smaller insects by the end of the experiment. EDS 1-2 and TCP Triple had the lowest average weight at .009 grams per insect. EDM 1-1, SRFR 1-4 was the failure of a genotype, as it was more susceptible to feeding than wild type. The rest of the genotypes had lower average weight than the wild type, which suggests an increase in resistance.

Plant performance comparison was qualitative in nature. The plants had varying amounts of tissue left after 1 week of insect feeding. The pictures and comments below serve as notes to aide visual comparison of the plant performance. Overall, TCP Triple and EDS 1-2 appeared to withstand the insect attack best while EDM 1-1, SRFR 1-4 did the worst. SRFR 1-1 was not the best performer, which is disappointing because its mutation held the greatest promise.

Figure 2: Edm 1-1. All leaves are eaten away, and plant will die as a result of feeding.

Figure 3: Colombia. Significant damage to the leaves, and some completely eaten away. Plant may not produce viable seeds.

Figure 4: RLD. Significant damage, though the impact will be less severe due to the large size of the leaves.

Figure 5: Srfr 1-1. Several leaves have significant damage, but the plant will be able to recover.

Figure 6: Edm 1-1. The damage to this plant appears severe due to the overall small size of the leaves. The plants may not recover due to the proportion of material lost.

Figure 7: Eds 1-2, srfr 1-4. The plant leaves are all injured, however none of them are eaten away. The plant is likely to recover from the feeding.

Figure 8: Tcp triple. The leaves have relatively minor injuried, and some leaves remain untouched. Despite the small leaf size, the damage is mild to moderate.

Figure 9: Eds 1-2. Fewer than half of the leaves are damaged, and the plant's growth is not likely to be hindered.

Experiment two's results differ significantly across the two assays. The Infected assay exhibited high rates of cannibalism after only 48 hours of starvation (the day the insects were removed from the plants and placed in dishes was Day 1 of this experiment).

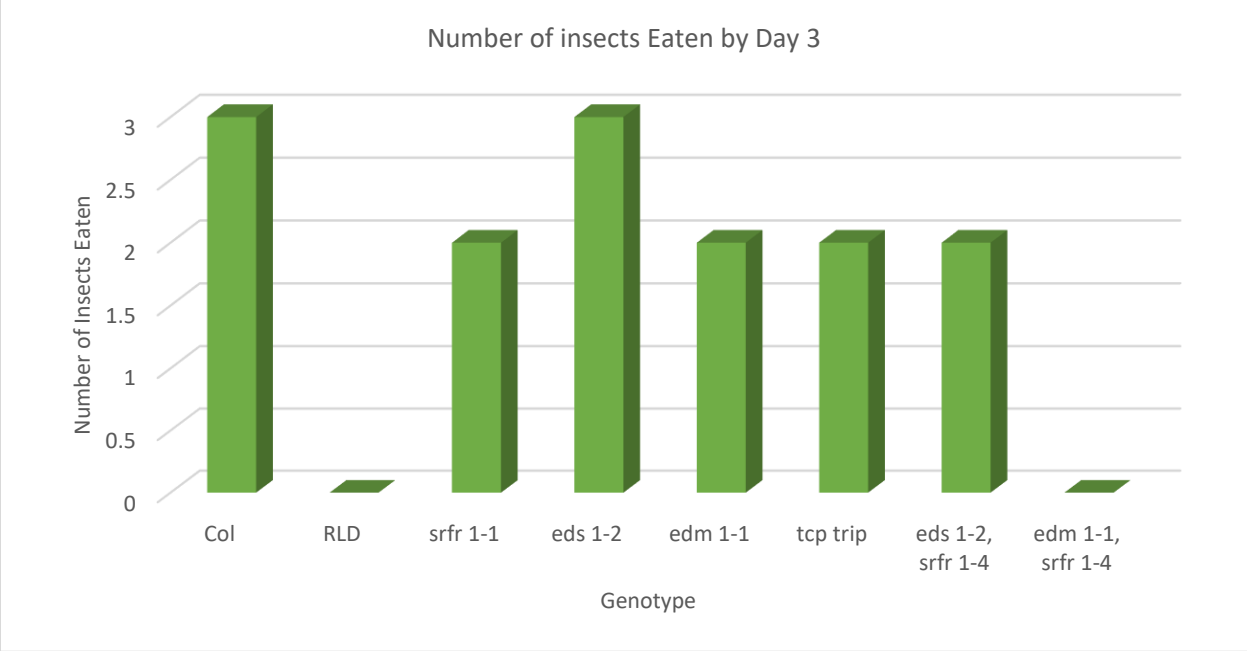

Table 4: Infect Insect Cannibalism

In addition to tracking the number of insects eaten, the insects were weighed on Day 3. The only two genotypes to evidence insect growth were Colombia and EDS 1-2, which also were the only groups to have a single caterpillar remaining in the petri dish.

Infected Insect Cannibalism Assay Statistics

Genotype	Average mass of insects on Day 1 (g/insect)	Average mass of insects on Day 3 (g/insect)	Change in average mass of insects (g/insect)	Cannibalism by Day 3
Col	0.015	0.025	0.010	3
RLD	0.015	0.010	-0.005	0
srfr 1-1	0.012	0.008	-0.004	2
eds 1-2	0.009	0.016	0.007	3
edm 1-1	0.012	0.010	-0.002	2
tcp trip	0.009	0.006	-0.003	2
eds 1-2, srfr 1-4	0.012	0.006	-0.006	2
edm 1-1, srfr 1-4	0.021	0.008	-0.012	0

Table 5

The Fresh insect assay was characterized by low rates of cannibalism. The control group was the only cup to have more than one insect eaten. These insects were not weighed for this experiment.

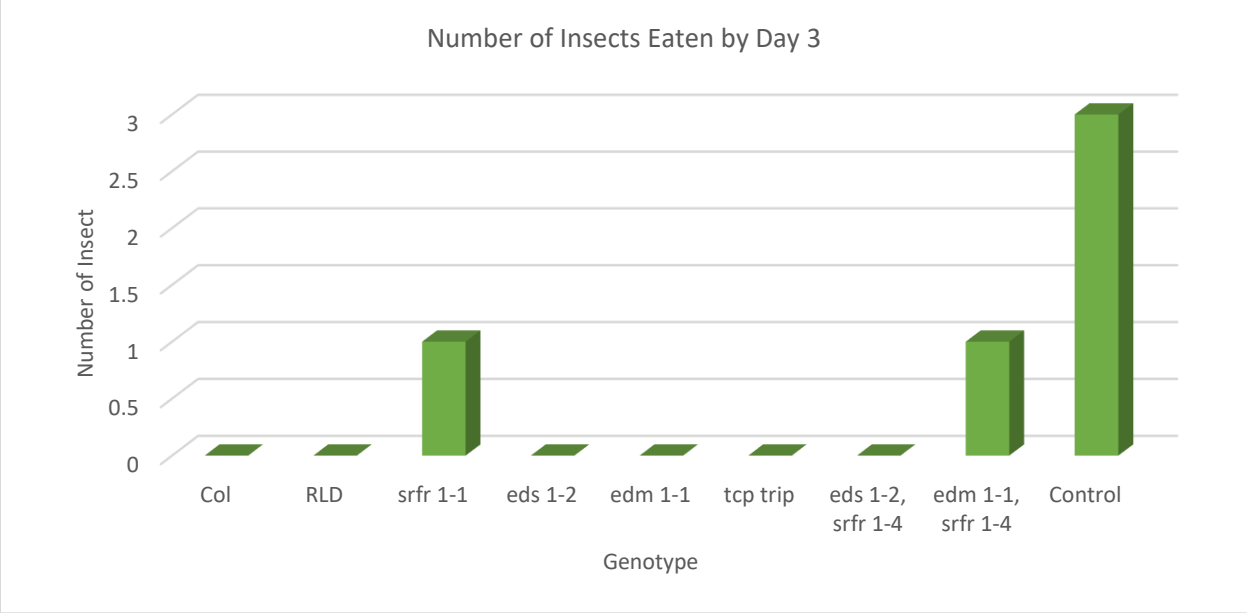

Table 6: Fresh Cannibalism Assay

The third and final experiment has a few missing pieces in the data. Due to issues with primers and the thermocycler, not every genotype was able to be analyzed. However, the data I do have is represented graphically below.

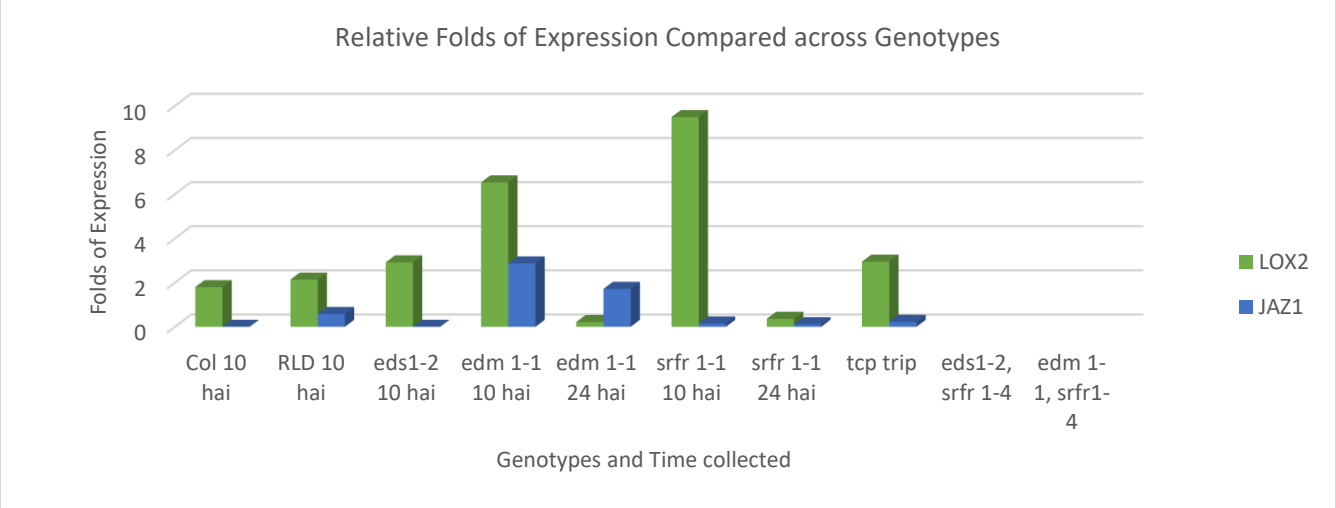

Table 7: HAI= hours after insect

JAZ1 for Colombia 10 HAI had a 102 fold increase, the largest increase in gene expression by far. This data for Colombia was omitted from the graph because it would render the rest of the data generally unreadable. With the exception of samples that were taken at 24 hours after insect removal (HAI), all the mutants had higher LOX2 expression than the wild types.

The table below summarizes the data from this experiment.

Genotype	Insect growth	Cannibalism: Infected		Cannibalism Fresh	Gene expression		
	Mean final weight per insect (g)	Number eaten by Day 3	Mean Insect mass Δ (g)	Number eaten by Day 3	Sample collection time (hours after insect)	Relative Folds of Expression for LOX2	Relative Folds of Expression for JAZ1
Col	0.015	3	0.010	0	10 hai	1.80	-
RLD	0.015	0	-0.005	0	10 hai	2.14	0.58
srfr 1-1	0.012	2	-0.004	1	10 hai	9.5	0.15
					24 hai	0.37	0.12
eds 1-2	0.009	3	0.006	0	10 hai	2.92	-
edm 1-1	0.012	2	-0.002	0	10 hai	6.54	2.87
					24 hai	0.22	1.72
tcp trip	0.009	2	-0.003	0	10 hai	2.95	0.23
eds 1-2, srfr 1-4	0.012	2	-0.006	0			
edm 1-1 srfr 1-4	0.021	0	-0.012	1			

Table 8: Summary table

Discussion

These preliminary results are oddly conflicting for most of the mutations. TCP Triple and EDS 1-2 performed the best in the first experiment, however their gene expression analysis failed to match that, and their cannibalism performance was not notable. EDM 1-1, SFRR 1-4 had an exceptionally poor showing in experiment one and three, yet in the fresh cannibalism assay, it was one of only two mutants to show cannibalism. SRFR 1-1 is the only genotype that performed moderately well at all three experiments. EDM 1-1 comes in second with a decent showing in experiments one and three. The overall inconsistency makes it difficult to draw conclusions about which mutations show the most promise. Additionally, due to size constraints,

the number of biological replicates was low. Therefore, it is difficult to tell whether the plants or the experimental setup is to blame for the discrepancy between experiments.

In a previous study by Dr. Nguyen in 2015, experiment one was completed using only SRFR 1-1 and RLD. Similar results were achieved in that SRFR 1-1 was more resistant to plant feeding than RLD in the same approximate ratio as this study. However, that study also included a choice assay where RLD and SRFR 1-1 were grown in the same pot, and the larva could choose a plant. RLD was chosen above Additionally, this same study performed a gene analysis study with genes that included JAZ1 and LOX2. The results were similar to this study, with LOX2 being upregulated, and JAZ1 being downregulated. However, one difference was that the results for SRFR 1-1 at 24 HAI was still elevated relative to RLD (Nguyen et. al., 2015). SRFR 1-1 24 HAI data decreased at a greater rate in our study, though they note that at time periods beyond 24 hours, the defense response is over. This difference may be due to the length of time the insects were on the leaves. In our study, 24 HAI is 34 hours after the insects were first placed on the leaves. However, Dr. Nguyen's study does not state the amount of time the cages were on the leaves. If it was a shorter time period, their 24 HAI samples may have been taken sooner than the samples in this study.

There was a fatal flaw in the fresh cannibalism experiment. As stated above, these insects were 4^{th} or 5^{th} instar. Beet armyworms pupate after the 5^{th} instar stage. Shortly before pupation, the insects stop eating, and obviously do not eat as pupae. Since many of the insects became disinterested in feeding, the experiment yielded poor rates of cannibalism. The worms for the control group were the left-overs after choosing equally sized worms for the experiments cups. As a result, three of them were smaller, and one was comparatively larger. This is likely due to them being a stage behind the larger worms who were still actively eating. However, while this mistake lessens the credibility of these results, it is interesting to note that SRFR 1-1 and EDM1-1, SRFR 1-4 did show cannibalism, despite the developmental stage. Additionally, the insects that cannibalized did not pupate in any of the three cups. Oddly, for the infected insect assay, the cannibalistic caterpillars died around the same time as the insects who had starved. This is interesting because it shows that cannibalism has biological disadvantages for these insects, increasing the chance that the drive to cannibalize each other is externally motivated (say, from the plant it eats), not a biological last resort. While conducting the cannibalism experiments, I

witnessed live cannibalism from both assays, showing that the insects do not wait to eat each other until death. The cannibal was always the largest larva in a cup. This effect is likely another contributor to the high amount of cannibalism in the control cup. Due to the drastic size difference of one larva, cannibalism might have been more easily achieved.

In a 2017 study published in Nature, Dr. Castells proved that a plant defense response increased cannibalism. However, their test pitted a normal plant against a plant that had been previously triggered to generate an immune response. Additionally, dead caterpillars were placed in the feeding area and the difference between the two groups was the timing of the cannibalism, not whether it occurred. In our study, the independent variable was genotype and the cannibalism was of live insects, not dead ones (Castelles, 2017). In my view, our study is more practical because it shows that we could theoretically o splice in a gene that gives an increased immune response, rather than assuming one is already taking place in crops. Also, cannibalism of live insects decreases the number of active feeders in a population. Instead of waiting for the insects to die, they might start eating each other. Also, our study tentatively shows that cannibalism is harmful to the insects' development, preventing reproduction.

One possible reason for the inconsistency in the study's overall results is that different genes could be responsible for the diverse effects measured by the three experiments. The pathway LOX2 and JAZ1 primers are a part of may not be the key reason for decreased feeding of insects on plants (Chung et. al., 2008). Also, the defense response that triggers cannibalism in insects may be a different pathway altogether. By expanding experiment one with more replicates, the most resistant plant types can be found. Then, a broader range of primers should be used on those plant types in an attempt to find the genes responsible for the increased resistance.

Conclusion

The first experiment is the truest test of plant susceptibility to insect attack, and is the one least prone to error. Consequently, I believe the results of that experiment are the most trustworthy. While replications are needed for all experiments, I believe the first experiment should be the focus of future studies, and the data from it should be used to prioritize the gene expression assay. Additionally, for future gene expression assays, the number of primers should be expanded to confirm that the suppression of JAZ is the main cause for increased resistance in

Arabidopsis mutants. In conclusion, this experiment shows that many *Arabidopsis* mutants do have enhanced resistance to insects relative to the wild types, however the mechanisms and specific mutations are not yet clear.

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