CHANGE IN PROTEASE GENE EXPRESSION IN AN INSECT PEST TO PLANTS IN THE POTATO/TOMATO FAMILY (*SOLANACEAE*)

By

Dana Morshed

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APPROVED

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Dr. Allison K Hansen
Department of Entomology

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Dr. Richard Cardullo, Howard H Hays Jr. Chair, University Honors

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Abstract

Lineage-specific gene expansions may allow eukaryotic insects to adapt and diverge functional traits. My lab's chromosomal assembly of the sap-sucking potato psyllid (Bactericera cockerelli) previously identified three gene expansions associated with protease expression. The current study further analyzed whether or not plant diet and insect life stage might impact protease expression. We fed insects different plant diets of either potato or tomato at two insect life stages (2nd instar and young adult) to test for differences in insect protease gene expression. First, RNA extractions were conducted followed by quantitative PCR (qPCR). The data were analyzed using the delta-delta Ct method and ANOVA test to calculate relative changes in gene expression. Preliminary data revealed a significant difference between the host plant diets potato and tomato for 2nd instar nymphs for two of the three different candidate genes. This project adds to our understanding of the putative function of species-specific protease genes that are evolving rapidly in the insect, and ultimately these genes may be involved in host plant adaptation.

Acknowledgments

I would like to begin by thanking my wonderful faculty mentor Dr. Allison K Hansen for allowing me this opportunity to learn and grow throughout my undergraduate career. Her dedication and expertise have allowed me to develop research skills and shaped me as a woman in science. Her continuous support has allowed me to learn the true meaning of research and inspired me for my future goals.

I would also like to thank my graduate student mentor Younghwan Kwak for her guidance and patience. Her help definitely allowed me to create a solid foundation for my research skills.

I would also like to thank the Mauck Lab for providing me with psyllids to begin my project.

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Introduction

Genetic variation ultimately leads to evolutionary change. Orthologous genes are passed down through vertical gene transfer through ancestors and encode the same function throughout different species. Paralogous genes evolve through events such as gene duplication or lineage-specific gene expansions and code for proteins that may have slightly different functions.

Gene duplication occurs through errors in DNA replication. It allows for eukaryotes to acquire new genetic material through the passing down of parts of or whole regions of DNA. This process increases the plasticity of the genome and allows for exaptation or the shift in the function of a gene (Magadum, 2013). Comparison of duplicated genes gives insight into diversification events and evolutionary history (Sogin, 1991).

Lineage-specific gene expansions are the new appearance of genes in a lineage that is not in a sister lineage (Jordan et al., 2001). Oftentimes these lineage-specific gene expansion events occur in response to pathogens or other stress (Lespinet et al., 2002). Lineage-specific gene expansions allow eukaryotes to adapt and are regarded as a key reason for organizational and regulatory diversity (Lespinet et al., 2002).

In particular, lineage-specific gene expansion impacts protein groups which can be seen through varying patterns in protein functions. This is important because these lineage-specific expansions impact structural proteins, enzymes involved in response to pathogens and environmental stress, and signaling pathways (Lespinet et al., 2002). They can lead to the proliferation of new biological functions in addition to increasing organizational diversity.

One insect that is known to both house and vector microbes is *Bactericera cockerelli*. This insect species belongs to the insect group called psyllids, which are small, plant sap-sucking insects that fly and are often recognized as pests on agricultural plants because of their ability to vector bacterial plant diseases (Trumble et al., 2011). This particular psyllid species feeds on the *Solanaceae* or nightshade plant family which includes potatoes and tomatoes (Pletsch, 1947).

Genome expansions, as well as genome reductions, are significant to note to properly understand the cellular functions of different species. Symbiotic relationships are essential to the function of life (Reece & Campbell, 2011). Humans unknowingly rely on these relationships across many disciplines such as within our own bodies or for the crops we eat. A microbiome is a community of microorganisms that can usually be found living together in any given habitat (Reece & Campbell, 2011). An insect host can house these microbes as symbionts and/or transmit them to other hosts (vector) (Reece & Campbell, 2011). All species of psyllids harbor a bacterial symbiont called *Carsonella ruddii* (referred to throughout as *Carsonella*). *Carsonella* is a symbiont that lives inside the psyllid's body and therefore is defined as an 'endosymbiont'. *Carsonella* exhibits a significant example of genome reduction (Nakabachi et al., 2006).

This ubiquitous psyllid endosymbiont is required for psyllid survival because it synthesizes essential amino acids for its insect host, which are lacking in the psyllid's diet (McCutcheon & Moran, 2011). In return, the psyllid provides its endosymbiont with shelter and nutritional resources. This obligate relationship is an example of a symbiosis in which the two organisms have a close association with each other (Reece & Campbell, 2011). In this case, the

psyllid and *Carsonella* both benefit each other, making their relationship a mutualism. In an obligate mutualism, neither organism can survive without the other.

Carsonella is detected in psyllids at all stages of life (Nachappa et al., 2011). The *Carsonella* cells are found within specialized cells in the psyllid called bacteriocytes (Baumann et al., 2005). Its genome is one of the smallest known genomes and exhibits gene reduction which is rectified by psyllid-encoded proteins due to their endosymbiotic relationship (Nakabachi et al., 2006). Nutrient provisioning is common in sap-sucking insects in which symbionts provide the hosts with necessary nutrients (Moran et al., 2003). This relationship is seen between the psyllid and *Carsonella*.

The plant pathogen *Candidatus Liberibacter psyllaurous* is only found in psyllids and not in other insect species that feed on infected host plants (Bové, 2006). Studies have been recently conducted finding the effects of the plant pathogen on plants and how it is vectored by psyllids which is important for pest management of our agricultural resources to ensure food security. These studies have confirmed *L. psyllaurous* as the cause of 'psyllid yellows' which is a disease that the psyllid vectors to its host plant (Hansen et al., 2008).

Past investigations of the psyllid such as by researching its endosymbionts like *Carsonella* which is found in the gut have given a better understanding of the psyllid's relationships with plants (Arp et al., 2014). Carboxypeptidases can also be found in the gut of the psyllid. Identifying these genes and deducing their functions can further our understanding of the psyllid's relationships with plants. Understanding this relationship is vital because of how psyllids impact agriculture since they are regarded as pests. This also highlights the importance

of the study of varying host plants because of slight differences in gene abundance that may occur based on the host plant. Variances can also possibly be impacted by the presence of the plant pathogen on these host plants.

Life stages and sex are also important factors that are considered when studying the psyllid as well. For example, in a study of the endosymbionts of *Bactericera cockerelli*, the presence of two endosymbionts were first confirmed in all life stages and sexes through the use of PCR analyses (Nachappa et al., 2011). This is because gene abundance can vary throughout different life stages and between sexes based on their physiological functions. Studies have also found different abundances of endosymbionts such as *Carsonella* between sexes (Cooper et al., 2015) which can possibly lead to variances because of physiological impact as well.

The first chromosomal assembly of the psyllid *Bactericera cockerelli* found several gene expansion events some of which are expected to be for genes associated with transcription factors, proteases, and odorant receptors (Kwak et al., 2022). This study found *B. cockerelli* to be the largest sequenced psyllid genome compared to other psyllid species. It also found *B. cockerelli* to have significantly more gene expansion events compared to other Hemipteran species. It noted that 75% of these gene expansions are transposable elements found throughout all of *B. cockerelli's* chromosome which may contribute to the large size of the chromosome (Kwak et al., 2022).

One of the gene expansions of protease families was predicted to be related to carboxypeptidase D because of NCBI BlastP hits from other insects (Kwak et al., 2022). Proteases are enzymes that break peptide bonds. Carboxypeptidases are proteases that remove

the C-terminal amino acid from proteins. These carboxypeptidases play a vital role in a variety of physiological functions in organisms. This expansion of protease gene families in *B. cockerelli* is important because of its impact on species-specific responses to host plant defenses. Both tomato and potato plants, psyllid host plants, produce carboxypeptidase inhibitor proteins in response to insect feeding (Díez-Díaz et al., 2004; Graham & Ryan, 1981). Since these inhibitors can cause antifeedant effects for insects (Zhu-Salzman & Zeng, 2015), *B. cockerelli* may compete with solanaceous host plant defenses and its rapidly evolving carboxypeptidase families (Kwak et al., 2022).

My study analyzes whether there are insect life stage and host plant rearing differences in insect gene expression for three rapidly expanding genes. I predict that these proteases are involved in host-plant interactions and are important in responding to host-plant feeding. Certain genes are expressed in different abundances throughout different life stages based on their function. Since the function of the genes I am studying are unknown, it is important to note whether there is a difference in gene expression. Furthermore, although *Bactericera cockerelli* feeds on both potatoes and tomatoes, it may respond differently to each host. I hypothesize that there will be a significant difference between expression between life stages for all genes on both host plants. To test my hypothesis, I will detect gene expression through quantitative polymerase chain reaction (qPCR).

Material and Methods

This approach involved first growing colonies of psyllids from the same genetic line on plant diets of potato and tomato plants. The psyllids were obtained from the Mauck lab at the University of California (UC), Riverside. The two established lines were maintained on 12-week-old *Solanum tuberosum* (Russet potato) and *Solanum lycopersicum* (Moneymaker tomato) plants at 25 °C under a 16L:8D light/dark cycle in an incubator. The materials needed included mesh cages, tomato and potato seeds, soil, pots, and planting materials to grow and maintain my colonies.

Plant Rearing

Plants were first grown by planting tomato seeds that were stored in a cool, dry, and dark refrigerator on seedling starter trays using soil purchased from a nursery. Potato tubers were planted in small pots with this same nursery soil. Potatoes were planted by slicing larger potatoes that were stored at 4° into sections. These sections were planted individually while making sure that each section has buds. The seedling starter trays and pots were kept on plastic trays which were kept in insect-rearing cages.

These plants were kept at 25 °C under a 16L:8D light/dark cycle under LED grow lights and daylight fluorescent lights in a separate insect-rearing room. Planting occurred biweekly. When first planted in the soil, the plants were top watered. After this first watering, when the topsoil was dry, the plants were bottom watered around twice a week. They were checked on every day to monitor moisture levels of the soil and ensure against overwatering.

After four weeks of age, the tomato plants were transferred to small pots with UCR soil mix which is composed of sterilized UCR soil, perlite, vermiculite, and Osmocote fertilizer. After around 6 to 8 weeks, both tomato and potato plants were transferred to large pots with the UCR soil mix. At this point, plants were used for psyllid colonies. This method ensured optimal plant growth and confirmed the plant ages were the same.

Colony Set Up

Colonies were kept in bread bag cages together in one tray in an incubator at 25 °C under a 16L:8D light/dark cycle as well. Backup colonies were also created in case of any unforeseen circumstances. These colonies were only visited while wearing lab coats and no other colonies were visited to ensure no contamination.

The psyllids were monitored closely to ensure they were of the same age. First, 15 psyllids were put on each plant and removed after three days after oviposition. This is done by using an aspirator to collect the psyllids. I would then repeat this by tracking these psyllids to adulthood and removing them to ensure the nymphs would be of the same generation. After three generations, my colony stabilized, and I began my experimental line.

Collection

The psyllids would be tracked daily by recording the number and life stage of each through knowledge of nymphal development. The psyllid life cycle goes from egg, to nymph, to adult. The nymphal stage has five instar stages. After hatching from the egg, the nymph is in the first instar stage for around 3 days and then stays in the second instar stage for around 2.5 days

(Knowlton and Janes, 1931). The first two instar stages are only visible under a microscope. After, around 13 days the psyllids would become young adults. The stages were monitored by looking at the leaves of the plants with nymphs on them under a microscope.

For each plant, I would select 3 samples of second instar nymphs as well as young adults, totaling 12 samples. For each biological sample, I would have three replicates. For second instar nymphs, 20 insects would be pooled and for young adults, 4 insects would be pooled. The life stages of the psyllids would be confirmed under the microscope before pooling.

DNA Extraction

In addition, prior to collection, it was confirmed that the colonies were free of the plant pathogen *Candidatus Liberibacter psyllaurous* which is only found with this species of psyllids (Hansen, 2008) through DNA extraction. This method involved the steps of lysing, binding, washing, and eluting. The Qiagen DNeasy Blood and Tissue kit manufactured in Maryland was used to perform DNA extractions. The samples were first lysed using Proteinase K and physically ground to disrupt cell tissue. This was then incubated overnight prior to using the lysate. Through centrifugation, the DNA was selectively bound to the membrane of the spin column provided by the kit while the contaminants pass through and are discarded. The contaminants and enzyme inhibitors that were left over were then removed through two washes and the DNA was eluted with a buffer (Qiagen, 2020).

RNA Extraction

I then began the molecular part of my experiment by first extracting and purifying RNA. The extractions were performed with the use of Zymo Quick- RNA Miniprep kit (Zymo

Research, Irvine, CA, USA). This method also involves the steps of lysing, binding, washing, and eluting in order to obtain usable RNA.

The samples were first lysed using a lysis buffer and the psyllids were physically ground to disrupt cell tissue. This was then centrifuged to only use the lysate. In the binding and washing steps, ethanol and wash buffer was used. Similarly to the DNA extraction method described before, through centrifugation, the RNA was selectively bound to the membrane of the spin column provided by the kit while the contaminants were discarded. Two more washes followed, and the RNA was finally eluted with nuclease-free water (Zymo Research, 2020). The samples were also DNase 1 treated to make sure they were clean. The concentration and cleanliness of the RNA were then checked using the SpectraMax QuickDrop Micro-Volume Spectrophotometer (Molecular Devices, San Jose, CA, USA). If the RNA was not clean, the RNA Clean & Concentrator kit -5 (Zymo Research, Irvine, CA, USA) was used and then the concentration and cleanliness were checked again until adequate.

All steps were done keeping the samples on ice due to the temperature sensitivity of RNA. The RNA extraction occurred on the same day as the collection of the samples. The extracted RNA samples were stored at −80 °C to prevent degradation.

Quantitative PCR

Reverse transcription was then used to create cDNA from the RNA. This cDNA was stored at –20°C. Reverse transcription involves primer annealing, DNA polymerization, and enzyme deactivation. This was done using the iScript™ Reverse Transcription Supermix for RT-

qPCR with the Bio-Rad C1000 Thermal Cycler w/ Dual 48 Well Block machine (Bio-Rad Laboratories, Hercules, CA).

The cDNA was then used for quantitative polymerase chain reaction (qPCR) to see gene expression. Quantitative PCR setup occurred on ice and under the biosafety hood. iQ™ SYBR® Green Supermix was used following the Bio-Rad reaction setup. This involved the use of negative and positive controls in addition to my samples.

For each plant, three biological samples for adults were analyzed as well as three biological samples for second instar nymphs. For each biological replicate, three technical replicates were run. Three separate reaction plates were run to analyze each gene of interest alongside the housekeeping gene individually.

A modified version of the Universal SYBR Green qPCR Protocol was used to perform analysis through qPCR. The qPCR steps involve isolation of DNA, denaturation, DNA annealing and extension, and DNA synthesis and fluorescence detection. SYBR Green emits a fluorescent signal when it comes between newly synthesized DNA. The detection of the fluorescence results in an amplification curve which is used for data analysis (Adams, 2020). The amplicons were disposed of immediately after the completion of the run to ensure no contamination occurs in the lab.

Analysis

In PCR, the Ct value refers to the cycle threshold. In literature, the Ct value is also synonymously referred to as the Cq value to indicate the quantification cycle. As described before, in qPCR fluorescence detection is used as quantification. The Ct value is the number of

cycles needed for the fluorescent signal to reach the threshold to exceed background levels. Ct values have an inverse relationship to the amount of target nucleic acid in the sample. In this case, a lower Ct value indicated a greater amount of target DNA in the sample.

To analyze the qPCR data results the delta-delta Ct method or comparative Ct method was used. The fold change of the gene expression is then calculated relative to the calibrator (Bookout, 2006). In this case, the calibrator was the adult treatment group to determine if there is a difference in expression in the second instars for each host plant. In addition, tomato adult was also used to determine if there is a difference in expression between all treatment types. IBM SPSS Statistics program was used to test for significance in the difference in change of Ct.

General Project Guidelines

General lab safety approvals as per university guidelines are required, but no special requirements or approvals are necessary to perform these methods. Proper protocol and microbiology techniques must be followed in order to ensure no cross-contamination in the lab. Challenges that may be encountered include dealing with dying colonies or impure results. This was corrected through trial and error and weekly meetings with my faculty mentor. Challenges were also addressed by tweaking colony care methods and reruns of qPCR to ensure primer efficiencies and proper analysis methods.

Results

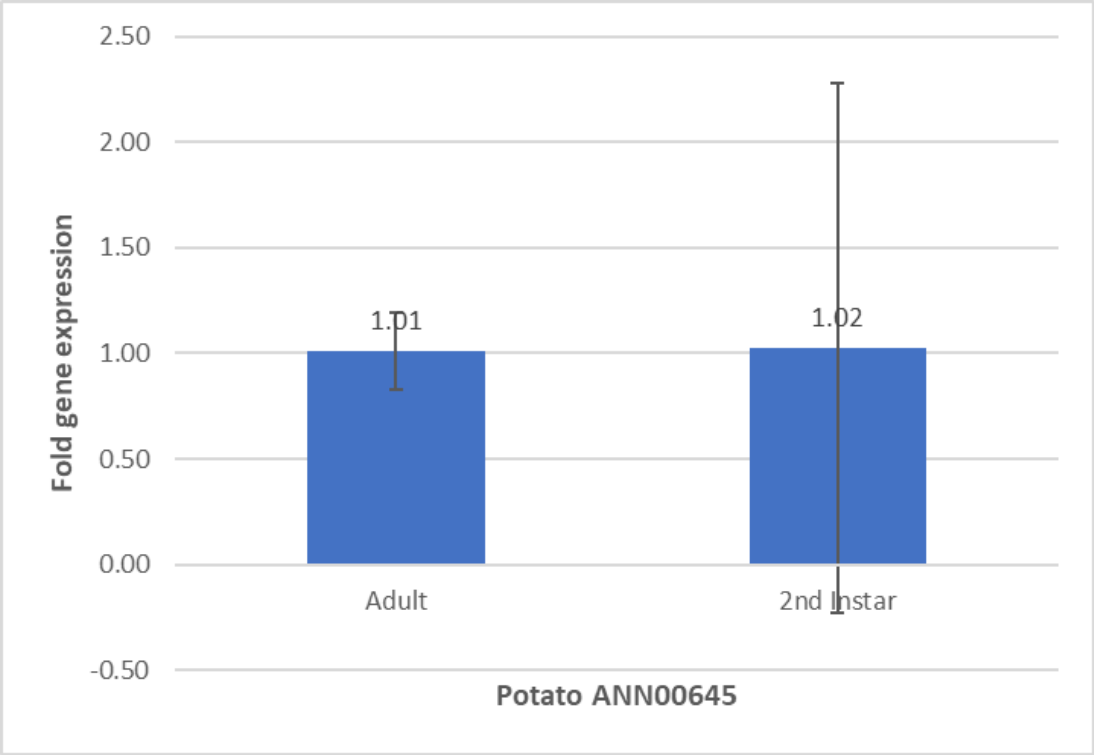
ANN00645

Average Ct Values:

Potato	Gene of Interest	Housekeeping Gene
Adult Sample 1	25.97	16.48
Adult Sample 2	26.1	17.04
Adult Sample 3	25.59333	16.61
Second Instar Sample 1	27.71667	16.65
Second Instar Sample 2	25.35	17.48
Second Instar Sample 3	25.94333	15.17

The average 2^-(ΔΔCt) for adults on potatoes was 1.01.

The average 2^-(ΔΔCt) for second instars on potatoes was 1.02.

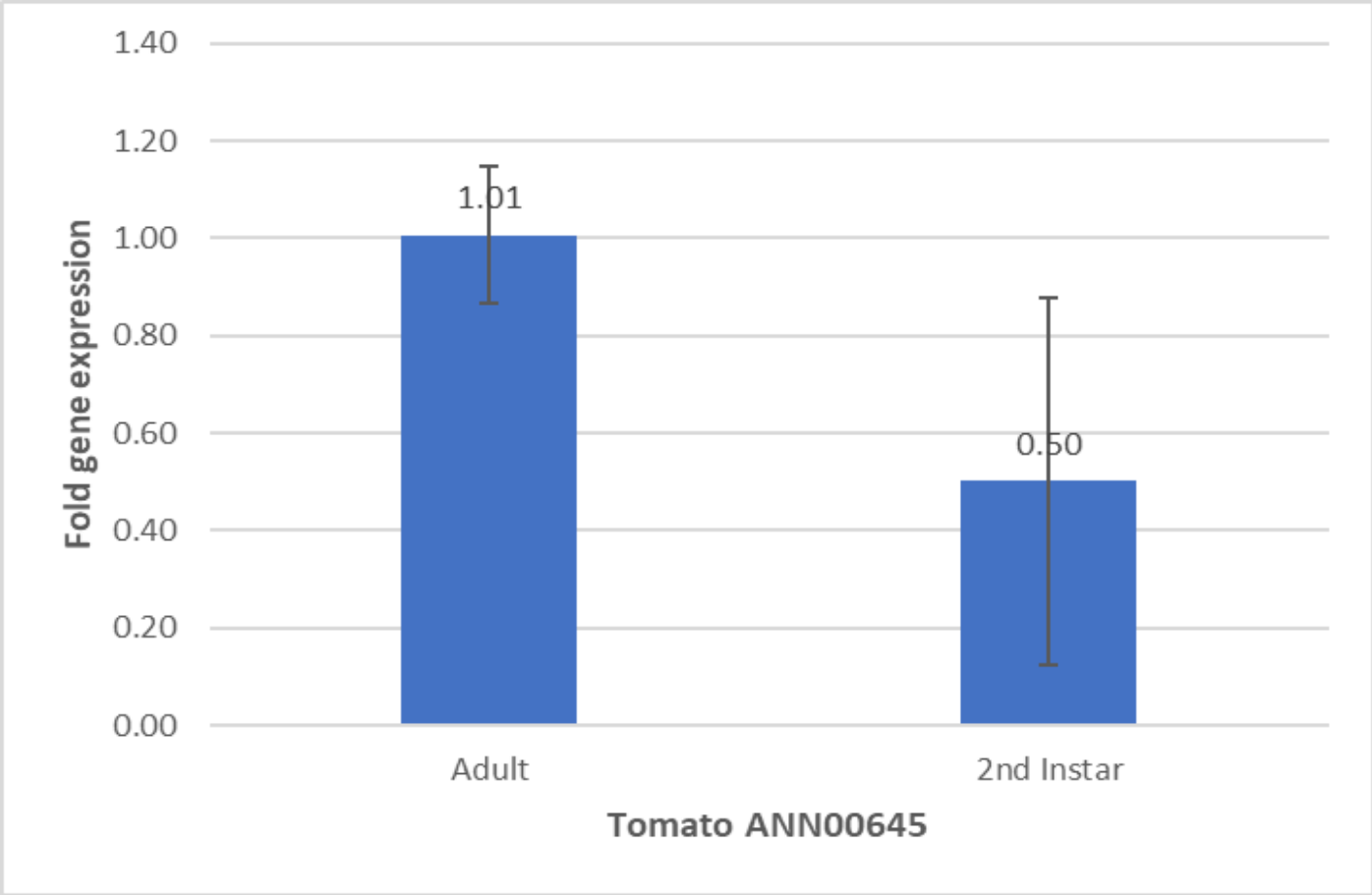


Average Ct Values:

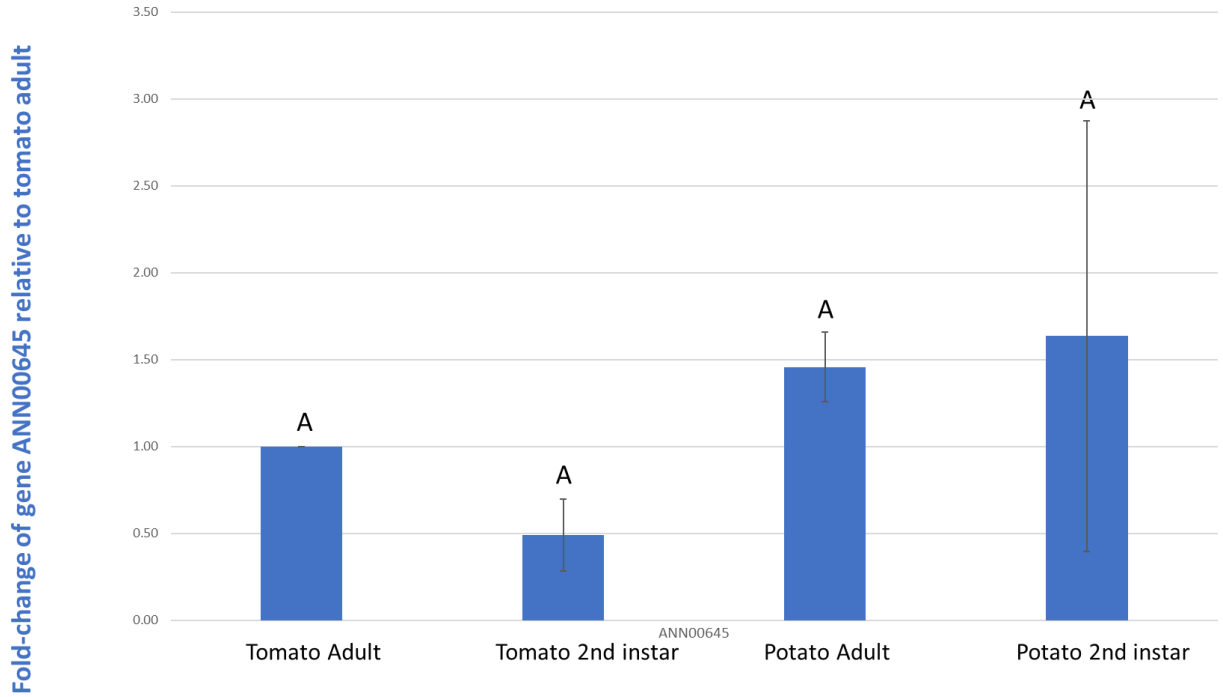
Tomato	Gene of Interest	Housekeeping Gene
Adult Sample 1	25.89333333	16.24
Adult Sample 2	25.79666667	15.88
Adult Sample 3	24.58	15.07
Second Instar Sample 1	27.13	17.32
Second Instar Sample 2	27.12	15.07
Second Instar Sample 3	27.72666667	16.68

The average 2^-(ΔΔCt) for adults on tomatoes was 1.01.

The average 2^-(ΔΔCt) for second instars on tomatoes was .50.



Fold Change relative to Tomato Adult between All Treatments (Figure 1)



Different letters denote significance between treatments (P<0.05 LSD Multiple comparisons)

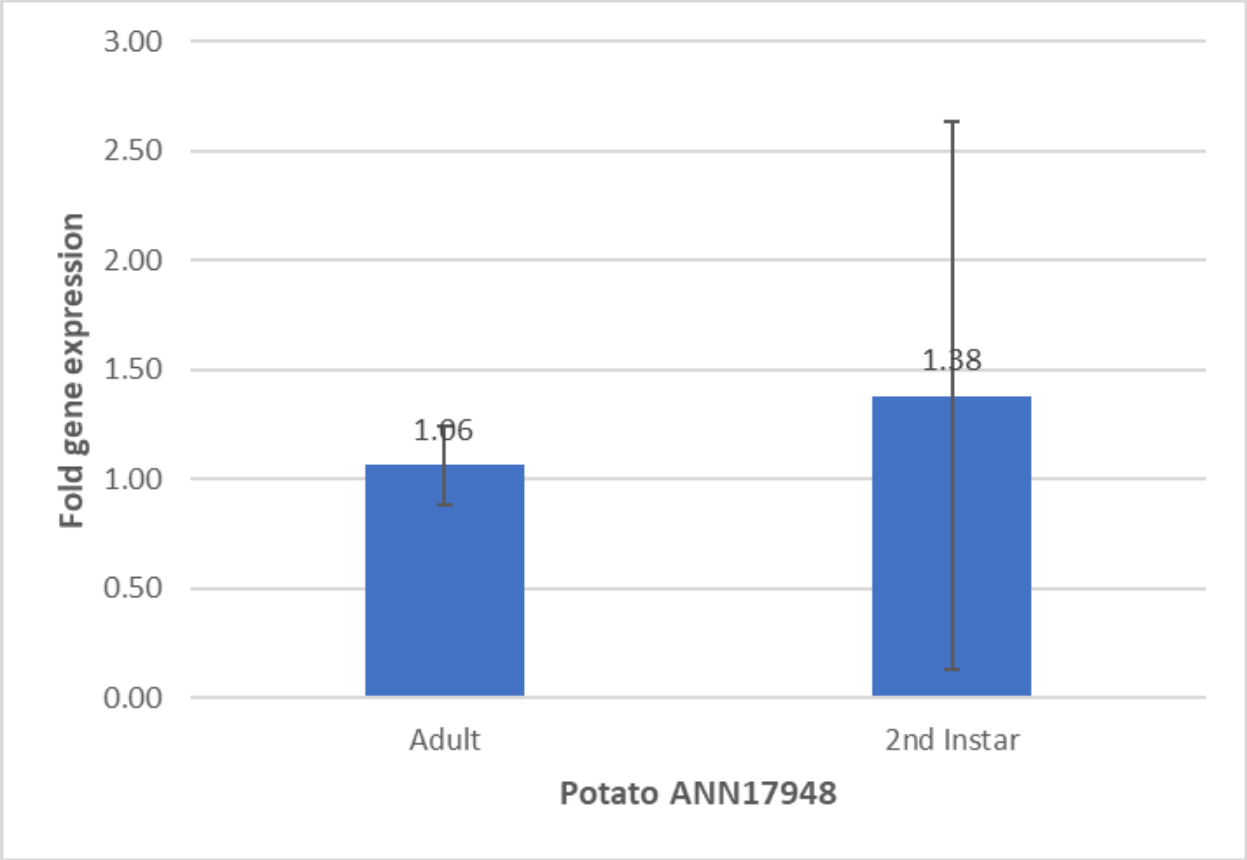
ANN17948

Average Ct Values:

Potato	Gene of Interest	Housekeeping Gene
Adult Sample 1	25.38333333	16.34
Adult Sample 2	25.95666667	17.37
Adult Sample 3	26.15666667	16.32
Second Instar Sample 1	25.00333333	16.20
Second Instar Sample 2	25.42	16.73
Second Instar Sample 3	23.89333333	15.31

The average 2^-(ΔΔCt) for adults on potatoes was 1.06.

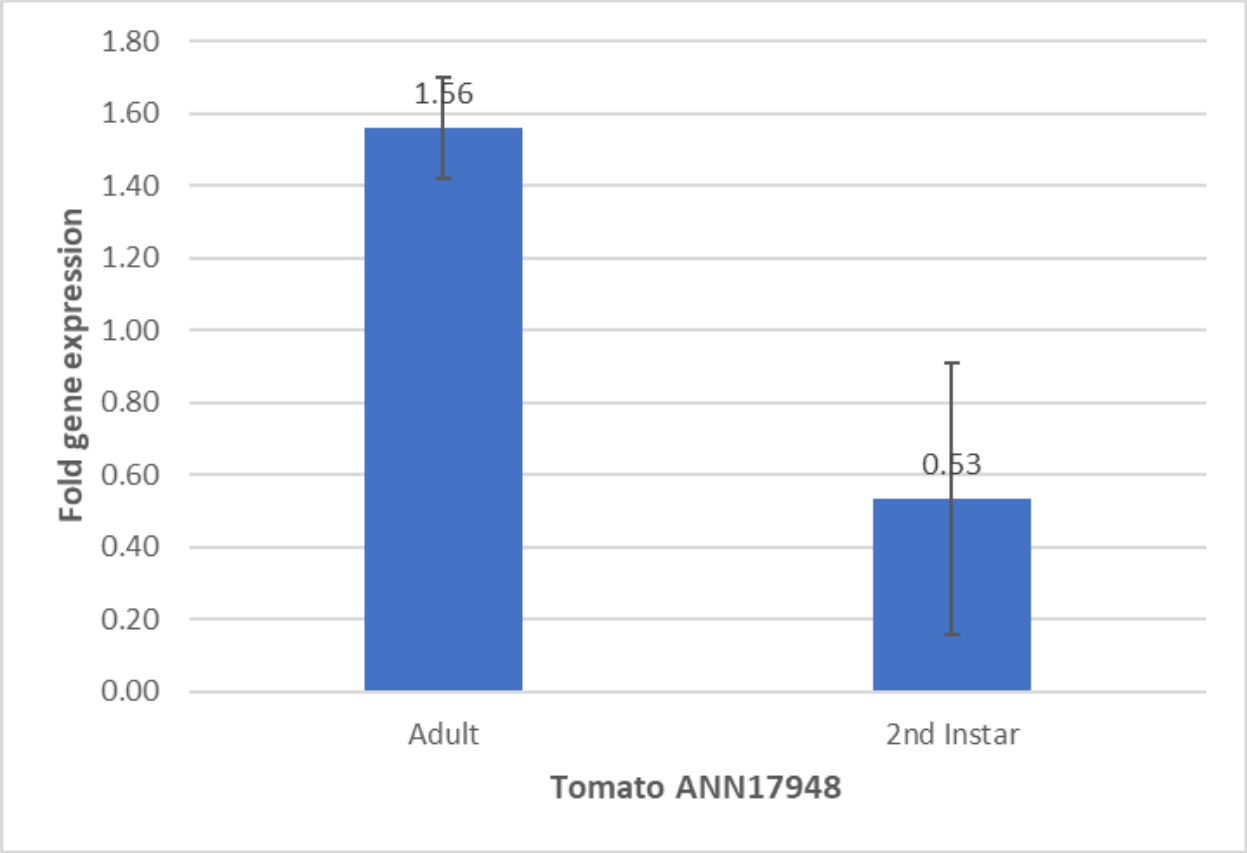
The average 2^-(ΔΔCt) for second instars on potatoes was 1.38.



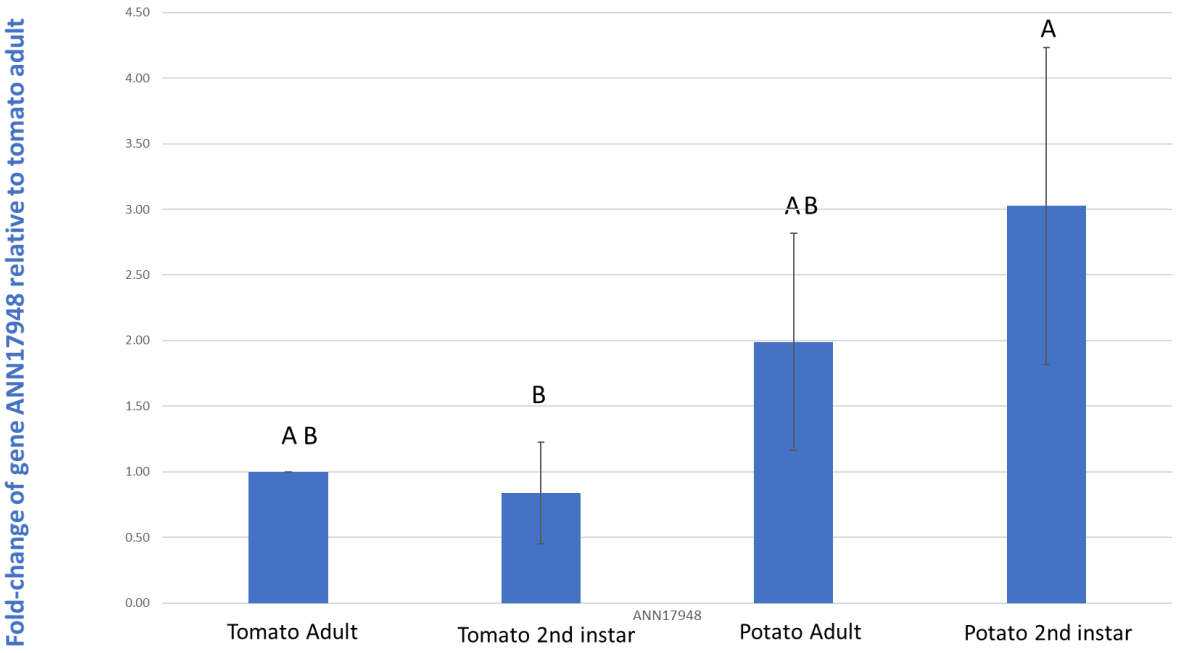
Tomato	Gene of Interest	Housekeeping Gene
Adult Sample 1	27.71666667	16.87
Adult Sample 2	25.19333333	17.21
Adult Sample 3	25.86666667	15.17
Second Instar Sample 1	27.33	16.95
Second Instar Sample 2	26.61	15.18
Second Instar Sample 3	27.69333333	17.05

The average 2^-(ΔΔCt) for adults on tomatoes was 1.56.

The average 2^-(ΔΔCt) for second instars on tomatoes was .53.



Fold Change relative to Tomato Adult between All Treatments (Figure 2)



* *Different letters denote significance between treatments (P<0.05 LSD Multiple comparisons)*

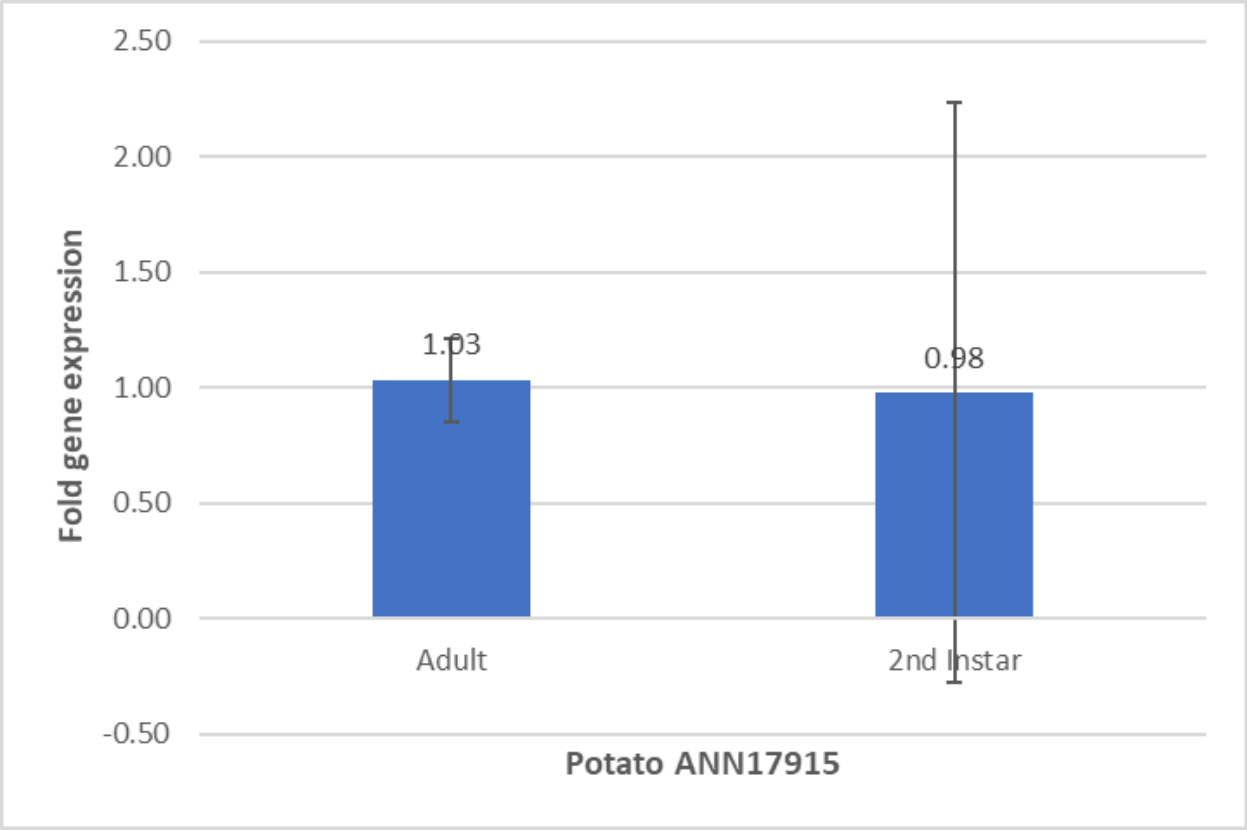
ANN17915

Average Ct Values:

Potato	Gene of Interest	Housekeeping Gene
Adult Sample 1	26.25251412	15.72
Adult Sample 2	26.58124786	16.87
Adult Sample 3	26.51077384	16.06
Second Instar Sample 1	26.3266585	15.83
Second Instar Sample 2	26.0707359	15.57
Second Instar Sample 3	24.40063757	14.52

The average 2^-(ΔΔCt) for adults on potatoes was 1.03.

The average 2^-(ΔΔCt) for second instars on potatoes was .98.

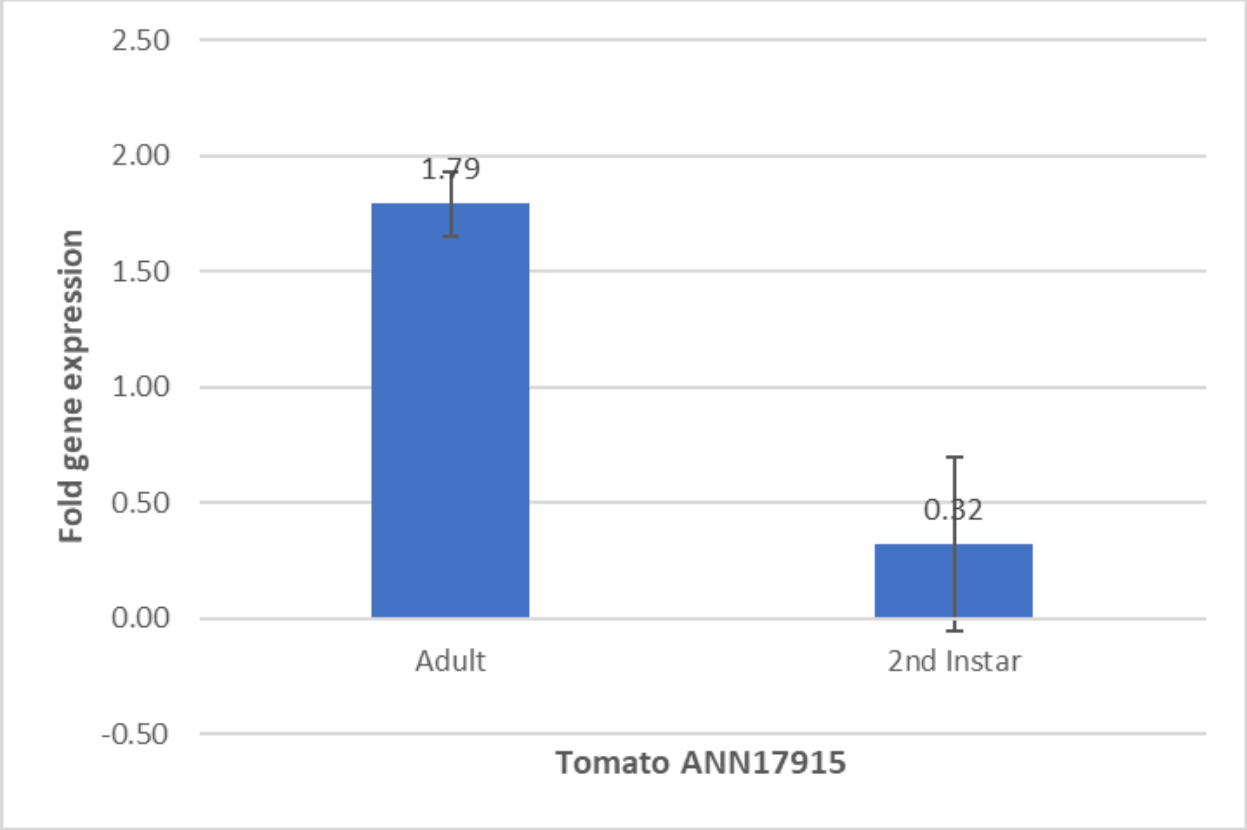


Average Ct Values:

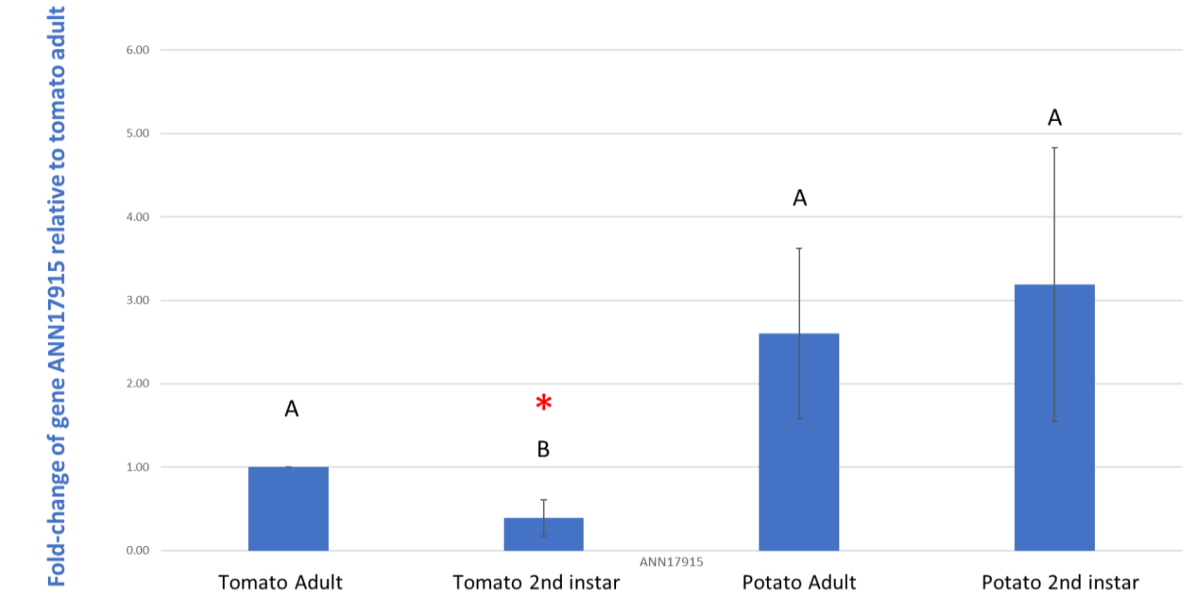
Tomato	Gene of Interest	Housekeeping Gene
Adult Sample 1	28.32341712	16.17
Adult Sample 2	26.25876434	17.16
Adult Sample 3	27.30815131	14.83
Second Instar Sample 1	28.26623102	15.83
Second Instar Sample 2	28.07320457	15.57
Second Instar Sample 3	28.93437076	14.52

The average 2^-(ΔΔCt) for adults on tomatoes was 1.79.

The average 2^-(ΔΔCt) for second instars on tomatoes was .32.



Fold Change relative to Tomato Adult between All Treatments (Figure 3)



* *Different letters denote significance between treatments (P<0.05 LSD Multiple comparisons)*

Discussion

ANN00645

Looking at the fold change of gene expression relative to the average of the adult treatment group for each plant host, there is a difference, although not significant, in gene expression between the adults and second instar when comparing the potatoes and tomatoes. This could possibly be due to variation when extracting RNA or during other molecular steps. On the potato plant, the expression of the adults and the second instar are relatively similar.

In comparison, on the tomato plant, the second instar expression value is almost half that of the adult expression value. When looking at the fold change of gene expression relative to tomato adults between all treatment groups (Figure 1), using a two-way ANOVA test, there was no significant difference ($P>0.05$) in the expression of the gene (ANN00645) between host-plant treatments or life stages.

ANN17948

Looking at the fold change of gene expression relative to the average of the adult treatment group for each plant host, there is a difference, although not significant, in gene expression between the adults and second instar when comparing the potatoes and tomatoes. This could also possibly be due to variation when extracting RNA or during other molecular steps. On the potato plant, the expression of the adults is around 3/4 of the expression of the second instar.

In comparison, on the tomato plant, the second instar expression value is around a third of the adult expression value. When looking at the fold change of gene expression relative to

tomato adults between all treatment groups (Figure 2), using a two-way ANOVA test, there is a significant difference in the expression of the gene (ANN17948) between host-plant treatments ($P=0.028$), but not life stages ($P>0.05$). A lower expression of the gene when feeding on tomato at 2nd instar compared to the potato at 2nd instar was found.

ANN17915

Looking at the fold change of gene expression relative to the average of the adult treatment group for each plant host, there is a difference, although not significant, in gene expression between the adults and second instar when comparing the potatoes and tomatoes. Again, this could also possibly be due to variation when extracting RNA or during other molecular steps. On the potato plant, the expression of the adults and the second instar are relatively similar.

In comparison, on the tomato plant, the second instar expression value is less than a fifth of the adult expression value. When looking at the fold change of gene expression relative to tomato adults between all treatment groups (Figure 3), using a two-way ANOVA test, there is a significant difference in the expression of the gene (ANN17915) between host-plant treatments ($P=0.018$), but not life stage ($P>0.05$). A lower expression of the gene when feeding on tomato at 2nd instar was found compared to all other treatments.

When looking at the fold change of gene expression relative to tomato adults between all treatment groups, initial preliminary data suggest a significant difference between host plant treatment for gene ANN17948 and ANN17915. The difference in expression between the two plants shows that these genes may have a more specific host response for feeding, but not life

stage. Ultimately, these genes may be involved in host plant adaptation which has further implications across other disciplines such as agriculture.

This project adds to our understanding of the putative function of species-specific protease genes that are evolving rapidly. It is important to deduce what these genes do to follow and maintain psyllids. Their role as pests makes them even more important to study because of how they vector certain diseases. The difference in expression among life stages is also important to note as expression changing throughout their life cycle can impact insect behavior and interactions.

Conclusion

Lineage-specific gene family expansions contribute to the rapid evolution of eukaryotes. They are important to study in organisms as they can lead to new functions. Studying the expansion of protease gene families *B. cockerelli* shows that these specific genes are involved in host-plant interactions and are important in responding to host-plant feeding.

There is a significant difference in gene expression between the host plant treatment for 2 of the genes studied. This difference can possibly be attributed to the evolutionary history of the tomato and potato plants. Although research is still being conducted on their history, differences can be credited to past hybridization events and varying speciation rates (Rodriguez et al., 2009). Furthermore, new discoveries have been made finding new defensive constituents of the potato plant (Liu., 2017).

Ongoing studies of the defenses of both plants can possibly explain the differences in gene expression noted because of the host plant responses to insect feeding. Noting this difference, future studies can aim to further narrow down the function of these genes through methods such as RNA sequencing or studies involving CRISPR. The study can be continued with a larger sample size to reduce variation, as well.

Hopefully, further investigation can identify the function of all three of these genes. Understanding these gene functions can ultimately help better understand species interactions. Since the psyllid is a vector of disease, this will positively impact agriculture and can also help with disease control because if we are able to learn more about the physiological functions of the

psyllid, we can control it more effectively. This also gives more insight into the necessary defenses of tomato and potato plants.

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