Project Title: Can we get codling moth females to stop laying eggs on apple?

Report Type: Final Project Report

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Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 56,110
Total Project Request for Year 2 Funding: $ 58,817
Total Project Request for Year 3 Funding: $ 61,610

Other related/associated funding sources: Awarded
Funding Duration: 2018
Amount: $35,000
Agency Name: USDA-ARS, Pacific West Area Office
Notes: Area Office awarded money to previous project manager, Dr. Stephen Garczynski, to purchase a flight tunnel and Track3D system. The Track3D system is comprised of cameras and software to monitor insect behavioral responses in a flight tunnel. No other funds have been sought for this project.

WTFRC Collaborative Costs: None

Budget 1
Primary PI: William B. Walker III
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Item	(Type year of project start date here)	(Type year start date of year 2 here if relevant)	(Type year start date of year 3 here if relevant)
Salaries	$37,306.00	$39,282.00	$41,322.00
Benefits	$13,804.00	$14,535.00	$15,288.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$5,000.00	$5,000.00	$5,000.00
Travel			
Plot Fees			
Miscellaneous			
Total	$56,110.00	$58,817.00	$61,610.00

Footnotes: Salaries and benefits are requested for a full-time GS-6 Lab Technician. Supplies are for cloning ORs, oligonucleotide primer synthesis, Cas9 mRNA and kits for making CRISPR guide RNAs.

AMENDMENT

This section serves notification that there was a change in PI for this project. After completion of the first year of the project, the lead PI, Dr. Stephen F. Garczynski passed away in February 2019. Dr. Garczynski had drafted the first continuation report for this proposal. There was an agreement between USDA-ARS and WTFRC that this project could be paused while Dr. Garczynski's Research Geneticist position was vacant. New PI William B. Walker was indicated on the first continuation report as a "Cooperator" and was hired in October 2020 to fill the vacant Research Geneticist position. Dr. Walker has been working to complete the project as planned and was advised to submit the continuation report as drafted by Dr. Garczynski. During the time this project was paused, no further project work was performed. The equipment items mentioned in "other funding sources" were obtained by Dr. Garczynski and are now available to facilitate completion of the project.

OBJECTIVES

1) Identify and clone Odorant Receptors expressed in the abdomen tip of codling moth females.
Over thirty odorant receptors (OR) were identified in a transcriptome generated from abdomen tips of codling moth females. A majority of ORs identified in the transcriptome have been confirmed to be expressed by molecular cloning and DNA sequencing. Gene expression assays have also been conducted to compare OR expression in mated versus non-mated codling moth females. In the coming years, several of the cloned ORs will be used in expression assays (not funded by this proposal) to determine activating plant compounds; this will facilitate future odorant-based lure research and development.

2) Knock-out OR genes using CRISPR/Cas9 genome editing.
CRISPR/Cas9 genome editing is fully developed for codling moth, and is being used to knock-out genes expressed in the female abdomen tip. CRISPR/Cas9 genome editing of ORs facilitates basic research in the laboratory on the function of the codling moth's olfactory system. Information derived from this line of research may be utilized by integrated pest management strategies including odorant lure development and push-pull applications. A single OR gene that is expressed in female antennae and abdomen tip has been genome edited with the CRISPR/Cas9 approach to induce inactive, functionally deleted OR protein. Laboratory populations of codling moth with the inactive OR gene are being generated for use in oviposition assays.

3) Determine which genes are essential for oviposition site selection.
The hypothesis tested here is that inactive OR genes for specific plant volatiles will alter female codling moth oviposition site selection. Oviposition assays for codling moth are under development and will be used to assess the effects of OR gene knock-out populations. Identification of ORs that directly mediate oviposition behavior will facilitate behavioral testing odorants that are detected by the OR.

SIGNIFICANT FINDINGS

- In addition to the odorant receptors (ORs) identified in the female abdomen transcriptome, several other odorant receptors of interest, including candidate pheromone receptors, were PCR amplified, cloned, and confirmed to be expressed in female abdomen tips
- Three ORs showed expression differences in abdomen tip of mated versus unmated codling moth females
- CRISPR-mediated editing has been achieved for a female specific OR that is expressed in female antennae and abdomen tip, but not in male codling moth.
- Functional assays of this OR resulted in identification of activating fruit odorants not previously known to be detected by codling moth

RESULTS AND DISCUSSION

Identification and cloning odorant receptors expressed in female codling moth
We have prepared a transcriptome (a compilation of all genes expressed in a particular tissue) from abdomen tips dissected from pre-adult (pupal) and unmated and mated codling moth female adults. Transcripts encoding 38 different ORs were discovered. In year two of this project, during the past year, attempts were made to clone and sequence full length transcripts of all of these ORs. Of the 38 ORs identified in the transcriptome, we were able to confirm full-length transcript expression of 24 from female codling moth abdomen tips of either unmated, mated or both (24/38 = 63%). These transcripts were cloned to verify expression and DNA sequence for the design of guide RNAs for use in future CRISPR genome editing experiments to determine functional roles for these receptors.

We were not able to detect or confirm expression in adult female abdomen tips for 14 of the 38 ORs (36%) identified in the abdomen tip transcriptome. It is possible that some of these ORs are not expressed in adult female abdomen tip, but rather only in the abdomen tip of pupal stage, since pupal

abdomen tips were also used to generate the source transcriptome. Given the overall objectives of this project, we did not conduct any PCR screening of ORs in the pupal stage. Alternatively, failure to detect OR expression by PCR in the female adult abdomen tips may be due to technical reasons, for example faulty oligonucleotide primers, or expression below detection thresholds. To rule these out, for ORs that we were unable to detect during initial PCR assays, we designed and tested additional oligonucleotide primers, and moreover, each primer set was subjected to serial rounds of PCR amplification in order to detect expression of transcripts that were otherwise below threshold detection levels after the first round of detection.

It was previously reported that a candidate pheromone receptor, OR1, was detected in female codling moth abdomen tips (Garczynski et al., 2017). Interestingly, we did not observe OR1 transcripts in our female abdomen tip transcriptome. It may be the case that OR1 displays restricted expression in codling moth abdomen tip neurons below thresholds levels for detection in our transcriptome. Because of this, we decided to conduct additional PCR screening of all candidate pheromone receptors, regardless of whether they were identified in our female abdomen tip transcriptome. In our previously published codling moth antennal transcriptome (Walker et al., 2016), a majority of these candidate pheromone receptors displayed either male-specific or female-specific antennal expression and are thus hypothesized to influence sex-specific behaviors such as egg-laying in case of the female specific ORs. Furthermore, one candidate pheromone receptor, OR3 was previously reported to be activated by host-plant volatiles, not pheromones (Bengtsson et al., 2014), opening up the possibility that other candidate pheromone receptors may also respond to host-plant odorants, and mediate behaviors such as egg-laying.

A total of 15 candidate pheromone receptors were screened, regardless of their identification-status in the female abdomen tip transcriptome. Of these, 13 were confirmed to be expressed in abdomen tip of unmated or mated female codling moth, including OR1 which was not identified in the female abdomen tip transcriptome, and OR3, which was. In total, we confirmed 34 ORs expressed in female codling moth abdomen tip tissue, regardless of mating status. While a sizable percentage of ORs identified in the transcriptome were not confirmed by this approach, we nonetheless confirmed and sequenced a substantially large repertoire of ORs in adult female abdomen-tip to provide ample substrate to pursue the main objective of this proposal.

Further analysis of the abdomen tip transcriptome has revealed expression of at least 23 gustatory receptors (GRs), which function in the sense of taste, and 17 ionotropic receptors (IRs), which are known to function in either the sense of smell or the sense of taste. Due to the importance of the sensory systems of both smell and taste in influencing insect oviposition behaviors, future research will examine in greater detail the expression and function of GRs and IRs in codling moth abdomen tip physiology and oviposition behaviors.

Expression analysis of odorant receptors in abdomen tip of unmated and mated female codling moth

For all odorant receptor transcripts that were successfully PCR amplified and cloned, PCR amplification attempts were made on biological samples containing abdomen tips of both unmated and mated codling moths. For some of the odorant receptors, we were initially only able to successfully generate PCR products from either unmated or mated samples but not both. These observations led us to hypothesize that for some of the ORs, expression might be regulated by mating, and furthermore, ORs for which expression is induced or increased by mating may have special relevance to detection of odorants that influence egg-laying behavior. We thus sought to utilize quantitative real-time PCR (qRT-PCR) assays on a subset of ORs that displayed preliminary indications of expression differences in abdomen tip samples of unmated versus mating codling moth. This methodology allowed us to more precisely assess whether mating could induce or restrict expression of specific ORs in female abdomen tip.

Oligonucleotide primers were designed for qRT-PCR assay to assess relative expression levels of 19 ORs in abdomen tip of unmated versus mated codling moth females. Initial testing using multiple

sets of primers for each OR revealed that for 10 of these 19 ORs (53%), expression was not consistently detectable with the qRT-PCR assay. For these ORs, it is likely that their expression levels in adult female abdomen tip are below the threshold of detection by the qRT-PCR assay. We thus pursued fully replicated qRT-PCR assays of the remaining 9 ORs (47%) from this subset to determine if mating affects their expression. It was determined that for one of the ORs, expression was significantly increased in abdomen tip of mated versus non-mated female codling moth, and for two other ORs, expression was significantly decreased in abdomen tip of mated versus non-mated female codling moth (Figure 1).

These ORs will be subjected to further research on their role in codling moth egg-laying behavior through assays on OR protein function as well as CRISPR knock-out experiments. None of the other seven ORs that we assayed showed statistically significant differences in expression in abdomen tip of mated versus unmated codling moth females.

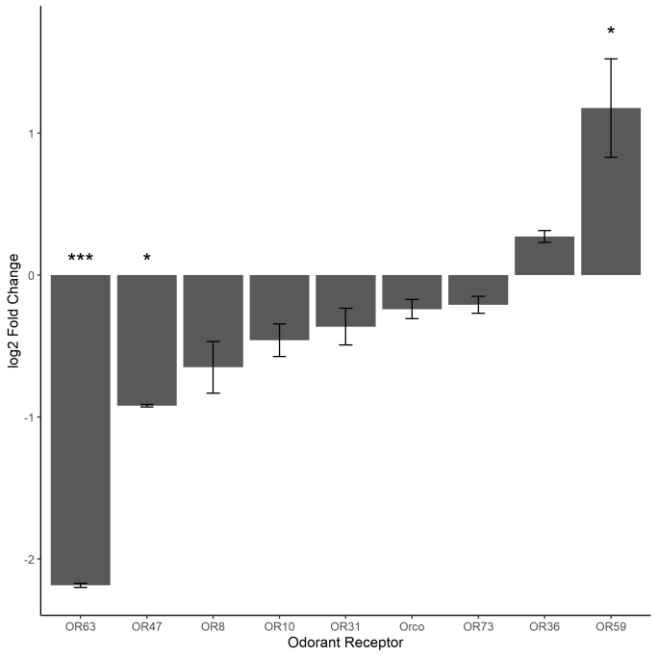

Figure 1. Relative expression of odorant receptors (ORs) in female abdomen tips of mated codling moths versus unmated codling moths. For each OR, three replicate biological samples each from mated and unmated codling moth females were used in quantitative real-time PCR assays. OR gene expression was normalized to gene expression of two control genes and binary log differences in expression values are shown in mated relative to unmated samples. Asterisks indicate statistically significant differences indicated with p value < 0.05 (*) or $p < 0.005$ (***). Error bars indicate standard error values.

Due to the fact that a majority of ORs initially screed by qRT-PCR assay were not detected by this methodological approach, this study is currently being expanded to test expression for all ORs identified in the abdomen tip transcriptome or otherwise detected through molecular cloning assay. Experiments are underway, and results are thus not yet available. Furthermore, GRs and IRs also identified in the abdomen tip transcriptome will also be screened. These additional experiments will provide an expanded view of the effects of mating on smell and taste function in the codling moth female abdomen tip.

Functional characterization of receptors expressed in female abdomen tip

Prior to initiation of this project, research was being conducted on functional characterization of ORs that displayed female-specific expression in antennae of codling moth. One of these female-specific antennal ORs, was determined to be activated by aldehyde odorants present in apple volatile collections, including nonanal (Walker et al., unpublished data), which has been shown to stimulate egg-laying behavior in codling moth (Witzgall et al., 2005). Further research on the functional capacity of this OR, in collaboration with the laboratory of Dr. Jason Pitts at Baylor University, has revealed that it is also strongly activated by specific lactone compounds, in addition to activation by aldehydes present in apple volatile collections. Lactones are known to provide distinct odor characteristics present in other fruit that codling moth can infest, such as peaches and plums, however detection of these types of compounds by codling moth has not been previously reported.

Furthermore, our research on abdomen tip expression of ORs in female codling moth revealed that the aldehyde/lactone sensitive OR is also expressed in female abdomen tip, in addition to the antennal female-specific expression profile previously reported (Walker et al., 2016). Therefore, it is hypothesized that this OR may have a dual role in detection of odorants that mediate both host-seeking behavior at as distance as well as close-range oviposition behavior. Current research efforts are focused on CRISPR editing of this female-specific OR to disrupt its functionality and evaluate specific roles it plays in mediating olfactory behaviors in codling moth females.

Preliminary CRISPR experiments on female-specific odorant receptors

Five different guide RNAs (sgRNAs) were designed to target editing of different genomic regions of the codling moth female-specific OR. Approximately 200 freshly laid codling moth embryos were injected with each sgRNA combined with the CAS9 mRNA. 40-60% of all injected embryos survived injections through at least neonate larval hatching. Larvae were provided with unrestricted access to standard artificial diet. In order to assess relative efficacy of CRISPR editing for each sgRNA, two different biological samples containing approximately 10 pooled third-instar larvae were taken from each sgRNA cohort. Genomic DNA was extracted from each sample, the appropriate genomic region was PCR amplified and subjected to high-throughput DNA sequencing in order to evaluate degree of editing induced by each sgRNA.

CRISPResso analysis revealed that substantial CRISPR editing was induced by three of the five sgRNAs in one or both of the biological samples selected for each sgRNA. These three sgRNAs have been selected for further experimentations. One of the sgRNAs is present in the exon 2 region of the OR gene, while the other is present in the exon 3 region (Figure 2). For initial CRISPR editing experiments, the exon-2 sgRNA was co-injected with either of the two exon-3 sgRNAs. The objective was thus to induce deletion of the genomic region across exon 2 and exon 3, resulting in a non-functional OR protein. PCR analysis of genomic DNA across exon 2 and exon 3 in CRISPR-injected individuals revealed the presence of several genotypic variants that contained genomic deletions across the targeted region (Figure 2). Furthermore, analysis of the sequence of mRNA for the target OR in the offspring of CRISPR-injected individuals revealed partial or whole deletions of entire exons including exon 2 and exon 3. These results provide further support that CRISPR-based genome editing of odorant receptors is robust in codling moth, and will serve as a viable approach to study gene function directly within codling moths.

Current efforts are focused on crossing CRISPR-edited codling moth insects that contain deletions of the target OR gene in order to generate codling moth colonies that are homozygous, or fully edited, with non-functional copies of the OR gene. Once these colonies have been generated and shown to be stable, CRISPR-edited insects will be subjected to oviposition behavioral assays that are currently under development.

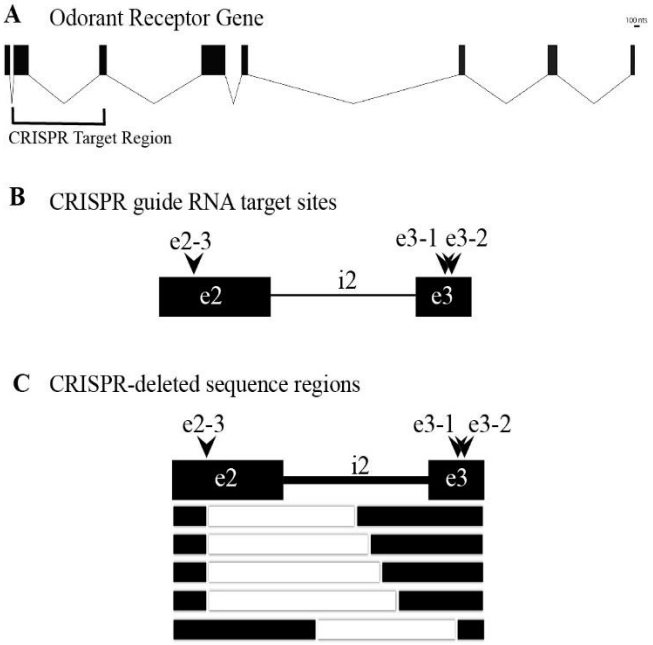

Figure 2. CRISPR editing of female odorant receptor (OR). **A)** Gene structure of target OR, with exons shown as black bars and introns shown as stick lines. Target region for CRISPR-editing encompasses exon 2 and exon 3. **B)** target site for selected guide RNAs (sgRNA) in exon 2 (e2) or exon 3 (e3). The aim is to delete intervening sequence between e2 and e3, including the second intron (i2). One sgRNA from each exon are both injected simultaneously. **C)** Five examples of CRISPR editing genotypes from injected individuals. The black bars (below exon/intron structure) indicate genomic regions not deleted, while intervening white bars indicate deleted regions.

Preliminary female codling moth oviposition behavioral assays

Initial efforts at developing a codling moth oviposition assay have not been successful. As the central aim of this project is to divert codling moth egg laying behavior from apple, we attempted oviposition assays using a plastic container with an apple wedge in it. Codling moth females that had been mated with males were placed into the contain and allowed to lay eggs freely within the container during an 8-hour period largely during their preferred hours for egg-laying, including the 3-4 hours prior to darkness. A majority of moths tested laid their eggs, however, not on the apple, but rather on the plastic container. This behavior may be attributed to the fact that moths used were from our codling moth colony, which has been maintained in our laboratory for more than a decade; behaviors of colony-kept insects is well known to deviate from those of wild insects due to a variety of factors including lack of natural conditions and inbreeding effects from restricted population size.

An attempt was made to assay wild-caught codling moths with our behavioral oviposition assay, and also introgress them into our colony. Codling moth larvae were harvested from a source of apples from a Yakima neighborhood tree. The intent was to rear the larvae to adulthood, then use a subset in behavioral assays and introduce the remainder into the colony. However, this population was heavily infested with parasitic wasps and very few adult moths emerged. Greater efforts will be made during the next growing season to obtain varied sources of wild codling moth. Additionally, optimization of the behavioral assay is underway, using colony insects, for example, with different types/sizes of containers.

Conclusions

Analysis of odorant receptor (OR) expression in the female codling moth abdomen-tip transcriptome has been completed. A repertoire of over thirty ORs expressed in the abdomen tip has been confirmed through molecular cloning and sequencing analysis. Rigorous quantitative expression analysis has resulted in identification of three ORs that display expression patterns in the abdomen tip that are modulated by mating. Stemming from this project, other chemosensory gene families that function in the sense of smell and taste are also being investigated. Odorant and tastant receptors that display increased or reduced expression after mating are strong candidates to have a prominent role in pre- or post-mating behaviors, such as food- or mate-seeking and egg-laying.

A single OR that is expressed in both female antennae and abdomen tip, and is not expressed in males, is observed to respond to fruit-derived odorants, including some that have not been previously reported to be detected by codling moth. Ongoing and future research is aimed at evaluating how these odorants affect codling moth behavior in both the laboratory and apple orchards. Current efforts in the laboratory are focused on CRISPR editing of this female-specific OR gene and subsequent consequences on olfactory behaviors when this gene is disrupted. Behavioral oviposition assays are under development in order to facilitate this aim. Future research is planned for CRISPR-editing disruption of other OR genes that display mating-affected expression patterns in the abdomen tip. Collaborative research efforts will be aimed at identification of odorant ligands that activate these receptors, as well as testing the effects of CRISPR-based disruption of them on codling moth oviposition behaviors.

Executive Summary

Project Title: Can we get codling moth females to stop laying eggs on apple?

Keywords: codling moth, olfaction, oviposition, CRISPR, lures

Abstract: Despite its prominence in the USA for over 200 years, codling moth remains a primary pest in apple cultivation to this day. In recent decades, control of codling moth in orchard production systems has been greatly aided by targeting the codling moth's olfactory system, exploiting the insect's keen sense of smell. Until now, this has largely been focused on disrupting male moth attraction to female through use of pheromone dispensers to induce mating disruption. However, it is the female moth that lays the eggs to ultimately propagate the next generation. Therefore, further attention to the female olfactory system is necessitated, ultimately aimed at disrupting female codling moth olfactory-based behaviors and thus complementing strategies that disrupt male codling moth behaviors. Research on insect olfactory systems has largely investigated the function of the insect's nose, the antennae. However, recent discoveries in other insects have cultivated an appreciation for an olfactory role for the female ovipositor, located on the abdomen tip, in guiding behaviors such as egg-laying, known as oviposition. In recent decades, much attention has been given to insect odorant receptors (ORs) due to their central role in the detection of odorants from the insect's environment.

For this project, analysis of OR expression in the female codling moth abdomen-tip has been completed. A repertoire of over thirty ORs expressed in the abdomen tip has been confirmed through molecular cloning and sequencing analysis. Rigorous quantitative expression analysis has resulted in identification of three ORs that display either increased or reduced expression in the abdomen tip subsequent to mating. These odorant receptors are strong candidates to have a prominent role in pre- or post-mating behaviors, such as food- or mate-seeking and egg-laying.

A single OR that is expressed in both female antennae and abdomen tip, and is not expressed in males, is observed to respond to fruit-derived odorants, including some that have not been previously reported to be detected by codling moth. Ongoing and future research is aimed at evaluating how these odorants affect codling moth behavior in both the laboratory and apple orchards. CRISPR-based editing of this female-specific OR gene has been achieved, and future efforts will be focused on the consequences of disruption of this gene on olfactory behaviors of female codling moths. Behavioral oviposition assays are under development in order to facilitate this aim. Future research is planned for CRISPR-editing disruption of other OR genes that display mating-affected expression patterns in the abdomen tip. Collaborative research efforts will also be aimed at identification of odorant ligands that activate these receptors, as well as testing the effects of CRISPR-based disruption of them on codling moth oviposition behaviors.