FINAL PROJECT REPORT

Project Title: Study of molecular mechanisms to preserve codling moth control agents

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Other funding sources: *None*

Total Project Funding: $126,000

Budget History:

Item	(2013)	(2014)	(2015)
Salaries[1]	26,100	27,000	28,000
Benefits	1,900	9,000	9,000
Wages			
Benefits			
Equipment			
Supplies	11,000	6,000	8,000
Travel			
Miscellaneous			
Plot Fees			
Total	39,000	42,000	45,000

ORIGINAL OBJECTIVES

1) Determine the effects of Altacor, Delegate, Calypso and granulosis virus on gene expression levels of codling moth heat shock (stress response) proteins and detoxification enzymes. The purpose of this objective is to identify gene transcripts that are elevated in response to sublethal doses of Altacor, Delegate, Calypso, and granulosis virus. We will focus on the most likely candidates based on studies performed in other moths, which include transcripts encoding detoxification enzymes (cytochrome P450s, esterases, and glutathione *S*-transferases), or increased expression of stress response proteins (heat shock proteins). Induced expression of gene transcripts encoding these proteins has been correlated with their potential as "insecticide resistance" factors. To complete this objective we will first clone gene transcripts encoding codling moth heat shock proteins and detoxification enzymes. From previous codling moth transcriptome data, we have identified gene transcripts encoding 24 different heat shock (stress response) proteins, 20 cytochrome P450s, seven esterases and 10 glutathione *S*-transferases. Once these transcripts are cloned and their nucleotide sequences verified, we will design oligonucleotide primers for use in quantitative PCR (qPCR). Once qPCR conditions are established, we will then quantify the expression levels of gene transcripts encoding heat shock proteins and detoxification enzymes from untreated eggs, neonates, and adults or those exposed to heat, cold, or sublethal doses of Altacor, Delegate, Calypso and granulosis virus. This will allow us to determine if any of these proteins have a potential role in resistance.

2) Determine the effectiveness of PBAN antagonists to inhibit codlemone production by codling moth females. Another way to prevent insecticide resistance is to use control agents that utilize a different mode of action to help control insect pests. Dr. Ron Nachman (USDA-ARS, Texas) has developed and synthesized several PBAN antagonists that reduce or eliminate pheromone biosynthesis in the tobacco budworm, *Heliothis virescens*. Because of the amino acid diversity of codling moth PBAN vs tobacco budworm PBAN (only 45 % similarity), it will be prudent to determine the effectiveness of the PBAN antagonists **before** they are fully developed into commercial products. This collaboration provides us the unique opportunity to test the PBAN antagonists on codling moth; first to determine if they work, then to determine dosage and timing of applications. To complete this objective, we will clone gene transcripts encoding codling moth PBAN receptors (PBANR) and then to express the cloned receptors in mammalian cell lines. We will then use cell based assays to verify PBANR activity and determine if PBAN antagonists block receptor activity. We will also test PBAN antagonists on female codling moth to determine biological activity in pheromone biosynthesis inhibition and to see if these compounds disrupt mating.

SIGNIFICANT FINDINGS (ACCOMPLISHMENTS)

- Additional detoxification enzymes/heat shock proteins were identified from a transcriptome
  - 50 transcripts encoding putative esterases
  - 80 transcripts encoding putative cytochrome P450 monooxygenases
  - 22 transcripts encoding putative glutathione *S*-transferases
  - 45 transcripts encoding putative heat shock proteins
- Protein targets for most classes of insecticides, past and present, were identified
  - Nicotinic acetylcholine receptor subunits (targets of neonicotinoids and spinosads)
  - Ryanodine receptor (target of rynaxypyr – Altacor)
  - Acetylcholinesterases (targets of Carbamates and Organophosphates)
  - GABA-gated chloride channels (targets of Organochlorines and phenylpyrazoles)
  - Sodium channels (targets of Pyrethroids and Indoxacarb)
  - Glutamate-gated chloride channels (targets of Avermectins and Milbemycins)
- A putative PBAN receptor has been cloned

- Cloned and confirmed detoxification enzymes/heat shock proteins
    - 8 transcripts encoding putative esterases
    - 10 transcripts encoding putative cytochrome P450 monooxygenases
    - 26 transcripts encoding putative glutathione *S*-transferases
    - 12 transcripts encoding putative heat shock proteins
- Cloned and confirmed protein targets of insecticides
    - Nicotinic acetylcholine receptor subunits (targets of neonicotinoids and spinosads)
    - Ryanodine receptor (target of rynaxypyr – Altacor)
- Preliminary quantitative real-time PCR analyses completed
    - Determined relative expression levels of 3 heat shock proteins in various life stages
    - Determined relative expression levels of 11 Glutathione *S*-Transferases
    - Analyzed Nicotinic Acetylcholine, Ryanodine and PBAN receptors

RESULTS AND DISCUSSION

Analysis of Nicotinic Acetylcholine and Ryanodine Receptor mediated resistance

Neonicotinoid (Calypso) Resistance

Nicotinic acetylcholine receptors (nAChR) are the protein targets of neonicotinoids (Calypso). nAChRs are nerve membrane proteins composed of a combination of 5 alpha and beta subunits, and the subunits can be all the same (homomeric) or any combination of multiple subunits (heteromeric). Resistance to neonicotinoids has been observed in the field, mainly through mutations in particular nAChR subunits. For the brown leafhopper, a single amino acid change in either nAChR subunit α1 or nAChR subunit α3 has been associated with neonicotinoid resistance (Fig. 1A). We have cloned and sequenced nAChR subunits α1 and α3 from our codling moth colony and have not seen any clones carrying the point mutation which leads to neonicotinoid resistance (Fig. 1A). Because our colony was derived from wild codling moth populations collected from various regions in eastern WA, it is likely that the point mutations in nAChR subunits α1 and α3 are not present at this time. However, it is possible that these mutations could occur in the future and we have developed a PCR assay to monitor for this occurrence. There is also another nAChR point mutation that can lead to target site resistance. For the green peach aphid, a single amino acid change in nAChR subunit β1 has been associated with neonicotinoid resistance in the field (Fig. 1A). We have cloned and sequenced nAChR subunit β1 from our codling moth colony and have not seen any clones carrying the point mutation which leads to neonicotinoid resistance (Fig. 1A). We have developed a PCR assay to also monitor the nAChR subunit β1 for occurrence of point mutations.

Spinetoram (Delegate) Resistance

Nicotinic acetylcholine receptors are also the protein targets of spinosads (Delegate). Two different types of mutations in nAChR subunits have been found to confer resistance to spinosads. For the Western flower thrip, a point mutation in nAChR subunit α6 has been associated with spinosad resistance (Fig. 1A). We have cloned and sequenced nAChR subunit α6 from our codling moth colony and have not seen any clones carrying the point mutation which leads to spinosad resistance (Fig. 1A). Another type of mutation in nAChR subunit α6 has been associated with spinosad resistance in the silkworm, *Bombyx mori*. A deletion of 15 amino acids in the silkworm nAChR subunit α6 was determined to be the cause of spinosad resistance (Fig. 1A). We did not detect this deletion in our sequenced codling moth nAChR subunit α6 clones (Fig.1A). We have also developed a PCR assay so that we can monitor for the occurrence of this mutation in codling moth.

Anthranilic diamide (Altacor) Resistance

The ryanodine receptor, a protein that is important in nerve and muscle function, is the protein target of Altacor. The gene transcript that encodes this extremely large protein (4000 – 5000

amino acids) is over 15,000 nucleotides in length. For the diamondback moth, a point mutation causing a single amino acid change has been associated with field resistance to chlorantraniliprole (Fig. 1B). We identified the ryanodine receptor from our codling moth transcriptome and cloned and sequenced the region containing the point mutation found in diamondback moth. We have not detected any clones of the ryanodine receptor from our codling moth colony that carry the point mutation that could lead to Altacor resistance (Fig. 1B). We are in the process of developing a PCR assay that we can use to monitor for the occurrence of a point mutation in codling moth that could lead to Altacor resistance.

A) Nicotinic Acetylcholine Receptor Subunits

Amino acids associated with susceptibility and resistance to neonicotinoids

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Brown planthopper α1 (sus)     ...CEIDVRYFPFDQQKCFMKFGSWTYDGNHVDLRHM...
Brown planthopper α1 (res)     ...CEIDVRYFPFDQQKCFMKFGSWTSDGNHVDLRHM...
Codling moth      α1 (sus)     ...CEIDVEYFPFDEQTCFMKFGSWSYDGYTVDLRHL...

Brown planthopper α3 (sus)     ...CEIDVEYFPFDEQKCVMKFGSWTYNGAQVDLLKH...
Brown planthopper α3 (res)     ...CEIDVEYFPFDEQKCVMKFGSWTSNGAQVDLLKH...
Codling moth      α3 (sus)     ...CEIDVEYFPFDQQTCVMKFGSWTYDGFQVDLRHI...

Green peach aphid β1 (sus)     ...GLAFVQLINVNEKSQIMKSNVWLRLVWRDYQLQW...
Green peach aphid β1 (res)     ...GLAFVQLINVNEKSQIMKSNVWLTLVWRDYQLQW...
Codling moth      β1 (sus)     ...GLAFVQLINVNEKNQIMKSNVWLRLVWMDYQLMW...
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Amino acids associated with susceptibility and resistance to spinosads

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Western flower thrip α6 (sus)     ...TILLSLTVFLNMVAESMPTTSDAVPLIGTYFNCI...
Western flower thrip α6 (res)     ...TILLSLTVFLNMVAESMPTTSDAVPLIETYFNCI...
Codling moth         α6 (sus)     ...TILLSLTVFLNLVAETLPQVSDAIPLLGTYFNCI...

Silkworm     α6 (sus)   ...QIIDVDEKNQLLITNIWLSLEWNDY...
Silkworm     α6 (res)   ...QIIDV---------------EWNDY...
Codling moth α6 (sus)   ...QIIDVDEKNQILTTNVWLNLEWNDY...
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B) Ryanodine Receptor

Amino acids associated with susceptibility and resistance to chlorantraniliprole

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Diamondback moth (sus)   ...FFFAAHLLDVAVGFKTLRTILQSVT...
Diamondback moth (res)   ...FFFAAHLLDVAVEFKTLRTILQSVT...
Codling moth     (sus)   ...FFFAAHLLDVAVGFKTLRTILQSVT...
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Figure 1. Target-site amino acids associated with insecticide susceptibility and resistance. Insects susceptible to the indicated insecticide are labeled with (sus). Resistant insects are labeled with (res). Amino acids indicated in the relevant mutations are high-lighted in gray.

Relative Quantification of Heat Shock Proteins and Glutathione* S*-Transferases

Glutathione S*-Transferase Expression*

An increase in glutathione S-transferases (GST) expression has been implicated in insect resistance to spinosads and rynaxypyr. To monitor codling moth for potential resistance to these compounds, we wanted to develop a quantitative real time PCR (qPCR) assay to look at transcript expression levels of individual GST enzymes. Basal GST expression levels relative to Actin (CpAct), a gene that is expressed at similar levels in all tissue types, for 11 GSTs, CpGST1, CpGST3, CpGST5, CpGST6, CpGST9, CpGST11, CpGST12, CpGST14, CpGST17, CpGST18 and CpGST19 were determined for neonates, adult male heads, adult female heads and a pool of RNA from all codling moth life stages (eggs through adults) using qPCR. In the RNA sample derived from all life stages, basal transcript expression of all 11 CpGSTs was detected, and ranged from 1.34×10^{-4} times that of Actin (CpAct) for CpGST6 to the highest level of 0.22 times that of CpAct for CpGST18 (Fig 2). Similarly, expression of all CpGSTs was detected in neonates, with levels ranging from 3.3×10^{-5} times that of CpAct for CpGST6 to 4.55×10^{-2} times that of CpAct for CpGST11 (Fig 2). No expression for CpGST3 was detected in adult male and female heads, and CpGST17 expression was also not detected in male heads (Fig 2). We observed that some GSTs display biased expression. For example, expression levels of CpGST3, CpGST12, CpGST14, CpGST17 and CpGST18 were higher in neonates than adult males and females while CpGST5, CpGST6, CpGST9 and CpGST11 were expressed higher in male and female heads than in neonates (Fig 2). Three GSTs, CpGST17, CpGST18 and CpGST19, displayed sex-biased expression with higher transcript levels detected in female heads compared to males. In the next two months, we will be determining the effects of sublethal doses of Altacor, Delegate and Calypso on GST expression levels. Additionally, we will finalize our development of a qPCR assay to monitor expression levels of cytochrome P-450 monooxygenases, another class of detoxification enzymes involved in insecticide resistance.

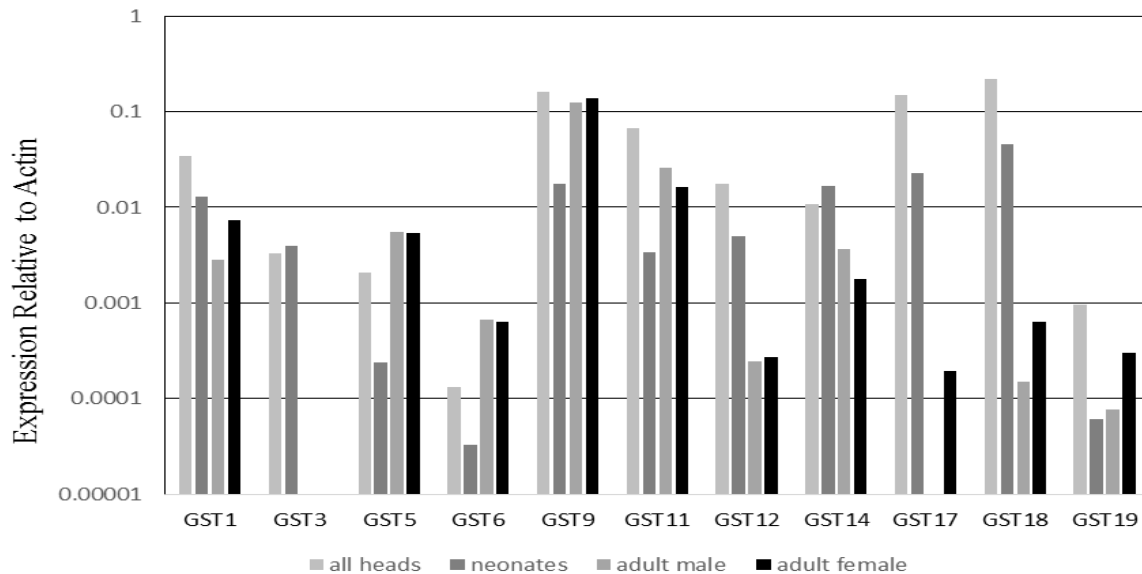

Figure 2. Relative expression of glutathione *S*-transferase transcripts in various life stages of codling moth as determined by quantitative real-time PCR. Expression levels of 11 CpGSTs relative to the Actin control.

Determining the basal expression levels for detoxification enzymes such as GSTs is an important first step in identifying potential roles of these enzymes in insecticide resistance. Detoxification enzyme mediated insecticide resistance usually occurs when the expression levels of one enzyme is substantially increased. Traditionally, microplate enzyme assays have been used in efforts to detect detoxification enzyme mediated resistance. These assays have often been found to be unreliable because they measure the activity of all isoforms of an enzyme in a single reading, even though resistance is usually the result of the overexpression of only one isoform. For example, when we perform a GST enzyme assay on codling moth extracts, we are measuring the activity of at least 26 unique GST proteins. The overexpression of just one of the GSTs may not be detected in a microplate assay. However, determining the expression levels of transcripts for individual GSTs (or other classes of detoxification enzymes) is more likely to provide us with the identity of the one enzyme isoform that is responsible for resistance.

Heat Shock Protein Expression

Insecticide resistance has also been correlated with expression levels of heat shock proteins. We have developed a qPCR assay to determine relative expression levels of three small heat shock proteins in codling moth. Relative expression levels of CpHsp19.8, CpHsp19.9, and CpHsp22.2, were determined for several developmental stages of codling moth (Fig. 3). In each stage, basal (without heat shock or insecticide treatments) transcript expression of all three CpHsps was detected, and ranged from 0.13 times that of CpAct for CpHsp19.8 in female pupae to 1.9×10^{-6} times that of CpAct for CpHsp22.2 in 3rd instar larvae (Fig.3). For CpHsp19.8, expression levels were significant between stages, with the highest expression found in pupal and adult females (Fig. 3), CpHsp19.9 expression was highest in 5th instars and adult males (Fig. 3). Significantly, very low expression of CpHsp22.2 was found in 3rd instars. These results provide us with an assay that can be used when trying to determine if heat shock proteins are involved in insecticide resistance mechanisms in codling moth.

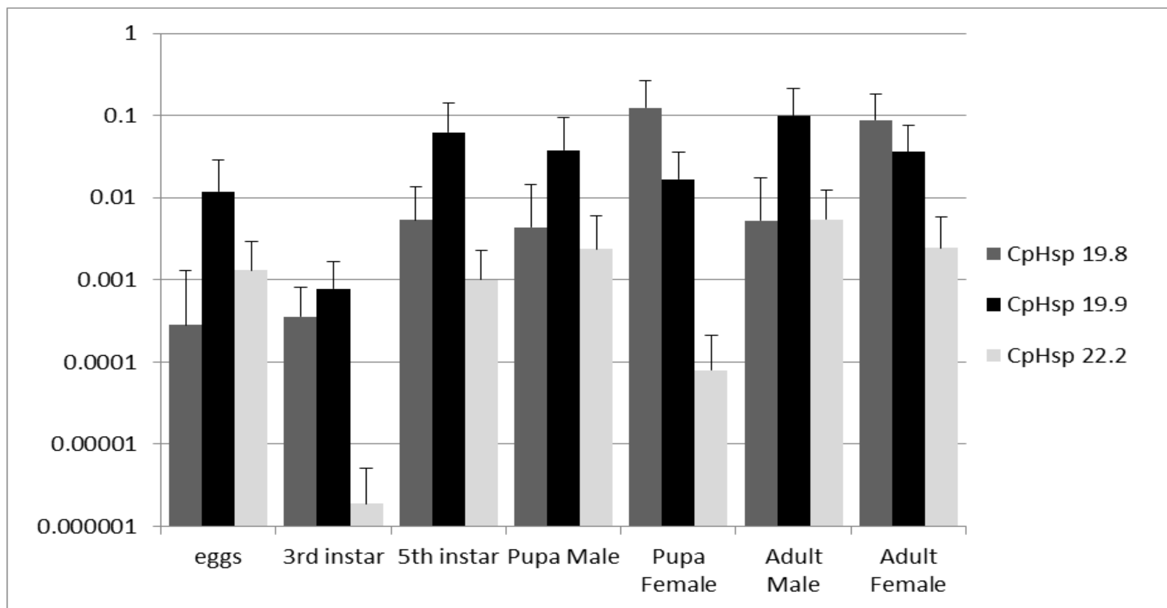

Figure 3. Relative expression of heat shock transcripts in various life stages of codling moth as determined by quantitative real-time PCR. Expression levels of CpHsp19.8, CpHsp19.9, and CpHsp22.2 relative to the Actin control.

EXECUTIVE SUMMARY

The use of neonicotinoids (Calypso), spinosyns (Delegate) and anthranilic diamides (Alatacor) have been effective in codling moth control programs. The possibility of codling moth becoming resistant to these control agents is of major concern to orchardists in the State of Washington. Insecticide resistance can occur by a couple of different mechanisms, target site resistance, and enzyme mediated detoxification. Therefore, the main goal of this project was to identify and characterize the protein targets of each of these insecticides, and to identify and characterize potential detoxification enzymes that could lead to resistance.

In previous WTFRC-funded projects, we prepared several transcriptomes from codling moth heads collected from all life stages. For this project, we used the transcriptome information to identify the protein targets of Calypso, Delegate and Altacor. Calypso and Delegate both target nicotinic acetylcholine receptors disrupting nerve function. Nicotinic acetylcholine receptors (nAChR) are nerve membrane proteins composed of five subunits. In this project, we identified, cloned and characterized 12 nAChR subunits (9 α, 3 β) expressed in codling moth heads. Point mutations and deletions in four of these subunits (α1, α3, α6 and β1) have been associated with field resistance to neonicotinoids and spinosyns. Analysis of clones of codling moth orthologs of nAChR subunits α1, α3, α6 and β1 revealed that there are currently no mutations present that would lead to target site resistance. We have developed PCR assays to monitor these subunits so that in the future if resistance occurs we can quickly determine if this resistance is caused by known target site alterations. The protein target of Altacor is the ryanodine receptor. From our transcriptome we were able to identify the codling moth ryanodine receptor. In diamondback moth, a point mutation has been associated with field resistance to anthranilic diamides. We cloned and sequenced the region associated with Altacor resistance and found that there are currently no mutations present that would lead to target site resistance. We are currently developing a PCR based assay to monitor codling moth ryanodine receptor so that in the future we can quickly determine if a point mutation is responsible for any increase in resistance.

Another potential resistance mechanism is through enzyme mediated detoxification of chemical control agents. The major classes of detoxification enzymes are carboxyl esterases, glutathione *S*-transferases and cytochrome P450 monooxygenases. From our codling moth transcriptomes, we have identified 50 transcripts encoding putative esterases, 80 transcripts encoding putative cytochrome P450s and 22 transcripts encoding putative glutathione S-transferases. We have cloned and sequenced 10 cytochrome P450 transcripts and 26 glutathione *S*-transferase transcripts. Using the sequence information from our cloned glutathione S-transferases, we have developed quantitative real time PCR assays to determine basal expression levels for these transcripts. We are currently treating insects with sublethal doses of Calypso, Altacor and Delegate so that we can determine if treatment with these chemicals has any effect on expression levels of the glutathione S-transferases. In the future, our qPCR assays will be used to quickly determine if resistant insects are using these enzymes to detoxify insecticides.

The results produced from this project have provided us with sequence information of protein targets and detoxification enzymes expressed by codling moth. Using this information, we have developed PCR based assays that will allow us to quickly determine potential insecticide resistance mechanisms in codling moth. Control of codling moth and other insect pests is critical for the production of clean fruit. We will continue to pursue this line of research so that in the future, should the need arise, we will be positioned to assist in analysis of codling moth that become resistant to chemical control agents.