Development of molecular diagnostic method for the diamide resistance in soybean looper
Project # 32-2023

2023-2024 MSPB Annual Report

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Background and Objectives

The soybean looper (*Chrysodeixis includens*) is one of the most serious pests in soybeans, migrating from southern latitudes up through Mississippi typically in August and September and consuming massive amounts of foliage in soybean. Since the introduction of the diamide insecticides such as chlorantraniliprole, the soybean looper has been well managed without any resistance issues. Diamides belong to a class of insecticides that target ryanodine receptors (RyR), the intracellular calcium channels that play an important role in muscle and nerve functions. However, soybean loopers have a long history of resistance to other insecticides such as pyrethroids, carbamates, and organophosphates. Therefore, diamide resistance in soybean looper is expected to develop, and growers should be prepared to manage loopers without diamides in the future. Resistance to diamides has already been reported in some lepidopteran pests, including diamondback moth, tomato leaf miner, rice stem borer and beet armyworm from different regions in the world. Although diamide resistance in soybean loopers hasn't been reported in the scientific literature yet, we have tested two collections from Puerto Rico that have had elevated resistance to a diamide in laboratory bioassays. Furthermore, diamide efficacy has been lower than expected at times in the southeastern USA, possibly due to loopers migrating from Puerto Rico. Therefore, it is important to regularly monitor populations in Mississippi in order to help farmers decide what kind of insecticide chemistries will be most efficacious. We have monitored soybean looper populations for resistance to diamides since 2017 by collecting larvae from the field, rearing them in the laboratory, and testing them with various concentrations of diamide insecticide. Although bioassays provide direct evidence of resistance, the bioassay method takes time (minimum of 1 month and typically 2-3 months) and rearing insects for 1 or more generations is expensive in labor and materials. However, if there are known molecular markers that distinguish the resistant and the susceptible strains, it would be faster and easier to detect resistance, because individual insects could be tested immediately upon collection. Also, the molecular method would not require live insects, but any developmental stage of the insect, or even any part of the body could be used to detect the resistance, because the resistance marker would be imprinted in DNA of every cell. Therefore, a molecular diagnostic method would provide a proactive detection tool for resistance and help farmers choose an insecticide without suffering from low efficacy due to resistance. Over the previous years, we screened two DNA sequence sites in the RyR gene using more than 250 individual samples from populations collected from Puerto Rico, Mississippi, and other southern states. Multiple bioassays were performed for their susceptibility/resistance to chlorantraniliprole. Additionally, we have established a resistant strain in the laboratory and completed a molecular cloning of the full-length of RyR gene. This project is a continuation to complete the following objectives. (1) Maintain a susceptible lab colony and the resistant strain of soybean looper from Puerto Rico, (2) Conduct diamide bioassays with synergists to test for metabolic resistance, (3) Perform enzymatic assays for candidate detoxification enzymes, (4) Evaluate a real-time PCR method to detect resistance marker genes, and (5) Monitor resistance in field populations with a molecular diagnosis method.

Report of Progress/Activity

<u>Objective 1: Maintain a susceptible lab colony and the resistant strain of soybean looper from Puerto Rico</u>
Following the loss of our resistant strain of soybean looper last year, we have been actively pursuing a new population from Puerto Rico to continue our genetic screening work. However, we have encountered

delays in the shipment due to a technical issue, despite successfully renewing the import permit. Nevertheless, we have worked closely with our local collaborator to coordinate the field collection of the Puerto Rico population. We anticipated that the collection of field strains and their subsequent shipment to Mississippi would be completed by December of 2023. Recently, we have successfully obtained a field population from Puerto Rico, comprising over 200 larvae. These populations have been reared in the insect rearing facility at Mississippi State University, where efforts were underway to establish a stable resistant strain. Concurrently, we are also maintaining a susceptible strain in the laboratory to serve as a reference control for our experiments. Despite the setbacks with the shipment delays, we are dedicated to continuing our research and are optimistic about the progress we will make once the new population from Puerto Rico is fully integrated into our study.

Objective 2: Conduct diamide bioassays with synergists to test for metabolic resistance

Before the loss of the resistant strain, we conducted a diamide bioassay, which revealed a mild resistance. To investigate the potential mechanism behind this diamide resistance, we conducted an induction experiment instead of a synergism experiment. This experiment involved applying 0.1 ppm technical grade chlorantraniliprole to both the resistant and susceptible strains. RNA samples from this experiment were then used for subsequent transcriptome analysis. The induction experiment yielded valuable insights. We identified several genes with significantly higher expression levels in the resistant strain of soybean looper larvae, particularly detoxification genes such as cytochrome P450s (**Table 1**). Considering that cytochrome P450s are primary targets for commercial synergists like PBO, we extended our gene expression analysis to include six different tissues. Our findings indicated elevated expression levels of numerous genes within the CYP6 family in the larval guts (**Figure 1**), strongly suggesting their involvement in metabolic resistance processes. This accomplishment laid the groundwork for further experiments related to Objective 3 (outlined below).

Objective 3: Perform enzymatic assays for candidate detoxification enzymes

Through the transcriptome analysis, we successfully identified several differentially-expressed genes involved in detoxification processes, including cytochrome P450 (CYP), UDP-glycosyltransferase (UGT), glutathione S-transferase (GST), and carboxylesterase/cholinesterase (CCE). In the resistant strain induced by chlorantraniliprole, we observed 12 up-regulated detox genes and 3 down-regulated detox genes. Furthermore, we found other up-regulated genes associated with cuticle formation, molting, energy production, and protein biogenesis, while approximately 100 genes associated with fatty acid synthesis, chemical communication, and the ryanodine receptor were down-regulated. Among these detoxification genes, our primary focus was on five CYP genes that showed significant upregulation. These genes were cloned and subsequently expressed in the Sf9 insect cell line for enzymatic assays. The five CYP genes underwent initial *in silico* annotation, providing insights into their structural attributes, including length, size, and amino acid sequences. Subsequently, each of these genes was successfully cloned into an expression vector (pIB/V5) and then transfected into the Sf9 cells for further experimental testing. Currently, we are actively investigating their biochemical and enzymatic activities to assess their capacity to degrade or modify the insecticide substrate, chlorantraniliprole. Out of the five up-regulated CYP genes in the resistant strain, two have been successfully cloned and expressed in Sf9 cells, generating proteins for the enzymatic assay. The heterologously-expressed proteins are now poised for harvesting to conduct *in vitro* tests with the insecticide compound, chlorantraniliprole. Meanwhile, the cloning and expression of the remaining three CYP genes are currently in progress to establish the Sf9 cell lines (**Table 2**).

Objective 4: Evaluate a real-time PCR method to detect resistance marker genes

A preliminary test using qRT-PCR was initiated on the five candidate CYP genes. Unfortunately, the relocation of the laboratory to a new building in November-December interrupted the experiment's continuity. The study will resume once the necessary equipment is re-installed in the new location. We have already prepared primers and reagents and are poised to proceed with the experiments as soon as the laboratory setup is complete.

Objective 5: Monitor resistance in field populations with a molecular diagnosis method

We recently identified a pigmentation gene (called *scarlet*) in the soybean looper, in order to develop the CRISPR/Cas9-mediated genome editing technique. Knocking out the *scarlet* gene resulted in significant changes in eye color (**Figure 2**). This marks the first successful application of the CRISPR/Cas9 technique in the soybean looper, establishing the gene as a distinctive genetic marker with potential applications across various aspects of insect research, including studies on insecticide resistance. This discovery not only confirms the gene's involvement in insect eye pigmentation but also paves the way for further CRISPR/Cas9-based genome editing investigations in the soybean looper. We recently published this finding in a peer-reviewed journal, reporting that leveraging the *scarlet* gene as a visual marker holds significant promise for advancements in fundamental biology as well as applied pest management strategies.

Impacts and Benefits to Mississippi Soybean Producers

Given the migratory nature of the soybean looper, it is imperative to conduct ongoing monitoring to ascertain its resistance status to pesticides, particularly in Mississippi. The rise in reported resistance cases in South America serves as a cautionary signal for Mississippians to remain vigilant regarding the potential development of resistance in this migratory pest, especially towards newer insecticides like diamides. Characterizing the genetic and molecular foundations of resistance is essential in developing an effective diagnostic method. The insights gained from our current findings will contribute significantly to the creation of a novel diagnostic tool. This tool will aid growers in making informed and strategic decisions regarding pest management across a wide expanse of Mississippi, ensuring more effective and sustainable practices.

End Products–Completed or Forthcoming

Presentations at conferences

Isbilir, S., Catchot, L., Musser, F. R, Ahn, S.-J. 2023. Revealing interactions between soybean loopers (*Chrysodeixis includens*) and chlorantraniliprole by transcriptome analysis. Mississippi Academy of Sciences - Summer Science and Engineering Symposium, July 25, Starkville, MS.

Isbilir, S., Catchot, B., Catchot, L., Musser, F. R, Ahn, S.-J. 2023. Transcriptome analysis of the soybean looper (*Chrysodeixis includens*) exposed to a diamide insecticide, Chlorantraniliprole. 12th Annual Mississippi Association of Entomologists, Nematologists, and Plant Pathologists Meeting. October 30-31, Starkville, MS.

Isbilir, S., Catchot, L., Musser, F. R, Ahn, S.-J. 2023. Transcriptome analysis of soybean looper, *Chrysodeixis includens*, provides insight into the potential resistance mechanism to diamide insecticide. Annual Meeting of Entomological Society of America, November 4-8, National Harbor, MD.

Isbilir, S., Catchot, B., Musser, F. R, Ahn, S.-J. 2023. Understanding potential resistance mechanisms to diamides in soybean loopers, *Chrysodeixis includens*, via transcriptome analysis. The 88th Annual Mississippi Academy of Sciences Meeting, February 29 - March 1, Hattiesburg, MS.

Publications

Isbilir, S. Catchot, B. Catchot, L. Musser, F.R. Ahn, S.-J. 2023. Molecular characterization and expression patterns of a ryanodine receptor in soybean looper, *Chrysodeixis includens*. ***Archives of Insect Biochemistry and Physiology*** 114(3), e22047. https://doi.org/10.1002/arch.22047 *(Published on August 21, 2023)*

Lee, S. Ahn, S.-J. 2024. CRISPR/Cas9-mediated knockout of scarlet gene produces eye color mutants in the soybean looper, *Chrysodeixis includens*. ***Archives of Insect Biochemistry and Physiology*** 115(3), e22100 https://doi.org/10.1002/arch.22100 *(Published on March 19, 2024)*

Graphics/Tables

Table 1. List of detoxification genes that were upregulated in the soybean looper larvae upon feeding on 0.1 ppm chlorantraniliprole.

Expression rank	Expression level (fold change)	Gene length (bp)	Gene description
1	9.55	1694	cytochrome P450 9e2-like
2	5.64	1759	cytochrome P450 6a14
3	4.66	2942	cytochrome P450 6B1-like
4	3.68	334	cytochrome P450 9e2-like
5	3.35	1365	glutathione S-transferase 1-like
6	3.22	1472	cytochrome P450 9e2-like
7	2.71	952	cytochrome P450 9e2-like
8	2.39	1574	UDP-glucuronosyltransferase 2B15-like
9	2.14	974	carboxylesterase B-1-like
10	2.06	861	glutathione S-transferase 1-like
11	2.02	609	glutathione S-transferase 1-like

Table 2. Current status of the functional assay experiments of the up-regulated CYP genes in heterologous expression system using Sf9 insect cell line.

Cluster name	CYP gene homolog	PCR status	Cloning status	Sf9 cell status	Assay status
Cluster-6496.19165	cytochrome P450 9e2-like	done	done	done	in progress
Cluster-6496.19026	cytochrome P450 6a14	done	done	done	in progress
Cluster-6496.34024	cytochrome P450 6B1-like	done	done	done	in progress
Cluster-5852.0	cytochrome P450 9e2-like	done	done	done	in progress
Cluster-2037.0	cytochrome P450 9e2-like	done	in progress	not yet	not yet

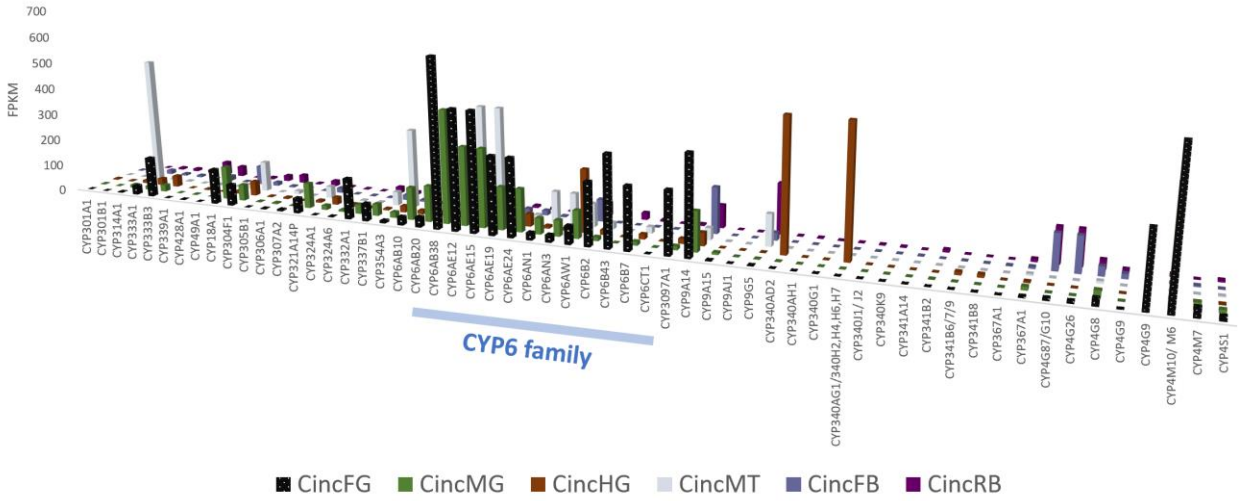

Figure 1. Expression levels of cytochrome P450 genes in six different larval tissues of the soybean looper (FG, foregut; MG, midgut; HG, hindgut; MT, Malpighian tubules; FB, fat body; RB, rest body). Noticeably, several genes within the CYP6 family exhibit significantly higher expression levels in comparison to other genes.

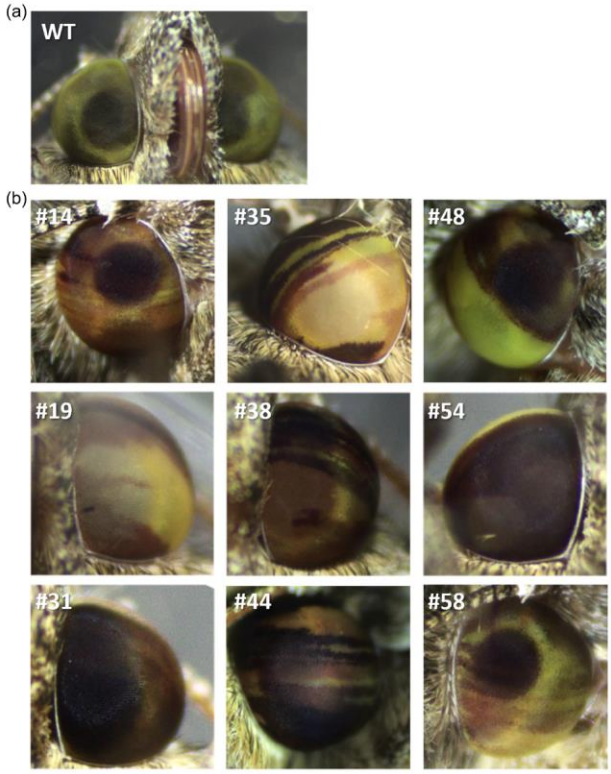

Figure 2. Eye color mutants identified in the G0 moths following CRISPR/Cas9-mediated mutagenesis of *scarlet* in *Chrysodeixis includens*. (a) Representative image of a wild type (WT) moth with the normal eye color. (b) Nine putative mutants exhibiting distinct eye colors when compared with WT.