GENE EDITING OF THE ABC TRANSPORTER/White LOCUS USING CRISPR/CAS9 MUTAGENESIS IN THE INDIAN MEAL MOTH (*Plodia interpunctella*)

By

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To my mother and father for their unwavering love and support
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<tr>
<td>ABC Transporter</td>
<td>ATP Binding Cassette Transporter protein</td>
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<td>ALP</td>
<td>Alkaline Phosphatase</td>
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<td>ANOVA</td>
<td>Analysis Of Variance</td>
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<td>APN</td>
<td>Aminopeptidase N</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>dNTP</td>
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<td>dsDNA</td>
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<td>G&lt;sub&gt;n&lt;/sub&gt;</td>
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<td>Homology Directed Recombination</td>
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<tr>
<td>kDa</td>
<td>KiloDaltons</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
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<td>µg</td>
<td>Microgram</td>
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<td>µM</td>
<td>Micrometer</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
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<tr>
<td>MDR</td>
<td>Multidrug Resistance</td>
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<td>NBDs</td>
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<td>NHEJ</td>
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<td>ORF</td>
<td>Open Reading Frame</td>
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<td>PAM</td>
<td>Protospacer Adjacent Motif</td>
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<td>PCR</td>
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<td>RT-qPCR</td>
<td>Quantitative Reverse Transcription Polymerase Chain Reaction</td>
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<td>sgRNA</td>
<td>Small Guide RNA</td>
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<td>SDS-PAGE</td>
<td>Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis</td>
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<td>UPGMA</td>
<td>Unweighted Pair Group Method with Arithmetic mean method of to produce an unweighted dendrogram</td>
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GENE EDITING OF THE ABC TRANSPORTER/White LOCUS USING CRISPR/CAS9 MUTAGENESIS IN THE INDIAN MEAL MOTH (Plodia interpunctella)  

By 
Bryce David Shirk  
May 2021  

Chair: Blair D. Siegfried  
Major: Entomology and Nematology  

ATP binding cassette (ABC) proteins are involved in transport of substrates across membranes. White, brown and scarlet genes code for ABC transporters that are involved in movement of ommochrome and pteridine precursors into insect eye pigment granules. Mutations of these genes result in observable eye color phenotypes and a white-eye strain of the Indian meal moth, Plodia interpunctella, (Piw-) has been identified. Null mutants created in Plodia white using CRISPR/Cas9 gene editing was employed to determine the impact on Bacillus thuringiensis (Bt)-derived Cry1Ac protein susceptibility. The white locus was identified and characterized in a genomic DNA assembly of P. interpunctella which extended for 17048 bp comprising 13 exons. Guide RNAs (sgRNA) were designed to target sites in exon 1 (sgRNA242) or exon 2 (sgRNA332). Microinjection of Cas9/sgRNA242 complex into newly laid Plodia wild type eggs produced 156 larvae. Of these, 81 eclosed as adults. Forty-five (56%) adults were wild type, while 26 females (32%) and 10 males (12%) showed full or partial white-eye phenotype (Pi_C9242w-). The white eye females were mated with Piw- males and 21 matings resulted in G_1 white-eye progeny. Thirteen Pi_C9242w- lines have been carried beyond G_{20} and were DNA sequenced demonstrating deletions and insertions.
mutations did not impart resistance to Cry1Ac suggesting that this ABC transporter gene differs from others involved in Cry1Ac mode of action and resistance. These studies demonstrate the utility of gene editing in this moth which can be used to characterize roles of genetic elements in acquired pesticide or toxin resistance.
Arthropod pests acquire pesticide resistance due to strong selective pressures resulting from recurring applications of insecticidal treatments intended to control these crop pests. Current estimates show that approximately 20% of all crop yields are lost due to arthropod pests infesting the five major crops worldwide, wheat, rice, maize, potato and soybean (Savary et al. 2019). Another 5%-10% of grain crops are lost in post-harvest storage (Perkin et al. 2016). With the projected expansion of the human population to 9.1 billion by 2050, the necessity for continued and expanded use of biopesticides is a critical component of future sustainability (Le Mouël and Forslund 2017). However, resistance to these biopesticides can erode their efficacy and diminish the benefits from employing them. Insecticide resistance can be established through a multitude of mechanisms that involve biochemical, physiological, genetic and ecological factors (Brattsten et al. 1986). One of the more important biopesticides where resistance has presented an emerging problem involves toxins derived from the soil dwelling Gram-positive bacterium, *Bacillus thuringiensis* (Bt).

**Review of BT-Toxin Derived Biopesticides**

Bt is a soil dwelling, spore-forming, Gram-positive bacterium that has been registered as an insecticide in the United States since 1961 and is the most commonly used biopesticide. With the advent of transgenic crops in 1996 (Adang et al. 2014; James 2017), the pesticidal proteins derived from Bt have made a significant contribution to pest management. There are many strains of Bt that upon ingestion by an insect, cause lethal pathology that ends with bacterial sporulation that facilitates the spread of the disease. Concurrent with sporulation, crystalline insecticidal proteins are
produced, including Cry (Crystal) and Cyt (Cytotoxic) toxins, pore-forming toxins (PFT). These toxins insert into the cell membranes of midgut cells creating pores, which kills the cells through osmotic shock (Adang et al. 2014; Canton et al. 2014). However, the complete mechanism resulting in toxicity is not fully understood.

**Resistance to BT-Toxin Biopesticides**

Resistance occurs at multiple levels of toxin action as identified from laboratory and field exposure to the various forms of Bt-toxins (Huang et al. 2014; Jurat-Fuentes and Crickmore 2017; Peterson et al. 2017; Tabashnik et al. 2009). The first report of Bt resistance was observed under laboratory conditions. Within fifteen generations of continuous feeding on Bt (bacterium) treated food, the Indian meal moth, *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae), showed significant resistance (McGaughey 1985). The first field population discovered to have acquired resistance was the diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae), exposed to the use of Bt microbial spray formulations in 1986 (Tabashnik et al. 1990). The appearance of evolved resistance to Cry1Ac used in transgenic crops was first reported in the corn ear worm, *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae), which displayed resistance to transgenic Bt cotton after only six years following widespread deployment of the plants (Tabashnik et al. 2005). There have been seven proposed stages in the mode of action of the three-domain Bt-toxins (including Cry1Ac) from ingestion through activation of toxin by midgut proteases, binding of toxin to receptors on the brush border membrane of midgut cells, to a conformational shift in the cell walls producing pores that lead to cell death (Jurat-Fuentes and Crickmore 2017). However, binding of the toxin and pore formation are the only widely accepted steps in toxicity, with the other stages in the mode of action for the array of Bt toxins being less known. The operational
paradigm has been that modification of any step could lead to resistance or a change in toxin specificity (Banerjee et al. 2017; Jurat-Fuentes and Crickmore 2017). The prevailing perspective is that mutations in the proteins produced in the midgut that are responsible for Cry toxin sensitivity, which includes aminopeptidase N (APN), alkaline phosphatase (ALP), cadherin (CAD) and ATP Binding Cassette (ABC) genes. The ABC genes are major receptor involved in Bt toxicity for specific toxins including Cry1Ac (Liu et al. 2018).

**ABC Transporters and the Role of ABCC2 in BT Susceptability**

There are various mechanisms for substrates to enter or exit cells through passive or active transport. One of the largest classes of active transporters is the ATP-binding cassette superfamily (Higgins 1992). This superfamily of transporters utilizes the binding and hydrolysis of ATP to control the translocation of a wide array of substrates, ranging from ions to macromolecules, back and forth across membranes (Rees et al. 2009). ABC transporters have a conserved architecture of four domains. Two transmembrane domains (TMDs) that are entrenched in the membrane bilayer, and two nucleotide-binding domains (NBDs) that are in the cytoplasm (Rees et al. 2009; Li et al. 2016) (Figure 1-1). The NBDs are responsible for binding ATP. The sequence of the TMDs are often variable, while the NBDs are highly conserved within the superfamily (Rees et al. 2009). Various ABC transporters can be involved in bringing nutrients and other molecules, such as drugs, into cells or can be responsible for the removal of drugs and toxins from the cell. Because of these roles, ABC transporters remain key players in resistance to some pesticidal proteins (Li et al. 2016).

The ABC transporter genes have been associated with insect species resistant to Cry1, Cry2 and Cry3 toxins (Banerjee et al. 2017). The major ABC transporter gene that
is responsible for resistance in Lepidoptera is in the subfamily C2, named ABCC2 (Liu et al. 2018). ABCC2 has been identified as a Cry1A receptor and facilitates toxin oligomerization and membrane insertion in susceptible insects. Therefore, disruption of expression of the ABCC2 gene disables one of the steps needed for the insecticidal protein to be functional and result in toxicity (Banerjee et al. 2017; Liu et al. 2018).

A significant test establishing a functional role for ABCC2 in Bt susceptibility was conducted in a heterologous system using *Drosophila melanogaster* Meigan (Diptera: Drosophilidae) (Stevens et al. 2017). The genome of *Drosophila* does not contain an ortholog of the ABCC2 gene and consequently *Drosophila* are not susceptible to Cry1Ac toxins. *Drosophila* were genetically transformed with the *P. xylostella* ABCC2 coding sequence regulated by *Drosophila* spp. promoters for either midgut or salivary glands and then tested for susceptibility to Cry1Ac, which was purified from the *Bacillus thuringiensis* strain HD73 (Stevens et al. 2017). *Drosophila* transformed with the PxABCC2 coding sequence that was expressed in larval midgut cells was 100 times more susceptible to Cry1Ac compared with non-transformed controls, while those that expressed PxABCC2 in the salivary glands were not susceptible (Stevens et al. 2017). This study demonstrates that the ABCC2 transporter is a critical component responsible for, Cry1Ac, susceptibility.

The importance of the cadherin and ABCC2 interaction to Cry1A susceptibility was addressed in Sf9 cell cultures, which are not susceptible to Cry1A toxins (Tanaka et al. 2017; Tanaka et al. 2016; Tanaka et al. 2013). To test the interaction, Sf9 cells were initially transformed with the *Bombyx mori* L (Lepidoptera: Bombycidae) ABCC2 gene alone. The transformed cells were then exposed to Cry1Aa and Cry1Ac which
produced a limited toxic response involving cell swelling. However, when the Sf9 cells were transformed with the *B. mori* cadherin like protein (CaLP) gene, another protein that has been associated with Cry toxin susceptibility, and subsequently exposed to Cry1Aa and Cry1Ac, there was very little effect on the cells (Tanaka et al. 2017; Tanaka et al. 2016; Tanaka et al. 2013). However, a synergistic effect was observed between the ABCC2 and CaLP proteins when the Sf9 cells were transformed with both genes; the toxic response was greater than in cells transformed with individual genes (Tanaka et al. 2013). The interaction of cadherin with *SIABCC3* in *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) the ovarian Li-HP cell line showed similar effects (Chen et al. 2015). Over expression of *SIABCC3* in Li-HP cells led to high levels of Cry1Ac sensitivity. Co-expression of *SIABCC3* with Hacadherin from *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) resulted in synergistic cytotoxic effects greater than either expressed alone. Using transformed cell lines to assess the impact of various mutations to cadherin, extracellular and intracellular domains of the protein were found to be critical for Cry1Ac cytotoxicity mediated by the *H. armigera* cadherin (Zhang et al. 2017). However, intracellular sequences of CaLP from *B. mori* did not contribute to the cytotoxicity of Cry1Aa and Cry1Ab to transformed Sf9 cell lines (Endo et al. 2018).

**White/ABC Transporter Gene**

The *White* gene is a fundamental cornerstone to modern day genetics, with its discovery transforming the field (Morgan 1910; Green. M 2010). In 1910, Thomas Hunt Morgan noticed a white-eyed male *Drosophila melanogaster* mutant, that strayed away from the expected wild type eye phenotype of red. From this, he designated this mutant gene *White* and through selective breeding experiments, was able to resolve that this gene resided on the X chromosome, which was the first concrete evidence for a gene
being localized to a specific chromosome (Green, M 2010). Since this discovery, eye phenotype has been a staple in the field of genetics for tracking mutations.

Subsequent research equated pigmentation in the eyes of *D. melanogaster* to the synthesis and deposition of drosoperins (red pigment), synthesized from guanine, and ommochromes (brown pigment), synthesized from typtophan, into the pigment cells in the organisms eyes (Summers et al. 1982). These pigment precursors need to be actively transported into the pigment cells and thus selective membrane transporters play a crucial role in eye pigmentation. The specific membrane transporters discovered for eye pigmentation in *D. melanogaster* and subsequently found in other arthropod species belong to the ABC transporter superfamily and encode *White, Scarlett* and *Brown* (Sullivan et al. 1974). Genetic and biochemical studies demonstrate that *White* forms a dimer with either *Scarlett* or *Brown* to form the tryptophan transporter (*White* and *Scarlett*) or the guanine transporter (*White* and *Brown*) (Dreeson et al. 1988; Tearle et al. 1989) (Figure 1-2).

Although, classically these ABC transporter genes were thought to only be involved in generating eye pigmentation in arthropods, there is evidence that these genes, highlighted by *White*, play a more dynamic role in arthropod physiology. These genes influence accumulation of pigments not only in the compound eyes but the ocelli, Malpighian tubules and general body tissues (Sullivan et al. 1979; Pirotta et al., 1985; Brent and Hull, 2019)

In *P. xylostella*, a naturally occurring mutant of a novel white ortholog, *Pxwhite*, led to transcript down regulation that resulted in Cry1A toxin resistance (Guo et al. 2015). Furthermore, RNA interference (RNAi)-mediated suppression of the *White* gene
in *P. xylostella* resulted in significantly reduced larval susceptibility to Cry1Ac, which was purified from Bt *var. kurstaki* strain HD-73 (Guo et al. 2015). This result suggests that additional studies are needed to illustrate any potential roles the white protein may play in Cry1A resistance in *P. interpunctella*.

**CRISPR-Cas9 Gene Editing**

Gene editing is a critical tool for establishing functional genomics to assess biochemical mechanisms for important metabolic pathways and can be used to identify specific genes associated with insecticide mode of action and resistance mechanisms. The Clustered Regularly Interspaced Short Palindromic Repeats/(CRISPR)-associated protein 9 (CRISPR/Cas9) system is a novel genome-editing tool that is less time consuming and labor intensive than zinc finger nucleases and transcription activator-like effector nucleases (Miller et al. 2007; Miller et al. 2011; Porteus and Baltimore 2003). The CRISPR/Cas9 system targets a specific DNA sequence of 2 to 6 base pairs termed the Protospacer Adjacent Motif (PAM) that is incorporated as a component of a small guide RNA (sgRNA) that directs the complex to a DNA site and leads to the cleavage of the double stranded DNA by the Cas9 nuclease (Sander et al. 2011) (Figure 1-3). The site-specific double-stranded DNA break is repaired either by non-homologous end joining (NHEJ) or by homology directed recombination (HDR) in the presence of donor template DNA. CRISPR/Cas9 has been used to precisely edit the genomes of numerous eukaryotic organisms (Cho et al. 2013; Cong et al. 2013; Ding et al. 2013; Hwang et al. 2013; Ran et al. 2013; Wood et al. 2011) including insects in the orders Coleoptera (Gilles et al. 2015), Diptera (Bassett et al. 2013a; Hall et al. 2015), and Lepidoptera (Li et al. 2015; Wang et al. 2013; Wei et al. 2014). The CRISPR/Cas9 biotechnology has led to functional genomic studies of model and non-model species.
and the development of novel genetics-based pest control approaches (for review, see (Taning et al. 2017)).

**Model Organism: Plodia interpunctella**

The model organism used in this study, Indian meal moth, *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae) is a major economic pest of stored products and processed foods world-wide (Mohandass et al. 2007). Control of this pest has become more difficult with the selection of resistance to most chemical pesticides. This resistance has extended to biopesticides in bioengineered crops. The first observation of evolved Bt resistance was found under laboratory conditions where continuous feeding of *P. interpunctella* on Bt treated food led to significant resistance within fifteen generations (McGaughey 1985). The utilization of gene editing in this moth will provide a means of conducting functional genomics in the moth as well as lead to new avenues to identify new targets for control.

**Hypothesis**

Gene editing to create mutations in the *white* ABC transporter in the Indian meal moth, *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae), will produce phenotypes resulting in Cry1Ac resistance that mimic naturally occurring resistance.

**Research Objectives**

To validate CRISPR/Cas9 gene editing and to assess its role in Cry1Ac resistance, the *white* gene in *P. interpunctella* was selected as the initial target. The *white*, *brown* and *scarlet* genes code for ABC transporter proteins which are primarily involved in the movement of ommochrome and pteridine pathway precursors into pigment granules in the eye (Bretschneider et al. 2016; Denecke et al. 2017; Ewart and
Dimerization of White and Scarlet proteins in *D. melanogaster* (Mackenzie et al. 1999) and the silk moth, *B. mori* (Osanai-Futahashi et al. 2016; Quan et al. 2002; Tatematsu et al. 2011), leads to the transport of the ommochrome precursors while dimerization with Brown protein affects transport of the pteridine precursors. Null mutations of the *white* gene result in distinct observable eye color phenotype and *white* was selected as the initial target for gene editing in *P. interpunctella*.

The objectives of this research explored whether CRISPR-Cas9 gene editing is a viable technique to study the functional genomics of insecticide resistance in alepidopteran pest. These objectives were:

1. Utilize CRISPR-Cas9 gene editing in *P. interpunctella* to cause a loss of functionality to the ABC transporter, *white*.
2. Isolate somatic CRISPR-Cas9 mutants with a white eye phenotype and mate them to confirm germ line mutation.
3. Characterize the created indels, demonstrating the utility of gene editing in this moth.
4. Perform a bioassay on the *white* mutant strain to determine susceptibility to the Cry1Ac protoxin.
Figure 1-1. A cartoon representation of the mode of action an ABC transporter undergoes to move a substrate (Li et al. 2016).

Figure 1-2. A graphic representation of intramembrane proteins encoded by the White and Brown genes of D. melanogaster (Mackenzie et al. 1999).
Figure 1-3. A graphic representation of CRISPR/Cas9 gene editing. Mechanism of CRISPR/Cas9 system in genome editing. Cas9 recognizes PAM and binds to the target DNA fragment through base pairing between the crRNA and DNA. During interaction with the Cas9-gRNA complex, double-stranded DNA dissociates next to PAM, followed by the crRNA-DNA base paring. The crRNA-DNA binding activates the Cas9 endonuclease function, which generates breaks in both DNA strands by the HNH and RuvC domains. The double-strand break is fixed by either non-homologous endjoining (NHEJ) for deletion or insertion of variable numbers of nucleotides or by homology-directed recombination (HDR) when a DNA template is present for precise insertion. (Taning et al, 2017).
CHAPTER 2
CRISPR/CAS9 GENE EDITING OF WHITE IN Plodia interpunctella:
TREATMENT AND ANALYSIS

Introduction

Genome editing is a critical tool for establishing functional genomics to assess biochemical mechanisms for metabolic pathways involved in metabolizing toxins or insecticides in pest insects. The clustered Regularly Interspaced Short Palindromic Repeats/(CRISPR)-associated protein 9 (CRISPR/Cas9) system is a novel genome-editing tool with advantages over techniques such as zinc finger nucleases and transcription activator-like effector nucleases that are typically more time consuming and labor intensive (Miller et al. 2007; Miller et al. 2011; Porteus and Baltimore 2003; Sander et al. 2011; Wood et al. 2011). The CRISPR/Cas9 system targets and cleaves a specific DNA sequence using the Cas9 nuclease coupled with a single guide RNA (sgRNA) (Sander et al. 2011). The site-specific double-stranded DNA break is then repaired either by non-homologous end joining (NHEJ) or by homology-directed recombination (HDR) in the presence of a donor DNA template. The CRISPR/Cas9 system has been used to precisely edit genomes of numerous eukaryotic organisms (Cho et al. 2013; Cong et al. 2013; Ding et al. 2013; Hwang et al. 2013; Ran et al. 2013; Wood et al. 2011) including the insect orders Coleoptera (Gilles et al. 2015), Diptera (Bassett et al. 2013a; Hall et al. 2015), and Lepidoptera (Li et al. 2015; Wang et al. 2013; Wei et al. 2014). The CRISPR biotechnology has facilitated functional genomic studies of model and non-model species and has the potential to provide insight into novel genetics-based pest control approaches (for review, see Tanning et al. (2017)).
In order to conduct gene editing, it is essential to have a complete understanding of the DNA sequences that are to be modified (Graham and Root, 2015). This requires having reliable genomic sequence information from the organism as well as transcript sequences so that the design of target sites can be made that avoid crossover interference with intron/exon boundaries or variable regions within alleles. The analysis of the target site mutations requires the production of PCR primers that flank the target site but do not become subject to alteration by the repair mechanisms. There are numerous bioinformatic resources available for the analysis of the genomic DNA sequences as well as production of PCR primers and identification of CRISPR/Cas9 PAM sites.

The Indian meal moth, *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae) is a major economic pest of stored products and processed foods worldwide (Mohandass et al. 2007). Control of this pest has become more difficult with increasing prevalence of individuals and populations with resistance to most chemical pesticides. Resistance has also been observed against biopesticides in bioengineered crops. The utilization of gene editing in this moth will provide a means to assign gene function as well as provide new avenues for clarifying biochemical and physiological pathways unique to insects that may prove useful as novel target sites for insect control.

To validate CRISPR/Cas9-mediated gene editing in *P. interpunctella*, the white gene was selected as the initial target. The *white*, brown and scarlet genes encode for ABC transporter proteins that are involved in the movement of ommochrome and pteridine pathway precursors into pigment granules in the eye (Bretschneider et al. 2016; Denecke et al. 2017; Ewart and Howells 1998; Heckel 2012; Khan et al. 2017).
Dimerization of White and Scarlet proteins in *Drosophila melanogaster* (Mackenzie et al. 1999) and the silk moth, *Bombyx mori* (Osanai-Futahashi et al. 2016; Quan et al. 2002; Tatematsu et al. 2011), lead to the transport of the ommochrome precursors while dimerization of White with Brown protein affected transport of the pteridine precursors. These studies showed that mutations within the *white* locus are typically non-lethal although this is not the case for all Lepidoptera (Li et al. 2015; Wang et al. 2013; Wei et al. 2014). For example, gene editing of the *white* locus in *Helicoverpa armigera* with CRISPR/Cas9 led to an embryonic lethal phenotype while gene-edited mutation of the *brown* and *scarlet* loci produced viable offspring with the respective eye color phenotypes (Khan et al. 2017).

**Experimental Procedures**

**Insect Strains**

The Indian meal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) wild type (collected from a wild population in Gainesville, Florida; 2014), *Piw−*, white eye mutant, which was isolated in Dr. Paul Shirk’s lab at USDA-ARS CMAVE (Unpublished, 1986) and CRISPR Piw−242 strains at CMAVE were reared according to Silhacek and Miller (1972) in a 16 h light : 8 h dark cycle at 30 °C and 70% relative humidity.

**Genomic Organization of the White Locus in Plodia interpunctella**

A genome assembly of *P. interpunctella* (Unpublished) was generated by Dr. Erin Scully USDA-ARS and shared in collaboration. The nucleotide sequence for the entire *P. interpunctella White* locus, including 150bp up- and down-stream of the coding region, was submitted to GenBank under accession number MN379839.
CRISPR/Cas9-Mediated Gene Editing of White

Based on the genomic sequence for *P. interpunctella white* sgRNA candidate CRISPR-PAM sites within the exon I region of the *P. interpunctella white* gene were designed using ZiFiT 4.2 (Sander et al. 2010; Sander et al. 2007). The highly ranked sgRNA candidates were further evaluated for potential off-target effect using MacVector Align-to-Folder searches applied to the *P. interpunctella white* genome sequences. Candidate sgRNAs that matched to genomic regions other than *P. interpunctella white*, which perfectly matched the final 12 nucleotides of the target sequence and NGG PAM (protospacer adjacent motif) sequence, were discarded (Cong et al. 2013). One candidate that targeted nucleotides 242 to 264 of the *P. interpunctella white* transcript (exon I) was chosen for the current study.

A technique to synthesize sgRNAs (Bassett and Liu 2014; Bassett et al. 2013b) was modified for the production of sgRNA242 (Figure 2-1). Briefly, sgRNA templates were made by amplifying overlapping forward and reverse primers containing a T7 promoter and stem loop structures needed for Cas9 binding (see Table 2-1 for primer sequences) with Phusion High Fidelity DNA polymerase (New England Biolabs, Ipswich, MA) in 50 µl reactions containing 50 mM of Tris (pH9.2), 16 mM of ammonium sulfate, 1.75 mM of MgCl₂, 350 nM of each dNTP, and 0.5 µM of forward and reverse primers. PCR was conducted with three linked profiles in a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA): (1) one cycle consisting of denaturation at 98 °C for 30 s; (2) thirty-five cycles each consisting of denaturation at 98 °C for 30 s, annealing at 60 °C for 30 s and extension at 68 °C for 15 s; (3) one cycle consisting of polishing at 68 °C for 10 min. The amplified PCR products were analyzed on 2% agarose gels containing
ethidium bromide and bands of expected size (125 bp) were excised and purified using a Gel Extraction kit (Qiagen) according to the manufacturer’s protocol. sgRNA242 was synthesized (4 h at 37 °C) using 800 ng of the purified PCR product as template with a MEGAscript T7 kit (ThermoFisher) and purified according to manufacturer’s instructions. Concentrations of purified sgRNA242 were determined using a NanoDrop 2000 (ThermoFisher). Purified sgRNA242 was stored in elution buffer at −80 °C until used.

The Cas9/sgRNA242 complex was produced by mixing 0.5 μg/μl sgRNA242 with 1.0 μg/μl Cas9 (Integrated DNA Technologies, Coralville, IA) and incubated for 10 min at room temperature.

**Embryo Microinjections**

Microinjections of the Cas9/sgRNA242 solutions into CMAVE *P. interpunctella* wild type eggs were conducted as described previously for DNA solutions (Bossin et al. 2007; Shirk et al. 2007). To achieve optimal exposure of the embryonic nuclei to the Cas9/sgRNA242, the eggs were collected within 10 min after deposition. Syncytial embryos were mounted on cellophane (BioRad; Hercules CA) strips without dechorionation, microinjected with Cas9/sgRNA242, and then sealed with Superglue at the site of injection (Ontario, CA) within 20 minutes of deposition (Figure 2-2). This procedure resulted in 25-38% embryo hatch rates but assured that embryonic nuclei were exposed to the gene editing complex at point in development when only a limited number of nuclei were present so that the gene editing would have the maximal probability on producing germline mutations. Following injection, the embryos of *P. interpunctella* were placed in modular incubators (Billups-Rothenberg, Inc., Del Mar CA USA) at 23 °C and 95% humidity until hatching.
The pupae and adults were scored for white phenotype using a M165 FC fluorescent stereozoom microscope (Leica Microsystems, Buffalo Grove IL). Digital micrographs were made using a Leica DMC 4500 camera using LASX software (Leica Microsystems).

**Quantitative Reverse Transcription PCR (RT-qPCR)**

Total RNA was extracted from the heads of pharate adult *P. interpunctella, Piw-* and *Piw-242* CRISPR strains using a Ribopure kit (Ambion|ThermoFisher, Carlsbad CA) according to the manufacturer’s instructions. cDNA was produced from 1 µg total RNA extract for each treatment using an AffinityScript QPCR cDNA synthesis kit (Stratagene|ThermoFisher, La Jolla CA) as directed by the manufacturer. RT-qPCR was conducted on cDNAs using IQ SYBER green mix (Bio-Rad) with white primers (Integrated DNA Technologies) PiWh-RT1F and PiWh-RT1R. For reference transcripts, β-actin primers (Pi_Bac3F and Pi_Bac3R) and ribosomal protein 7S (Pi_RPS7AF and Pi_RPS7AR) transcript levels were also determined. Three independent biological samples were run in triplicate with triplicate RT-qPCR reactions conducted from each biological sample. RT-qPCR reactions were run in a C1000 Touch Thermal Cycler controlled by a CFX96 Real-Time System (BioRad) programmed for 35 cycles of 97° C, 30 sec; 56° C, 30 sec; 72° C, 30 sec. Quantitation of transcript copy number was based on comparison with a standard dilution series of a quantified plasmid containing the corresponding DNA sequence. For all plasmids used as standards, the linear regressions had $r^2$ greater than 0.9 and efficiencies of 100% (±5%). To assure that there was no interference from DNA carried over from the RNA extract, reactions were made using the RNA extract and none contained more than 0.1% of the cDNA measured. The relative levels of a specific transcript were standardized as a ratio to the geometric
mean of the reference transcripts (Vandesompele et al. 2002). The data were analyzed using the General Linear Model procedure using R (Team 2013). Tukey’s studentized range test was used to separate treatment means in those cases where the treatment analysis of variance (ANOVA) F was significant (P <0.05).

**Genomic DNA Extraction and Identification of Piw-242 CRISPR Mutations**

Genomic DNA was extracted from adult hind legs using a method adapted from a protocol of the DNeasy kit (Qiagen). Briefly, the samples were homogenized for 30 s in a 2 ml Eppendorf tube using an Omni TH tissue homogenizer (Omni International, Kennesaw, GA). After placing the sample on ice, 180 μl of ATL buffer and 20 μl of proteinase K solution were added. The sample was then incubated at 56º C for 10 min. After vortexing the sample for 15 s, 200 μl of AL buffer was added, followed by incubation at 56º C for 10 min. Following 3 min of centrifugation at 14,000 rpm at 25º C, the supernatant was transferred to a new 1.5 ml tube. Two hundred μl of 100% ethanol was added to the supernatant and the mixture was added to a DNeasy Mini spin column sitting on top a 1.5 ml collection tube. The genomic DNA was retained on the column after a 1 min centrifugation at 14,000 rpm. The columns were then washed with 500 μl of AW1 and AW2 buffer. The spin columns were transferred to a new 1.5 ml tube and 50 μl of AE buffer was added. After incubation at 25º C for 1 min, the genomic DNA was recovered by a 1 min centrifugation at 14,000 rpm. Concentrations of purified genomic DNA were determined using a NanoDrop 2000. Purified genomic DNA was stored at −20º C until used.

Primers for Exon 1 (PiWh-1F-133 and PiWh-5R-791) of *P. interpunctella* were designed to produce a 695 bp product that centered on the 242 PAM site. The PCR products were cloned into pGem-T Easy (Promega, Madison, WI) and sequenced.
Sequences were analyzed and aligned using MacVector V17.0.5.

**Cry1Ac Bt-Toxin Bioassay**

The Cry1Ac protoxin was produced essentially as described by Grove et al. (2001). Cry toxin was prepared from fermentation of recombinant *Escherichia coli* strain that was transformed with the plasmid ECE53 that expresses Cry1Ac. The ECE53 plasmid was obtained from the Bacillus stock center (Cry1Ac cloned in pKK223-3; in E. coli JM103; ampicillin resistant). The ECE53 plasmid was transformed into *E.coli* Mach1 (ThermoFisher, CA) and grown in 500 ml of Superbroth medium (Lech and Brent 1992) that has been supplemented with 2 g/l D-glucose and 50 µg/ml ampicillin in a shaking incubator set at 28° C for 10 days. The bacteria were collected by centrifugation at 11,000 x g and resuspended in 50 ml lysis buffer containing 49.5 mM tris base (tris (hydroxymethyl)aminomethane, Sigma), 55.3 mM EDTA (ethylenediaminetetraacetic acid, Sigma), 438 mM sucrose, and 0.1 g lysozyme (EC3.2.1.17, Sigma) and adjusted to pH 8.0 with 10 N HCl. Subsequently, the resuspended bacteria were incubated at room temperature for 12-14 h and stored at -20° C. The bacterial mixture was then sonicated in an ice water bath for 3 five-minute periods to completely lyse the cells. The lysed bacteria were pelleted and washed 3 times in 150 ml of 0.5% Triton X-100 in 0.5M NaCl (Sigma), 5x in 0.5M NaCl, followed by a final 3 washes in deionized water. After washing, the cell contents were pelleted and stored at -20° C.

The total protein concentration of the protoxin sample was quantified using the Pierce BCA Protein Assay Kit in accordance with the manufacturer’s instruction (ThermoFisher, CA) and the stock Cry1Ac extract was 442 mg/ml of total protein/ml. An SDS-PAGE was performed on the protein sample using the concentration determined in
the protein assay in accordance to the manufacturer’s instruction with a 10% Mini-
PROTEAN TGX Stain-Free Protein Gel (BioRad, Hercules, CA). The standard ladder
run with the sample was the Precision Plus Protein Dual Band (BioRad, Hercules, CA).
Visualization and imaging of the protein bands was performed using a Chemidoc MP
system (BioRad, Hercules, CA) which showed the presence of a 133 kDa Cry1Ac
protoxin band (Figure 2-3).

A bioassay to establish sensitivity to Cry1Ac protoxin was conducted for P. interpunctella wild type, Piw- and the CRISPR/Cas9 white mutants. The bioassay was
performed with individual P. interpunctella larvae housed in separate wells of a 96-well
culture plate. Each larva was provided 2-4 flakes of standard Plodia diet that were
treated with deionized water, 0.02 µg/well Cry1Ac protoxin or 2.21 µg/well Cry1Ac
protoxin, diluted in deionized water. A newly emerged P. interpunctella larva was placed
in each of 40 wells per treatment and covered with Breathe-Easy sealing membrane
(Sigma-Aldrich) and then monitored for growth at 7 and 14 days after initiation of the
bioassay. Caterpillers were checked for activity based on frass production or evidence
of silk production or mortality. On the 7-day inspection mortality was denoted if there
was no observed activity as described above and on the 14-day inspection mortality
was denoted if after 14 days there was not a 4th instar larva in the well. The data was
analyzed using the regression model found in the Analysis ToolPak on Microsoft Excel
365, where the treatment analysis of variance (ANOVA) was calculated based on
percent mortality. F statistic was significant at P<0.05.
Results

Genomic Organization of the Plodia White Locus

The white locus of *P. interpunctella* extends over 16,670 base pairs (bp) and the 3,030 bp transcript is comprised of 13 exons (Figure 2-5) which produces a putative 686 amino acid protein. The genomic organization of the white locus is similar to that of other Lepidoptera where the transcript is comprised of 13-14 exons extending over 16-26 kilobases.

A UPGMA dendrogram was constructed based on the complete putative amino acid sequences of the White protein from Lepidoptera and members of other orders as out groups (Figure 2-6). The putative amino acid sequence for *P. interpunctella* white shows the highest similarity (97.66%) with White from *Amyelois transitella* (XP_013190063.1), another pyralid moth. However, White from *Galleria mellonella* (XP_026757709.1), also a pyralid, has a lower similarity (91.69%) and is not grouped with these two pyralids in the phenetic dendrogram but is interceded by species from four other families of Lepidoptera. The alignment of the putative amino acid sequence for *P. interpunctella* white with that of the *P. xylostella* Pxwhite shows significant differences in the amino terminus with 35 of the first 100 amino acids mismatched or gapped which accounts for 45% of the differences over the full length of the sequences (Figure 2-7).

CRISPR/Cas9-Mediated Mutagenesis of the Plodia White Locus

Candidate guide RNAs (sgRNA) were identified by scanning the genomic sequence of the *P. interpunctella* white locus with ZiFit (Sander et al. 2010). CRISPR-PAM target sites were found throughout the sequence and one was selected from exon
1 (sgRNA242) for subsequent testing (Table 2-1). Guide RNA (sgRNA242) was produced according to Bassett and Liu (2014) and the Cas9/sgRNA242 complex was microinjected into newly laid *P. interpunctella* wild type eggs ($\leq$ 20 min). From 586 microinjected embryos, 156 larvae hatched (27%). Larvae were collected and placed directly on diet, but only 81 eclosed as adults (52%). Forty-five (56%) of the adults had wild type phenotype, while 26 females (32%) and 10 males (12%) of the G₀ adults showed full or partial white eye phenotype (Figure 2-8).

To select for germline transmitted mutations, the G₀ females showing the white eye phenotype were mated with males of the *P. interpunctella* (*Piw*- ) strain that exhibits the white eye phenotype that was previously isolated (Unpublished, 1986). Each of the 26 G₀ white eyed females were mated with two *Piw*- males. Five of the matings did not produce offspring while the remaining 21 matings of sgRNA242 white eyed females crossed with *Piw*- males resulted in white eye F₁ progeny (*Piw*-242). Thirteen of the *Piw*-242 strains were maintained through to G₃₈.

**Analysis of CRISPR/Cas9 Indel Mutations of the Plodia White Locus**

The DNA sequences surrounding the 242 PAM site in *Piw*-242 strains were compared with those from *P. interpunctella* wild type and *Piw*- strains (Figure 2-9). The sequences for the CGAHR-SPIERU wild type and *Piw*- strains were identical. However, there were differences at bases 12 and 92 when compared with the CMAVE *P. interpunctella* wild type strain. There was a deletion of a G at position 12 and a T substitution for an A at position 92, which provided convenient markers for identifying the parental origin of the sequences. PCR products from the strains were cloned and sequenced. Deletions of 1 to 24 bases were found in eight of the sequences shown from the different strains examined (Figure 2-9A). The 242B-BL0 strain carried at least 3
deletions and one large insertion (Figure 2-9B). Four of the sequences recovered from the 242B-BL0 did not exhibit altered sequences around the 242 PAM site. Because these sequences originated from a strain that had both white and black eye progeny, these sequences likely represent non-mutated alleles of the CMAVE *P. interpunctella* wild type parent.

The impact of the mutations on the amino acid sequence was assessed by aligning the putative translation products from these sequences (Figure 2-10). Five of the mutations resulted in in-frame deletions of the amino terminus (2ap, 3ap, 5ap, B-BLG0-2ap, and B-BLG0-3ap). Three of the mutations resulted in early translational termination for the sequence (6ap, B-BLG0-1ap and B-BLG0-9ap). The 1ap sequence resulted in a novel peptide that continued in frame into the adjoining intron.

**RT-qPCR determination of white Transcript Levels in CRISPR/Cas9 Indel Mutations of the *Plodia white* Locus**

The *white* transcript levels in the CRISPR wild type and mutant strains that exhibited the white-eye phenotype were assessed by RT-qPCR to assess transcript abundance. When compared with the CMAVE *P. interpunctella* wild type strain, the *Piw*-strain as well as most of the *Piw*-242 allele strains had significantly lower levels of transcript (Figure 2-11; \( p \leq 0.05 \) experiment-wise error rate (Df=14). Type I error rate was set at \( \alpha=0.05 \)). The *Piw*-242-1-2 strain that had both white and black eye progeny had transcript levels that were not different from the wild type strain. However, even though the *Piw*-242 A13 and B10 strains had a white-eye phenotype the transcript levels were intermediate to those of the wild type and *Piw*- strains. This result suggests that a non-functional transcript was produced in these two strains.
Sensitivity of *Plodia* to Cry1Ac Protoxin and the Influence of White Locus Mutations

Newly hatched caterpillars from *P. interpunctella* wild type, *Piw-* and three *Piw-\textsuperscript{242}* were placed on diet treated with either 0.02 µg/well or 2.21 µg/well Cry1Ac toxin and allowed to feed at will. On 7-day, there was less total activity, as determined by frass and silk production, in the Cry1Ac treated groups when compared with the untreated groups for all *Plodia* strains tested (Figure 2-12A). The wild type *Pi+*, and the naturally occurring white eye mutant, *Piw-*, had 32.5% and 35% mortality in the untreated group respectively, while 90% mortality in the 2.21 µg/well toxin treated group for both groups and in the 0.02 µg/well toxin treated group 80% and 85% mortality, respectively. However, the F statistic did not show there was a significant difference between the three treatment groups, (0.0558 and 0.6092, respectively). In the three *Piw-\textsuperscript{242}* strains, *Piw-\textsuperscript{242-8}*, *Piw-\textsuperscript{242B1-3}*, and *Piw-\textsuperscript{332-2}*, tested there were similar results. For the untreated samples there was 17.5%, 52.5% and 35% mortality, respectively. For the 2.21 µg/well treated group there was 80%, 95% and 90% mortality, respectively. For the 0.02 µg/well treatment group there was 95%, 97.5% and 85% mortality, respectively. There was no statistical significance between mortality and treatment for any of these groups.

At 14-day after initiation, it was clear which individuals survived compared with those that had initial feeding, but subsequently perished due to the treatment. Most of the caterpillars that produced frass and were counted as alive in the 7-day inspection had subsequently died (Figure 2-12B). The *Pi++* and *Piw-*, had 37.5% and 47.5% mortality in the untreated group, respectively, while in the 2.21 µg/well toxin treated group 100% and 97.5% mortality, respectively, and in the 0.02 µg/well toxin treated group 100% mortality for both groups. In the three *Piw-\textsuperscript{242}* strains, *Piw-\textsuperscript{242-8}*, *Piw-\textsuperscript{242B1-3}*
and Piw-332-2, tested there were similar results. For the untreated samples there was 57.5%, 80% and 60% mortality, respectively. For the 2.21 µg/well treated group there was 95%, 100% and 97.5% mortality, respectively. For the 0.02 µg/well treatment group there was 97.5%, 100% and 100% mortality, respectively. There was no statistical significance between mortality and treatment for any of these groups.
<table>
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<td>GGGCGGTGATTTCCTTCTG</td>
</tr>
<tr>
<td>Sequecing of the Piw genomic fragment flanking the sgRNA242 target region</td>
<td></td>
</tr>
<tr>
<td>PiWh-1F-133</td>
<td>TGAAGTGACGTTTCACGTAAT</td>
</tr>
<tr>
<td>PiWh-5R-791</td>
<td>ATGTCGATCCATACGCACT</td>
</tr>
</tbody>
</table>

Table 2-1. sgRNA sequences for CRISPR/Cas9 mutagenesis and PCR primers for analysis of the *P. interpunctella white* locus and transcripts.
Figure 2-1. A graphic representation of the production of template DNA for in vitro transcription of sgRNA. DNA target site is indicated in orange, and PAM sequence in red. CRISPR Forward primer containing the T7 promoter (blue), target site (orange) and overlap with CRISPR sgR primer (purple) is shown. PCR is used to generate the template for in vitro transcription of the sgRNA. T7 polymerase transcription start site is indicated by an arrow, and mature RNA sequence is shown (Bassett and Liu, 2014).
Figure 2-2. Microinjection of newly laid eggs of *Plodia interpunctella*: microneedle pulled to an outer diameter of less than 1 µM (Panel A). Eggs are aligned and adheared to a strip of cellaphane (BioRad, Hercules, CA) with BSA (Panel B); microneedle filled with the transforming solution and used to puncture the egg in the posterior region (opposite of the micropyle) for delivery of the transforming solution (Panel C). Photo courtesy of Bryce Shirk. *Microinjection of P. interpunctella Embryos*. January 2020.
Figure 2-3. SDS-PAGE analysis of Cry1Ac protoxin extract. Lane A, 10-fold dilution of the Cry1Ac protoxin extract. Lane B shows Cry1Ac protoxin extract. Lane C, Precision Plus Dual Color protein standard. Arrows on the right point to the major bands of molecular mass markers. The arrow on the left points to the predicted position of the 133 kDa Cry1Ac protoxin. Photo courtesy of Bryce Shirk. October 2020.
Figure 2-4. Graphic representation of the genomic sequence of the white locus in *Plodia interpunctella*. The continuous line shows the 20,000 base pair region of the locus. Solid blocks above the line represent the position of the Exons (E1-E13). TATAAAT designates the position of transcription initiation sites. AUG designates the sequence position of the methionine translation initiation site.
Figure 2-5. Graphic representation of the white gene transcript in *Plodia interpunctella*. The continuous line shows the length of the 3,300 nucleotide base sequence of the transcript. The solid black arrow designates the position of the open reading frame (ORF) for the white protein. The diagonal hash arrows show the position of the exons (E1-E13).
Figure 2-6. Phenetic associations of white/ABC transporter proteins based on deduced amino acid sequences. The phenetic dendrogram shows an UPGMA bootstrap consensus (Poisson model; 1000 bootstrap replicates; pairwise deletions) constructed using Mega 7 to analyze a ClustalW alignment of full-length white amino acid sequences with a mid-point rooted tree. The scale bar represents the relative evolutionary distance as changes per amino acid residue. The branch supports are given as percent likelihood. Species names with accession numbers are given.
Figure 2-7. Graphic representation showing the alignments of the putative amino acid sequences of the predicted *Plodia interpunctella* white protein (Pl_in-white) and the *Plutella xylostella* Pxwhite protein (Pl_xy_Pxwhite) (GenBank accession no. KM667971). The dark gray boxes show regions of identical amino acids, Light gray boxes show homologous substitutions. Unshaded residues are non-identical.
Figure 2-8. *White* eye phenotype resulting from CRISPR/Cas9-mediated mutagenesis in *P. interpunctella*. Following microinjection of Cas9/sgRNA242 complex into early embryos, the G₀ pupae and adults were examined for white eye phenotype. A wild type *P. interpunctella* with normal eyes is shown in panel A. A pharate pupa with a variegated eye pattern is shown in panel B. Adults with only a left white eye phenotype are shown in panels D, E, H, and J; an adult with a right white eye phenotype is shown in panel C; and adults with both white eye phenotypes are shown in panels F, G, and I. Photo courtesy of Bryce Shirk. *White Knockout Mutant Eye Phenotypes*. September 2019.
Figure 2-9. Heritable CRISPR/Cas9-mediated mutations of the white gene in *P. interpunctella*. Various indel mutations were found in the progeny of G₀ mutant moths. Sequences in panel (A) show sequence deletions at the sgRNA242 PAM site Pi_white-gDNA with the wild type reference. The Piw-sequence matches the wild type reference. Sequences from Pi_242B-BGL0- were individual isolates from the same colony. The 242B-BGL0-5a, -6a, -7a and -8a do not show modifications at the PAM site. The solid red bar above the sequences shows the position of the sgRNA homology site. The sequence in panel B shows a sequence insertion at the sgRNA242 PAM site in sequence Pi_242B-BGL0-9a.
Figure 2-10. Mutations of the amino acid sequence resulting from the heritable CRISPR/Cas9 indels with white eye phenotype in *P. interpunctella* with white eye phenotype in *P. interpunctella*. The Pi_White-gDNA-p is the wild type sequence and the Piw-p is from the Piw-strain. The Pi_242-2ap, -3ap, -5ap, 242B-BLG0-2ap, and 242B-BLG0-3ap are read through that resulted in modification to only the extramembrane amino terminus. The 242-1ap mutation resulted in a frame-shift that produced a novel amino acid sequence. The 242-6ap, 242B-BLG0-1ap and 242B-BLG0-9ap mutations resulted in insertion of stop codons that led to truncated sequences. The 242B-BLG0-5ap, 242B-BLG0-6ap and 242B-BLG0-7ap show no alteration in the predicted amino acid sequence. White boxes with – represent deleted sequences. White boxes with * represent stop codons.
Figure 2-11. Relative transcript levels of white in sgRNA242/CRISPR/Cas9 deletion mutations with white eye phenotype in Plodia interpunctella. Transcript levels were measured from total RNA isolates of each strain. The relative levels determined by reference to the geometric mean of the β-actin and ribosomal protein 7S transcripts. The letters associated with each bar show the statistical equivalency as determined by Tukey’s studentized range test following significant ANOVA analysis. Pi+ = Plodia wild type strain; Piw- = Plodia white eye strain; 1-2 through B11 are Piw-242 recovered allele strains.
Figure 2-12. Sensitivities of *Plodia* strains to Cry1Ac protoxin. The newly hatched caterpillars were placed on Cry1Ac protoxin treated diet and assessed for mortality at 7-days (panel A) and 14-days (panel B) later. To test the level of sensitivity, the caterpillars were fed on either 0.02 µg/well or 2.2 µg/well of Cry1Ac protoxin. Data are presented as percent mortality for each of the times and treatment levels.
CHAPTER 3
DISCUSSION

The findings presented here show that the CRISPR/Cas9-mediated mutations in the white gene of *P. interpunctella* resulted in the expected white eye phenotype but did not cause embryonic lethality as observed in *Helicoverpa armigera* (Khan et al. 2017). Heritable CRISPR/Cas9 mutations in the *P. interpunctella* white gene are similar to other lepidoptera where white does not have a critical function during embryogenesis. This is consistent with findings of similar white eyed phenotypes in the silk worm, *B. mori* (Osanai-Futahashi et al. 2016; Quan et al. 2002; Tatematsu et al. 2011) and the diamondback moth, *Plutella xylostella* (Guo et al. 2015; Guo et al. 2019).

ABC transporters, such as the White protein, are characterized structurally by having two highly conserved functional domains: the nucleotide-binding domain (NBD) that binds and hydrolyzes ATP, and a transmembrane domain (TMD) that provides a platform to translocate various substrates (Rees et al. 2009). In the current study, the sgRNA242 targeted a site in exon 1 which led to the White phenotype. Mutations at the sgRNA242 PAM site should disrupt the NBD of the *P. interpunctella* White protein thus resulting in a non-functional transporter. In *Piw*\textsuperscript{242} mutations 2ap, 3ap, 5ap, B-BLG0-2ap, and B-BLG0-3ap directly disrupt the NBD due to in-frame deletions while mutations 6ap, B-BLG0-1ap, and B-BLG0-9ap caused a loss of White function by producing truncated peptides through early translation termination. In most of the CRISPR/Cas9-mediated mutations isolated, little or no transcript was detected (Figure 2-11). However, intermediate levels of transcript abundance were detected in two of the mutant lines suggesting that the mutation at the sgRNA242 PAM site resulted in a white phenotype due to a defective translational peptide product.
Members of the ABC transporter gene superfamily produce proteins involved in translocation of substrates across intra- and extracellular membranes in both prokaryotes to eukaryotes (Higgins 1992). However, they have a relatively indiscernible role in transporting not only beneficial substrates but also toxic or inhibitory substrates such that they have been implicated in multidrug resistance (MDR) phenotypes in prokaryotes as well as eukaryotes (Lage 2003). Consequently, the transport activities of a broad range of substrates by the ABC transporters can contribute to protecting insects from xenobiotics such as insecticides (Dermauw and Van Leeuwen 2014; Merzendorfer 2014). Recent studies in insects have demonstrated a role of the ABC transporters in toxin resistance. This includes a report in *P. xylostella* where a naturally occurring mutant as well as RNAi suppressed, led to the down regulation of a novel white ortholog, *Pxwhite*, that resulted in Bt Cry1Ac toxin resistance by 3500-, 4000- and 450-fold in the various strains tested (Guo et al. 2015).

The ABC transporter genes have been associated with resistance to Cry1, Cry2 and Cry3 toxins in other moth species (Park et al. 2014). The major ABC gene that is responsible for resistance in Lepidoptera is in the subfamily C2, named ABCC2 (Gahan et al. 2001; Liu et al. 2018; Park et al. 2014). ABCC2 functions as a Cry1A receptor and facilitates the toxin oligomerization and membrane insertion in susceptible insects so a disruption to the expression of this gene disables the receptor needed for the insecticidal protein to be functional (Banerjee et al. 2017; Liu et al. 2018; Park et al. 2014). In addition, ABCC2 and ABCC3 have been implicated in Cry1Ac resistance in *P. xylostella*, based on reduced Cry1Ac susceptibility following CRISPR/Cas9-mediated knockout for each of these genes (Guo et al. 2019).
A significant test establishing a functional role for ABCC2 in Bt susceptibility was conducted in a heterologous system using *D. melanogaster* (Stevens et al. 2017). The genome of *D. melanogaster* does not contain an ortholog of the ABCC2 gene and therefore, *D. melanogaster* are not susceptible to Cry1Ac toxins. In this study, genetically transformed *D. melanogaster* with the *P. xylostella* ABCC2 coding sequence regulated by promoters for either midgut or salivary glands were tested for susceptibility to Cry1Ac (Stevens et al. 2017). *D. melanogaster* transformed with the PxABCC2 coding sequence that was expressed in larval midgut cells was 100 times more susceptible to Cry1Ac toxin compared with non-transformed controls, while those that expressed PxABCC2 in the salivary glands were not susceptible (Stevens et al. 2017).

Previous studies have shown that field-resistant populations in *P. xylostella* which had a downregulation of the White gene, led to reduced sensitivity to the Bt-toxin Cry1A toxin (Guo et al. 2015). The hypothesis tested in these studies was that using CRISPR-Cas9 mutagenesis to create White knockout mutants in *P. interpunctella* would create mutants that would behave similarly to the naturally occurring mutation observed in *P. xylostella* and reduce sensitivity to the Bt-toxin Cry1Ac. As shown here, the Piw-strain as well as most of the Piw-242 strains had statistically significantly lower transcript levels when compared with the CMAVE *P. interpunctella* wild type strain (Figure 2-11; p≤0.05 experiment-wise error rate (Df=14). Type I error rate was set at α=0.05). From this assessment, the gene editing Piw- mutants produced in this study mimicked what was described for *P. xylostella* Pxwhite strain (Guo et al, 2015).

However, the hypothesis was not supported by the results shown here as the Piw- and Piw-242 mutants exposed to Cry1Ac Bt-toxin did not exhibit reduced
sensitivities and had high levels of mortality when exposed to the toxin. Our results indicate that the genetically modified White knockout mutants in *P. interpunctella* did not affect sensitivity of neonate larvae to the Cry1Ac Bt-toxin, in contrast to what had been observed in *P. xylostella*. There was not a statistically significant difference between the mortality of the different strains of *P. interpunctella* when exposed to Cry1Ac. When treated with the two dosages of Cry1Ac and all strains exhibited nearly 100% mortality at both concentrations. However, these results are inconclusive based on issues with experimental design. The untreated control group had high mortality. A potential cause of this could be the plastic 96-well plate with Breathe-Easy was not a suitable environment for conducting the assay. There could have been either toxic effects due to the coatings on the plastics or the Breathe-Easy, even with holes being punctured, did not allow enough air exchange and/or humidity to be too high or low. To test for this issue the assay could be performed in small glass tubes with cotton plugging the top. Another issue was the dosage of Cry1Ac toxin was very high compared with literature dosages for *P. interpunctella* treatment. Further experimentation will need to be performed at lower dosages to determine the LD$_{50}$ and with certainty confirm there is no change in susceptibility caused by the mutation.

The current study establishes the utility of CRISPR/Cas 9 gene editing as a tool to assess functional genomics in *P. interpunctella*. Employing current protocols for embryo microinjection coupled with targeting of genes critical to toxin resistance, such as the ABC transporters, or other potential target sites for control, will provide an efficient means assessing the functional genomics in this stored products pest insect.
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BIOGRAPHICAL SKETCH

Bryce David Shirk was born in Gainesville, Florida, in 1991 to Dr. Paul David Shirk and Lynna Kay Shirk. In 2010, Mr. Shirk graduated from high school, at which point he had the opportunity to further his education at Florida State University. Initially, he began his undergraduate degree studying Chemical Engineering and had his first experience in research as a summer volunteer in Dr. Christine Schmidt’s lab at the University of Florida, Department of Biomedical Engineering. From these experiences, Mr. Shirk realized he had a passion for biological sciences and felt he did not have the desired exposure to this education in Chemical Engineering. For this reason, he changed his undergraduate major to Biochemistry, where he finished his Bachelor of Science in 2017. Upon graduation, Mr. Shirk worked as a biological technician in Dr. David Oi’s lab at the USDA-ARS CMAVE, as well as volunteering in Dr. Paul Shirk’s lab. These lab experiences allowed Mr. Shirk to hone his technical skills in molecular genetic techniques, along with creating a passion for using molecular genetic techniques to study important agricultural issues. In November of 2018, Mr. Shirk was accepted into the graduate program at the University of Florida in the Entomology and Nematology Department advised by Dr. Blair Siegfried. In conjunction with performing his Master of Science program, he worked as a research assistant at the Florida Museum of Natural History under Dr. Caroline Storer where he studied functional genetics of silk genes in lepidopteran species. Bryce Shirk received his Master of Science from the University of Florida, Entomology and Nematology Department in 2021.