# Midgut-specific expression of *CYP321A8* P450 gene increases deltamethrin tolerance in the fall armyworm *Spodoptera frugiperda*

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## Abstract

The *piggyBac*-based germline transformation system was recently established in a global agricultural pest, the fall armyworm (FAW), *Spodoptera frugiperda*. Tissue-specific promoters are needed to apply this transformation system to express transgenes in a tissue-specific manner. Highly expressed genes in the midgut were identified by RNA sequencing and RT-qPCR. Promoter regions of 11 genes highly expressed in the midgut were identified and cloned. Baculoviruses expressing the luciferase gene under the control of these promoters were produced and tested in the FAW. These baculoviruses did not show significant luciferase activity in the FAW midgut. Four transgenic FAW lines expressing the luciferase gene under the control of the SfSP38/P2000, SfCalphotin/P2000, SfMG17/P2000, and SfCPH38/P2000 promoters were generated using *piggyBac*-based germline transformation methods. Significantly higher luciferase activity was detected in the midgut than in other tissues of transgenic FAW. The SfCPH38/P2000 promoter with the highest activity and midgut specificity was used to drive the expression of P450, *SfCYP321A8,* which is known to be involved in deltamethrin resistance. Higher mRNA levels of *SfCYP321A8* and P450 activity were detected in the midgut of transgenic larvae than in wild-type larvae. Bioassays showed that transgenic larvae expressing SfCYP321A8 in the midgut are tolerant to deltamethrin. Here, we presented methods for the identification of midgut-specific promoters in the FAW and used them to study the role of P450 overexpression in the midgut on insecticide resistance. These methods could also be used to identify other tissue-specific promoters for applications of *piggyBac*-based germline transformation in functional genomics in FAW and other non-model insects.



## Keywords

Midgut-specific genes, promoters, transgenesis, P450, deltamethrin, and *Spodoptera frugiperda*

## Introduction

The midgut is the second largest organ and site of digestion of ingested food and absorption of nutrients (Hakim et al. 2010). As an important target tissue for many insecticides, the insect midgut also plays a major role in insecticide resistance (Smagghe and Tirry 2001). It is also an immune organ acting as the first line of defense against pathogens, including bacteria and viruses (Hakim et al. 2010). The distinctive environment in the midgut also allows microbial colonization, which benefits host insects (Engel and Moran 2013). Additionally, remodeling of the midgut is critical for successful metamorphosis in insects (Parthasarathy and Palli 2007; Wu et al. 2006). It is believed that the products from midgut genes, especially highly expressed genes and midgut-specific genes, perform critical functions (Hakim et al. 2010). Studies on *Drosophila melanogaster* extended our knowledge on the insect midgut (Harrop et al. 2014; Hung et al. 2020; Lucchetta and Ohlstein 2012). However, these results may not apply to all insects with significant differences in the structure and function of the midgut. The transgene-based overexpression, knockdown, and knockout of midgut genes used routinely in *D. melanogaster* are still challenging in other insects. The availability of these methods in pest insects will promote studies on the midgut and benefit the management of these pests.

The fall armyworm (FAW), *Spodoptera frugiperda* (Lepidoptera: Noctuidae), is native to tropical and subtropical regions of the Americas. It has now successfully invaded most parts of the world (Gui et al. 2020). As a successful polyphagous pest, FAW feeds on more than 300 species of host plants, including some major food crops (Montezano et al. 2018). FAW has developed resistance to many chemical insecticides (Carvalho et al. 2013; Diez-Rodriguez 2001) and transgenic *Bt* corn (Omoto et al. 2016). The midgut is likely to play a central role in FAW feeding

on multiple plants and developing insecticide resistance. However, there are not many studies on FAW midgut.

Unlike in model insects, germline transformation systems are not well developed in lepidopteran pest insects. We recently established a *piggyBac*-based transformation system in the FAW (Chen et al. 2020b). Several highly active promoters have been identified in FAW (Bleckmann et al. 2015; Chen et al. 2020a), which will further facilitate the use of the *piggyBac* system in FAW. However, tissue-specific promoters are needed to conduct tissue-specific gain- or loss-of-function studies using the *piggyBac*-based transformation system. There are no reports on FAW tissue-specific promoters.

In model insects, including *D. melanogaster* and *Bombyx mori*, a few midgut-specific promoters have been identified and used for tissue-specific expression of transgenes (Fischer et al. 1988; Hu et al. 2015; Jiang et al. 2013; Park and Kwon 2011; Zeng et al. 2010). Midgut-specific promoters were also identified in several mosquito species (Moreira et al. 2000; Nolan et al. 2011; Skavdis et al. 1996; Zhao et al. 2014). Although some midgut-specific promoters showed cross-species activity (Skavdis et al. 1996), it is still not feasible to use midgut-specific promoters in non-host insects. Information on midgut-specific promoters in non-model pest insects is not available.

Transgenic insects have been used to evaluate the model insect promoters *in vivo* (Anderson et al. 2010; Deng et al. 2013; Jiang et al. 2013; Moreira et al. 2000; Totten et al. 2013; Xu et al. 2019; Xu et al. 2014). However, this method is not available to most insect species. Recently, baculoviruses were used to evaluate the performance of *B. mori* promoters *in vivo* (Tian et al. 2018; Zhang et al. 2015; Zhao et al. 2014). Conducting baculovirus transduction is much easier than manipulating transgenesis in insects. Whether the baculovirus transduction method is

feasible in evaluating the activity of FAW promoters, including the midgut-specific promoters, *in vivo* is unknown.

In our recent work, we found that a P450 gene, SfCYP321A8, is upregulated in the first instar FAW larvae fed on deltamethrin, and transgenic over-expression of this P450 gene by a strong constitutive promoter, SfPub promoter, confers deltamethrin resistance in FAW(Chen and Palli 2021). However, whether over-expression of SfCYP321A8 only in the midgut could confer deltamethrin resistance in FAW in unknown. To develop tools for tissue-specific gain- or loss-of-function studies in FAW, we analyzed RNA-Seq data from the midgut and other tissues, identified several genes uniquely, and highly expressed in the midgut. Promoter regions of identified genes were used to drive luciferase reporter gene expression in cells via the baculovirus system. In addition, transgenic reporter FAW lines were established to investigate candidate promoter activity *in vivo*. Finally, the promoter of the *SfCHP38* gene, which is highly active in the midgut compared to that in other tissues, was selected to drive the expression of a P450 gene, *SfCYP321A8*, involved in deltamethrin resistance. Deltamethrin tolerance was observed in transgenic animals expressing *SfCYP321A8* in the midgut.

## Materials and Methods

### Insect and cells

The laboratory strain of FAW was purchased from Benzon Research Inc. (Pennsylvania, USA) and reared for multiple generations without exposure to any pesticides. Larvae were fed an artificial diet from Southland Product Inc. (Arkansas, USA). Adults were provided with a 10% sucrose solution under the conditions of 23 ± 1 °C, 75 ± 5% relative humidity and a photoperiod

of 16:8 (light: dark). Sf9 cells were cultured at 27 ℃ in Sf-900 II medium (Thermo Fisher, USA). The SfMG_0617 (hereafter referred to as Sf17) cell line, generated from the FAW midgut (Zhou et al. 2020), was maintained in TNM-FH insect medium (Sigma-Aldrich, USA) supplemented with 10% FBS at 27 ℃.

**Plasmid construction**

The candidate midgut gene promoters were identified and cloned as described in our previous publication (Chen et al. 2020a). To generate plasmids for producing baculoviruses, the ORF of the luciferase gene was amplified from the pG5Luc vector using primers containing 20 bp homologous arms on either end of the EcoR I/Kpn I-digested pFastBac-S/His vector and cloned into the pFastBac-S/His vector by Gibson assembly (NEB, USA). Promoters of FAW midgut-specific genes containing 20 bp homologous arms on either end of the SnaB I/BamH I-digested pFastBac-Luc plasmid were amplified from FAW genomic DNA and inserted into the SnaB I/BamH I-digested pFastBac-Luc plasmid to generate pFastBac-promoter-Luc vectors as described above. All vectors were transformed into *E. coli* 10G cells (Lucigen, USA). Positive transformants were selected and cultured in 3 ml LB medium. Plasmid DNA was extracted using a plasmid mini kit (Qiagen, USA)

To prepare the plasmids for generating transgenic FAW, a *piggyBac* vector, pBac:hr5ie1-EGFP-SV40:hr5ie1-Cas9-SV40 (gifted from Dr. Tan, Shanghai Institute of Plant Physiology and Ecology, China), was digested with Aat II/Apa I to remove the Cas9 cassette. The ORF of luciferase or a P450 gene, *SfCYP321A8*, from FAW was inserted into the digested *piggyBac* vector as described above to produce the intermediate vectors pBac:hr5ie1-EGFP-SV40:hr5ie1-Luc-SV4 and pBac:hr5ie1-EGFP-SV40:hr5ie1-SfCYP321A8-SV40. The cloned promoters were then inserted into Kpn I/Aat II- or Xho I/Aat II-digested intermediate vectors to generate the final

vectors, pBac:hr5ie1-EGFP-SV40:hr5/Promoter-Luc-SV40, pBac:ie1-EGFP-SV40:Promoter-Luc-SV40, pBac:hr5ie1-EGFP-SV40:hr5/Promoter-SfCYP321A8-SV40, and pBac:ie1-EGFP-SV40: Promoter-SfCYP321A8-SV40. All constructs were transformed into *E. coli* 10G cells. Positive transformants were selected and cultured in 100 ml LB medium. The plasmid DNAs for transfection were prepared using the plasmid midi kit (Qiagen, USA).

The primers used to produce the constructs are listed in Table S1.

**Reporter baculovirus preparation**

pFastBac-promoter-Luc plasmids were extracted and transformed into *E. coli* DH10Bac cells (Invitrogen, USA) to produce recombinant bacmids, which were then used to generate reporter baculoviruses in Sf9 cells following the methods described in the bac-to-bac system (Invitrogen, USA). The viral nucleic acids of P2 viruses were purified using the PureLink$^{TM}$ Viral RNA/DNA Mini Kit (Invitrogen, USA). The plaque-forming units (PFU) of reporter baculoviruses were determined using the BacPAK qPCR Titration Kit (Takara Bio, USA).

**RNA isolation, cDNA preparation and RT-qPCR**

Total RNA was isolated using TRI reagent (Molecular Research Center Inc., Cincinnati, OH) and converted to cDNA using M-MLV Reverse Transcriptase (Invitrogen, USA). Real-time PCR reactions were conducted in a 10-μL total reaction volume containing 5 μL of 2xSYBR Mix (BioRad, USA), 0.4 μL of each primer, 0.8 μL of 20-fold diluted cDNA, and 3.4 μL of double-distilled water. Primers were designed using Primer3 (http://bioinfo.ut.ee/primers-0.4.0/) and are listed in Table S1. The reaction conditions used were as follows: 95 °C for 2 min, 40 cycles of 95 °C for 10 seconds, and 60 °C for 1 min. The expression levels of each gene were calculated as a fold change over the expression levels of a reference gene, 28S rRNA, which is stably expressed

in different tissues of both transgenic and wild-type FAW (Figure S1). All samples contain three biological replicates.

**Transformation of FAW**

The hyperactive transposase mRNA was prepared as described previously (Chen et al. 2020b) and stored at -80 ℃. A mixture of hyperactive transposase mRNA (400 ng/μL) and each final vector (300 ng/μL) was injected into fresh FAW embryos (less than 4 hr old) as previously described (Chen et al. 2020b). The surviving larvae (G0) were reared under normal conditions. Collection and screening of newly hatched 1$^{st}$ instar larvae (G1) were performed as described previously (Chen et al. 2020b). All transgenic larvae and adults were reared under normal conditions.

**Luciferase activity assay**

To test the luciferase activity of reporter baculoviruses in cell lines, Sf9 and Sf17 cells were seeded in 96-well culture plates at a density of 2x10$^5$ cells per well and incubated at 27 ℃ overnight. A total of 6x10$^4$ PFU of each virus was added to 100 μL medium of Sf17 and Sf9 cells. Cells were collected for luciferase activity assay at 24 hr postinfection, and the luciferase activity was quantified as described (Chen et al. 2020a). Four biological replicates were performed for each treatment.

To determine the luciferase activity in the midgut and other tissues of FAW larvae, newly molted 6$^{th}$ instar larvae were injected with 2x10$^5$ PFU of each virus. Midguts and other tissues dissected on the third day postinjection were homogenized in luciferase assay buffer and centrifuged at 12,000 rpm and 4 °C. Twenty microliters and 10 μL of each supernatant were used for luciferase activity assay and protein concentration determination, respectively, as described previously (Chen et al. 2020a). Three biological replicates were performed for each treatment.

To investigate the luciferase activity in tissues of transgenic reporter FAW larvae, four different tissues, including head, epidermis, fat body, and midgut, were dissected from transgenic 6$^{th}$ instar larvae. They were then processed and subjected to luciferase activity assays as described above. Three biological replicates were performed for each treatment.

**P450 activity assay in tissues of transgenic insects**

Midgut and fat body were dissected from wild-type and transgenic FAW 6$^{th}$ instar larvae and homogenized in 1.7-ml tubes containing ice-cold 0.1 M sodium phosphate buffer (pH 7.5) with 1 mM EDTA, 1 mM PMSF, 0.1 mM DTT, and 1 mM PTU. Then, the homogenates were clarified two times by centrifugation at 1,000 x *g* for 5 min at 4 ℃. The protein concentration in the supernatant was measured using the Bradford method. The supernatant was diluted with sodium phosphate buffer to give a final protein concentration of 1 mg/ml. Luminescent P450 activity assays were performed in all-white 96-well plates using the commercial P450-Glo substrate Luciferin-Be (Promega, Madison, WI, USA) as described (Inceoglu et al. 2009). To initiate the P450 activity assay, 50 μM luciferin-Be substrate was added to each well containing 40 μL of homogenate supernatant, and 0.1 M sodium phosphate buffer mentioned above was added to each reaction to reach a final volume of 100 μL. The luminescent reactions were incubated at room temperature for 1 hr. Then, 25 μL of the reaction mixture was transferred to a new well. After adding 25 μL of luciferin detection reagent, the reactions were incubated at 27 °C for an additional 20 min. The luminescence was quantified using SpectraMax i3x (San Jose, CA, USA). The average relative luminescence unit (RLU) values from wells containing all assay components except the tissue were used as a blank. Three biological replicates were performed for each treatment.

## Deltamethrin bioassay

Bioassays were conducted with newly molted 1st instar larvae using a standard leaf disk method (Yang et al. 2017). Deltamethrin (EMD Millipore Crop., USA) was diluted using 0.1% Triton X-100 to produce serial concentrations of solution. Brussels sprout leaves were washed and dried at room temperature, cut into small, ~1 x 0.5 cm pieces and dipped in deltamethrin solution for 10 seconds. These leaf discs were air-dried on food wrap film at room temperature for 20-30 min. Leaf discs dipped in sterile distilled water containing 0.1% Triton X-100 were used as controls. The treated leaf discs were then transferred to 2.0-ml Eppendorf tubes. Fifteen newly molted 1st instar larvae were gently transferred into each tube. The tubes were sealed with cotton and kept at 27 ± 2 °C, 65 ± 5% relative humidity, and a photoperiod of 16:8 (light: dark). Each treatment has three replicates. Mortality was recorded at 48 h after exposure to deltamethrin. Larvae were considered dead if they failed to move when prodded with a brush.

## Statistical analysis

$LC_{50}$ values were generated using GraphPad Prism 8. IBM SPSS Statistics 25 was used in the statistical analysis. All data are shown as the mean ± SD (standard deviation). Significant differences between the two groups were analyzed using independent samples *t* tests; $p<0.05$ was considered statistically significant.

# Results

## Identification of midgut-specific promoters in FAW

Several highly active promoters from FAW have been identified, and their activity *in vivo* was determined (Chen et al. 2020a). In this study, a group of eight highly expressed genes in the midgut and other tissues and a group of 16 genes (Table S2) that are expressed only in the midgut

were identified in RNA-Seq data. The mRNA levels of these genes were investigated by RT-qPCR. All eight highly expressed genes showed significantly higher expression levels in the midgut than in the other tissues. The expression levels of *SfCPH38* in the midgut were substantially higher than those in other tissues (Figure 1A). In the group of midgut-specific genes, two genes, *SfZCPase2* and *SfmALP*, were not expressed in other tissues. However, their expression levels in the midgut were much lower than those of the other 14 genes. Among these 14 genes, *SfTrypsinC* showed the highest specificity in the midgut, 1,842,596-fold higher than in other tissues (Figure 1B). The overall relative expression levels of 16 midgut-specific genes were much lower than those of eight highly expressed genes. NCBI accession numbers of these genes are provided in Table S2.

Following the method described in our previous paper (Chen et al. 2020a), the putative promoter regions of 11 midgut-specific genes, including SfTrypsinC/P844, SfSP/P415, SfLipase3/P1558, SfCBP-I/P360, SfCalphotin/P799, SfTrypsin/P1705, SfCptlike/P857, SfMG17/P2000, SfSP11/P2000, SfSP38/P2000, and SfCPH38/P2000, were amplified from the genomic DNA and cloned.

**Determination of the specificity of candidate FAW midgut promoters *in vivo* using baculovirus**

Two FAW ovarian cell lines, Sf9 and Sf21, and one FAW midgut cell line, Sf17, were used to investigate FAW promoter activity. The FAW promoters from highly expressed genes displayed weak or no activity in these cell lines (Bleckmann et al. 2015; Chen et al. 2020a). Cell lines are not ideal for investigating the activity of tissue-specific promoters, which is likely due to the lack of essential transcription factors required for the activation of tissue-specific genes. Additionally, the promoter activity in the cell line may not always match that in the insect *in vivo*.

To test whether the baculovirus could be used to measure the promoter activity in FAW, a reporter baculovirus expressing the luciferase gene under the control of the promoter region of an early baculovirus gene, *pag1*, was constructed. This reporter baculovirus, pag1:Luc, was then added to Sf9 cells and Sf17 cells. Both cell lines showed luciferase activity at 24 hr postinfection, and the luciferase activity in virus-infected Sf9 cells was much higher than that in virus-infected Sf17 cells (Figure S2). The virus was then injected into newly molted 6$^{th}$ instar larvae. Midgut and other tissues were dissected at 1, 2, 3, 4, and 5 days postinjection. In the midgut, the maximum luciferase activity was detected on day 3. In samples containing all other tissues, the maximum luciferase activity was detected on day 4 (Figure S3). Luciferase activity was not detected in uninfected wild-type larvae or those infected with another reporter baculovirus expressing EGFP (data not shown). These results indicated that the reporter baculovirus could be employed to evaluate FAW promoter performance *in vivo*. Since baculoviruses could quickly spread to different tissues of FAW, we hypothesized that the reporter baculovirus carrying a tissue-specific promoter may support tissue-specific transgene expression in infected FAW larvae. Reporter baculoviruses expressing luciferase controlled by 11 candidate midgut-specific promoters were generated. The plaque-forming units (PFU) of each reporter baculovirus were determined by RT-qPCR (Figure S4). Unlike the pag1:Luc baculovirus, most of these 11 reporter baculoviruses showed higher luciferase activity in Sf17 cells than in Sf9 cells (Figure 2A). These reporter baculoviruses were injected into the hemocoel of FAW larvae, and the luciferase activity in the midgut and other tissues was measured on day 3 after injection. The results showed that weak or no luciferase activity was detected in the midgut, while the other tissues showed some luciferase activity (Figure 2B). The reporter baculoviruses failed to show the expected midgut-specific expression of the reporter gene, which is likely due to the injection of the virus into the hemocoel. Additionally,

baculovirus infection may have altered cellular gene expression patterns (Blissard 1996; Clem and Passarelli 2013), making endogenous promoters inactive and/or changing their tissue specificity.

**Determination of the specificity of candidate FAW midgut promoters *in vivo* using transgenic insects**

The germline transformation technologies are not well established in nonmodel insects, likely due to the low transformation efficiency and difficulty of delivering transformation components into fresh embryos. We recently established a *piggyBac*-based transformation system in FAW (Chen et al. 2020b), which was used to test FAW promoters *in vivo*.

A *piggyBac* vector expressing the marker protein EGFP under the control of the ie1 promoter, a baculovirus immediate-early gene promoter, was used to generate reporter vectors. The promoters from the genes that are highly expressed in the midgut were cloned into this *piggyBac* vector to drive luciferase expression. The hr5 enhancer (Rodems and Friesen 1993), which can increase promoter activity, was placed between the ie1 promoter and the chosen midgut-specific promoter to enhance the expression of both EGFP and luciferase (Figure 3A). Four reporter vectors, hr5-SfSP38/P2000:Luc, hr5-SfMG17/P2000:Luc, hr5-SfCalphotin/P799:Luc, and hr5-SfCPH38/P2000:Luc, were produced. A mixture of each reporter vector and the mRNA of hyperactive transposase, which was reported to increase the transformation efficiency in several insect species (Eckermann et al. 2018; Otte et al. 2018), was injected into 2000 fresh embryos collected within 6 hr after oviposition. Forty-six transgenic hr5-SfSP38/P2000: Luc neonate larvae, 31 transgenic hr5-SfMG17/P2000:Luc neonate larvae, 56 transgenic hr5-SfCalphotin/P799: Luc neonate larvae and 41 transgenic hr5-SfCPH38/P2000:Luc neonate larvae were identified based on EGFP marker gene expression in the G1 generation (Table 1). The adults developed from G1-positive larvae were crossed with wild-type adults to produce the G2 generation. The positive

transgenic larvae from all four lines showed strong GFP signals in G2 (Figure 3B), indicating that the transgenic insertions are inheritable. We then tested the luciferase activity in four tissues, including the head, epidermis, fat body, and midgut dissected from each transgenic line. The results showed that the midgut has significantly higher luciferase activity than other tissues. The luciferase activity in the midgut was 1,731-, 1,558-, 2,337-, and 14,124-fold higher than that in the fat body of hr5-SfSP38/P2000:Luc, hr5-SfMG17/P2000:Luc, hr5-SfCalphotin/P799:Luc, and hr5-SfCPH38/P2000:Luc transgenic larvae, respectively (Figure 3C). The SfCalphotin/P799 promoter displayed higher activity in the midgut and fat body than the other three promoters tested. The SfCPH38/P2000 promoter showed the best specificity in the midgut. Low luciferase activity was also detected in other tissues, indicating the weak activity of these promoters in other tissues (Figure 3C).

As a promoter fused with the hr5 enhancer showed elevated activity (Chen et al. 2020a), we hypothesized that removing the hr5 enhancer might decrease promoter activity in other tissues and increase its specificity in the midgut. To test this hypothesis, a new reporter vector, SfCPH38/P2000:Luc, was constructed (Figure 3A) and injected into 2000 eggs. Twenty-nine EGFP-positive neonate larvae were obtained (Table 1). The EGFP signals in transgenic SfCPH38/P2000:Luc G2-positive larvae were not as bright as those in transgenic hr5-SfCPH38/P2000:Luc G2-positive larvae (Figure 3B), as the hr5 enhancer also boosted ie1 promoter activity, resulting in the production of more EGFP protein. The luciferase activity in the midgut was 13,152-fold higher than that in the fat body of SfCPH38/P2000:Luc larvae. Additionally, the luciferase activity in SfCPH38/P2000:Luc larval tissues was lower than that in the tissues of hr5-SfCPH38/P2000 Luc larvae (Figure 3C). These results suggest that the hr5

enhancer could significantly enhance the activity, but not the midgut specificity, of the CPH38 promoter.

**Midgut-specific promoter-mediated overexpression of P450 confers deltamethrin tolerance to *S. frugiperda***

P450 enzymes metabolize insecticides and plant toxins (Dermauw et al. 2020; Feyereisen 2012), and their overexpression is involved in insecticide resistance (Feyereisen 2012; Jiang et al. 2015). Although many P450 genes were found in the genome of FAW (Gui et al. 2020), little is known about their function in insecticide resistance. A recent study revealed that overexpression of *CYP321A8* in *Spodoptera exigua*, a close relative of *S. frugiperda*, conferred resistance to deltamethrin (Hu et al. 2021). The insect midgut is the major organ encountering plant toxins and insecticides (Hakim et al. 2010; Smagghe and Tirry 2001). Transgenic overexpression of P450 genes in the midgut likely confers tolerance to insecticides. We recently reported that transgenic over-expression of *SfCYP321A8* by a strong constitutive promoter conferred deltamethrin resistance in FAW (reference). To test whether the identified midgut-specific promoters could be used in transgenic FAW to study insecticide resistance, the SfCPH38/P2000 promoter was used to drive the expression of the *SfCYP321A8* gene in the midgut.

The *SfCYP321A8* gene was cloned. Two vectors, SfCPH38/P2000:CYP321A8 and hr5-SfCPH38/P2000:CYP321A8, were produced and injected into embryos (Figure 4A). Seventy-nine transgenic SfCPH38/P2000:CYP321A8 neonate larvae and 38 transgenic hr5-SfCPH38/P2000:CYP321A8 neonate larvae were identified based on EGFP marker gene expression in the G1 generation (Table 1). The EGFP signals in transgenic SfCPH38/P2000:CYP321A8 G2-positive larvae were less bright than those in transgenic hr5-SfCPH38/P2000:CYP321A8 G2-positive larvae (Figure 4B).

335 The relative mRNA levels of *SfCYP321A8* were determined in the head, midgut, fat body,
336 and epidermis dissected from the 6$^{th}$ instar larvae of wild-type and two G2 *SfCYP321A8* transgenic
337 insects. The highest expression levels of *SfCYP321A8* mRNA were detected in the midgut of both
338 transgenic lines. Increases of 155.5- and 287.6-fold in the SfCPH38/P2000:CYP321A8 and hr5-
339 SfCPH38/P2000:CYP321A8 mRNA levels in transgenic larvae, respectively, compared to their
340 levels in the wild-type larvae were detected (Figure 4C). The expression also increased by 6.3- and
341 10.6-fold in the fat body of SfCPH38/P2000:CYP321A8 and hr5-SfCPH38/P2000:CYP321A8
342 transgenic larvae, respectively. In the epidermis of transgenic hr5-SfCPH38/P2000:CYP321A8
343 larvae, the mRNA level of *SfCYP321A8* was slightly higher than that in wild-type and control
344 transgenic larvae. No significant changes in *SfCYP321A8* expression were detected in the
345 epidermis of either transgenic larvae (Figure 4C). To test whether overexpression of *SfCYP321A8*
346 in the midgut leads to an overall increase in total P450 activity in the midgut of the transgenic
347 animals, the midgut and fat body were tested for P450 activity. The total P450 activity in the
348 midgut dissected from SfCPH38/P2000:CYP321A8 and hr5-SfCPH38/P2000: CYP321A8
349 transgenic larvae increased by 1.41- and 1.48-fold, respectively, when compared to the P450
350 activity in the wild-type larvae. However, no increase in P450 activity was detected in the fat body
351 of either transgenic larvae (Figure 4D). These results suggest that the SfCPH38/P2000 promoter
352 is capable of driving midgut-specific expression of SfCYP321A8.

353 The expression levels of both *SfCPH38* and *SfCYP321A8* were investigated in the eggs and
354 1$^{st}$ instar larvae of wild-type and transgenic lines. The expression of *SfCYP321A8* in 1$^{st}$ instar
355 larvae of both transgenic lines gradually increased, similar to the *SfCPH38* gene expression (Figure
356 S5). Leaf-disc assays were performed to evaluate the efficacy of deltamethrin in wild-type and
357 transgenic 1$^{st}$ instar larvae. The results showed that the $LC_{50}$ of deltamethrin in

SfCPH38/P2000:CYP321A8 and hr5-SfCPH38/P2000:CYP321A8 larvae increased by 4.2- and 4.5-fold, respectively, when compared to the wild-type larvae (Figure 4E). The 95% confidence limits for the $LC_{50}$ values in wild-type, SfCPH38/P2000:CYP321A8 and hr5-SfCPH38/P2000:CYP321A8 larvae are 0.085~0.675 mg/L, 0.423~3.313 mg/L, and 0.512~5.307 mg/L, respectively. These data suggest that transgenic overexpression of *SfCYP321A8* in the midgut increased deltamethrin tolerance in FAW larvae.

## Discussion

Identification and characterization of midgut-specific promoters from a non-model insect, FAW, are included in this paper. The *de novo* mining of tissue-specific promoters requires gene expression data, which are not available for many insects. Since information on many tissue-specific promoters from model insects is available, it may be possible to identify homologous promoters in target non-model insects. In the lepidopteran model insect *B. mori*, the promoter of the *APN* gene is only active in the midgut (Jiang et al. 2015). We found six *APN* genes in *S. frugiperda*. RT-qPCR analysis revealed midgut-specific expression of all *APN* genes (Figure S6). The candidate promoter region of the *SfAPN6* gene was cloned and used to drive the expression of *SfCYP321A8*. Transgenic SfAPN6/P1949:CYP321A8 animals were obtained (Table S3, Figure S7A). However, the mRNA levels of *SfCYP321A8* only increased by 2.3-fold in the midgut of this transgenic line compared with the wild-type line (Figure S7B). Additionally, this transgenic line showed similar levels of deltamethrin susceptibility as the wild-type line (Figure S7C). The candidate promoter region of *SfSP11*, a homolog of a midgut-specific serine protease in *B. mori* (Liu et al. 2017), was also tested to drive the expression of *SfCYP321A8* in animals (Table S3, Figure S7A). A 16.7-fold increase in *SfCYP321A8* mRNA levels was detected in the midgut of transgenic animals compared with the wild-type animals (Figure S7B). However, no increase in

deltamethrin tolerance was observed (Figure S7C). Since these two midgut-specific promoters of FAW were identified from information that *B. mori* did not show good activity as the SfCPH38/P2000 promoter, our hypothesis on using information from model insects to identify promoters in non-model insects is not supported. We used an ~2 kp region upstream of the ATG of each gene as a candidate promoter region. The low activity of the *SfAPN6* and *SfSP11* promoters might be because this region did not include a complete promoter.

Transgenic reporter insects have been used to investigate promoter performance *in vivo*. However, germline transformation technologies are not available in most nonmodel insects. We tried to use baculoviruses to deliver reporter gene expression cassettes to different tissues of FAW larvae but failed to determine the activity of midgut-specific promoters *in vivo*. A recent report revealed that one baculovirus species, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), is an efficient vector for gene delivery into several mosquito species; it can transduce both larvae and adults with little or no tissue barriers and without obvious negative effects (Naik et al. 2018). It seems that baculovirus could be used to investigate promoter performance in nonpermissive hosts *in vivo*.

In conclusion, transgenic reporter insects were successfully used to determine the *in vivo* performance of midgut-specific promoters in an important agricultural pest, FAW. The midgut-specific promoter was used to investigate the role of P450 in insecticide resistance. This work could serve as a model for exploring other tissue-specific promoters, which will benefit functional genomic studies in FAW and other non-model insects.

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409

410 **Declarations**

411 All authors certify that they have no affiliations with or involvement in any organization or entity
412 with any financial interest or nonfinancial interest in the subject matter or materials discussed in
413 this manuscript.

414 All data generated or analyzed during this study are included in this published article [and its
415 supplementary information files].

# References


Anderson MA, Gross TL, Myles KM, Adelman ZN (2010) Validation of novel promoter sequences derived from two endogenous ubiquitin genes in transgenic Aedes aegypti Insect molecular biology 19:441-449

Bleckmann M et al. (2015) Genomic analysis and isolation of RNA polymerase II dependent promoters from spodoptera frugiperda PloS One 10:e0132898

Blissard GW (1996) Baculovirus-insect cell interactions Cytotechnology 20:73-93

Carvalho RA, Omoto C, Field LM, Williamson MS, Bass C (2013) Investigating the molecular mechanisms of organophosphate and pyrethroid resistance in the fall armyworm Spodoptera frugiperda PLoS One 8:e62268

Chen X, Chereddy SC, Gurusamy D, Palli SR (2020a) Identification and characterization of highly active promoters from the fall armyworm, Spodoptera frugiperda Insect Biochemistry and Molecular Biology 126:103455

Chen X, Koo J, Gurusamy D, Mogilicherla K, Reddy Palli S (2020b) Caenorhabditis elegans systemic RNA interference defective protein 1 enhances RNAi efficiency in a lepidopteran insect, the fall armyworm, in a tissue-specific manner RNA biology:1-9

Chen X, Palli SR (2021) Transgenic overexpression of P450 genes confers deltamethrin resistance in the fall armyworm, Spodoptera frugiperda Journal of Pest Science:1-9

Clem RJ, Passarelli AL (2013) Baculoviruses: sophisticated pathogens of insects PLoS Pathog 9:e1003729

Deng D, Xu H, Wang F, Duan X, Ma S, Xiang Z, Xia Q (2013) The promoter of Bmlp3 gene can direct fat body-specific expression in the transgenic silkworm, Bombyx mori Transgenic research 22:1055-1063

Dermauw W, Van Leeuwen T, Feyereisen R (2020) Diversity and evolution of the P450 family in arthropods Insect biochemistry and molecular biology 127:103490

Diez-Rodriguez G, Omoto, C. (2001) Inheritance of lambda-cyhalothrin resistance in Spodoptera frugiperda (JE Smith)(Lepidoptera: Noctuidae) Neotropical Entomology 30:311-316

Eckermann KN et al. (2018) Hyperactive piggyBac transposase improves transformation efficiency in diverse insect species Insect biochemistry and molecular biology 98:16-24

Engel P, Moran NA (2013) The gut microbiota of insects–diversity in structure and function FEMS microbiology reviews 37:699-735

Feyereisen R (2012) Insect CYP genes and P450 enzymes. In: Insect molecular biology and biochemistry. Elsevier, pp 236-316

Fischer JA, Giniger E, Maniatis T, Ptashne M (1988) GAL4 activates transcription in Drosophila Nature 332:853-856

Gui F et al. (2020) Genomic and transcriptomic analysis unveils population evolution and development of pesticide resistance in fall armyworm Spodoptera frugiperda Protein & Cell:1-19

Hakim RS, Baldwin K, Smagghe G (2010) Regulation of midgut growth, development, and metamorphosis Annual review of entomology 55:593-608

Harrop TW, Pearce SL, Daborn PJ, Batterham P (2014) Whole-genome expression analysis in the third instar larval midgut of Drosophila melanogaster G3: Genes, Genomes, Genetics 4:2197-2205

Hu B et al. (2021) Changes in both trans-and cis-regulatory elements mediate insecticide resistance in a lepidopteron pest, Spodoptera exigua PLoS genetics 17:e1009403

Hu C, Wang F, Ma S, Li X, Song L, Hua X, Xia Q (2015) Suppression of intestinal immunity through silencing of TCTP by RNAi in transgenic silkworm, Bombyx mori Gene 574:82-87

Hung R-J et al. (2020) A cell atlas of the adult Drosophila midgut Proceedings of the National Academy of Sciences 117:1514-1523

462 Inceoglu A, Waite T, Christiansen J, McAbee R, Kamita S, Hammock B, Cornel A (2009) A rapid luminescent
463 assay for measuring cytochrome P450 activity in individual larval Culex pipiens complex
464 mosquitoes (Diptera: Culicidae) Journal of medical entomology 46:83-92
465 Jiang L et al. (2013) Identification of a midgut-specific promoter in the silkworm Bombyx mori Biochemical
466 and biophysical research communications 433:542-546
467 Jiang L et al. (2015) The 5′-UTR intron of the midgut-specific BmAPN4 gene affects the level and location
468 of expression in transgenic silkworms Insect biochemistry and molecular biology 63:1-6
469 Liu HW et al. (2017) A midgut‐specific serine protease, BmSP36, is involved in dietary protein digestion
470 in the silkworm, Bombyx mori Insect science 24:753-767
471 Lucchetta EM, Ohlstein B (2012) The Drosophila midgut: a model for stem cell driven tissue regeneration
472 Wiley Interdisciplinary Reviews: Developmental Biology 1:781-788
473 Montezano DG et al. (2018) Host plants of Spodoptera frugiperda (Lepidoptera: Noctuidae) in the
474 Americas African Entomology 26:286-300
475 Moreira LA, Edwards MJ, Adhami F, Jasinskiene N, James AA, Jacobs-Lorena M (2000) Robust gut-specific
476 gene expression in transgenic Aedes aegypti mosquitoes Proceedings of the National Academy of
477 Sciences 97:10895-10898
478 Naik NG, Lo Y-W, Wu T-Y, Lin C-C, Kuo S-C, Chao Y-C (2018) Baculovirus as an efficient vector for gene
479 delivery into mosquitoes Scientific reports 8:1-14
480 Nolan T, Petris E, Müller H-M, Cronin A, Catteruccia F, Crisanti A (2011) Analysis of two novel midgut-
481 specific promoters driving transgene expression in Anopheles stephensi mosquitoes PloS one
482 6:e16471
483 Omoto C et al. (2016) Field‐evolved resistance to Cry1Ab maize by Spodoptera frugiperda in Brazil Pest
484 Management Science 72:1727-1736
485 Otte M, Netschitailo O, Kaftanoglu O, Wang Y, Page Jr R, Beye M (2018) Improving genetic transformation
486 rates in honeybees Scientific reports 8:1-6
487 Park J-H, Kwon JY (2011) Heterogeneous expression of Drosophila gustatory receptors in enteroendocrine
488 cells PLoS One 6:e29022
489 Parthasarathy R, Palli SR (2007) Developmental and hormonal regulation of midgut remodeling in a
490 lepidopteran insect, Heliothis virescens Mechanisms of development 124:23-34
491 Rodems SM, Friesen PD (1993) The hr5 transcriptional enhancer stimulates early expression from the
492 Autographa californica nuclear polyhedrosis virus genome but is not required for virus replication
493 Journal of Virology 67:5776-5785
494 Skavdis G, Sidén‐Kiamos I, Müller H, Crisanti A, Louis C (1996) Conserved function of anopheles gambiae
495 midgut‐specific promoters in the fruitfly The EMBO journal 15:344-350
496 Smagghe G, Tirry L (2001) Insect midgut as a site for insecticide detoxification and resistance. In:
497 Biochemical sites of insecticide action and resistance. Springer, pp 293-321
498 Tian S, Zhang Z, Zhang R, Liu Q, Xu S, Gong J, Hou Y (2018) Analysis of cysteine protease inhibitor gene
499 (BmCPI) promoter activity in silkworms using bac-to-bac baculovirus systems Bioscience,
500 biotechnology, and biochemistry 82:1488-1496
501 Totten DC, Vuong M, Litvinova OV, Jinwal UK, Gulia‐Nuss M, Harrell RA, Beneš H (2013) Targeting gene
502 expression to the female larval fat body of transgenic Aedes aegypti mosquitoes Insect molecular
503 biology 22:18-30
504 Wu Y, Parthasarathy R, Bai H, Palli SR (2006) Mechanisms of midgut remodeling: juvenile hormone analog
505 methoprene blocks midgut metamorphosis by modulating ecdysone action Mechanisms of
506 development 123:530-547
507 Xu J et al. (2019) Identification of a germline‐expression promoter for genome editing in Bombyx mori
508 Insect science 26:991-999

509 Xu J et al. (2014) Functional characterization of the vitellogenin promoter in the silkworm, B ombyx mori
510 Insect molecular biology 23:550-557
511 Yang F, Kerns DL, Head GP, Price P, Huang F (2017) Cross‐resistance to purified Bt proteins, Bt corn and
512 Bt cotton in a Cry2Ab2‐corn resistant strain of Spodoptera frugiperda Pest management science
513 73:2495-2503
514 Zeng X, Chauhan C, Hou SX (2010) Characterization of midgut stem cell‐and enteroblast‐specific Gal4
515 lines in Drosophila Genesis 48:607-611
516 Zhang K et al. (2015) Identification and characterization of three novel hemocyte-specific promoters in
517 silkworm Bombyx mori Biochemical and biophysical research communications 461:102-108
518 Zhao G et al. (2014) Activation of BmGSTd1 promoter and regulation by transcription factor Krüppel (Kr)
519 in silkworm, Bombyx mori Gene 551:152-159
520 Zhou K, Goodman CL, Ringbauer J, Song Q, Beerntsen B, Stanley D (2020) Establishment of two midgut cell
521 lines from the fall armyworm, Spodoptera frugiperda (Lepidoptera: Noctuidae) In Vitro Cellular &
522 Developmental Biology-Animal 56:10-14

523

524 **Table 1. Germline transformation of transgenic constructs.**

| Plasmids | Injected eggs (n) | G0 Larvae (n) | G0 Pupae (n) | G1 Positive (n) |
|---|---|---|---|---|
| hr5-SfSP38/P2000: Luc | ~2200 | ~800 | ~600 | 46 |
| hr5-SfMG17/P2000: Luc | ~2400 | ~900 | ~700 | 31 |
| hr5-SfCalphotin/P799: Luc | ~2400 | ~1000 | ~700 | 56 |
| hr5-SfCPH38/P2000: Luc | ~2000 | ~800 | ~600 | 41 |
| SfCPH38/P2000: Luc | ~2000 | ~700 | ~600 | 29 |
| hr5-SfCPH38/P2000: CYP321A8 | ~2400 | ~600 | ~500 | 38 |
| SfCPH38/P2000: CYP321A8 | ~2400 | ~800 | ~600 | 79 |

525

526

## Figures

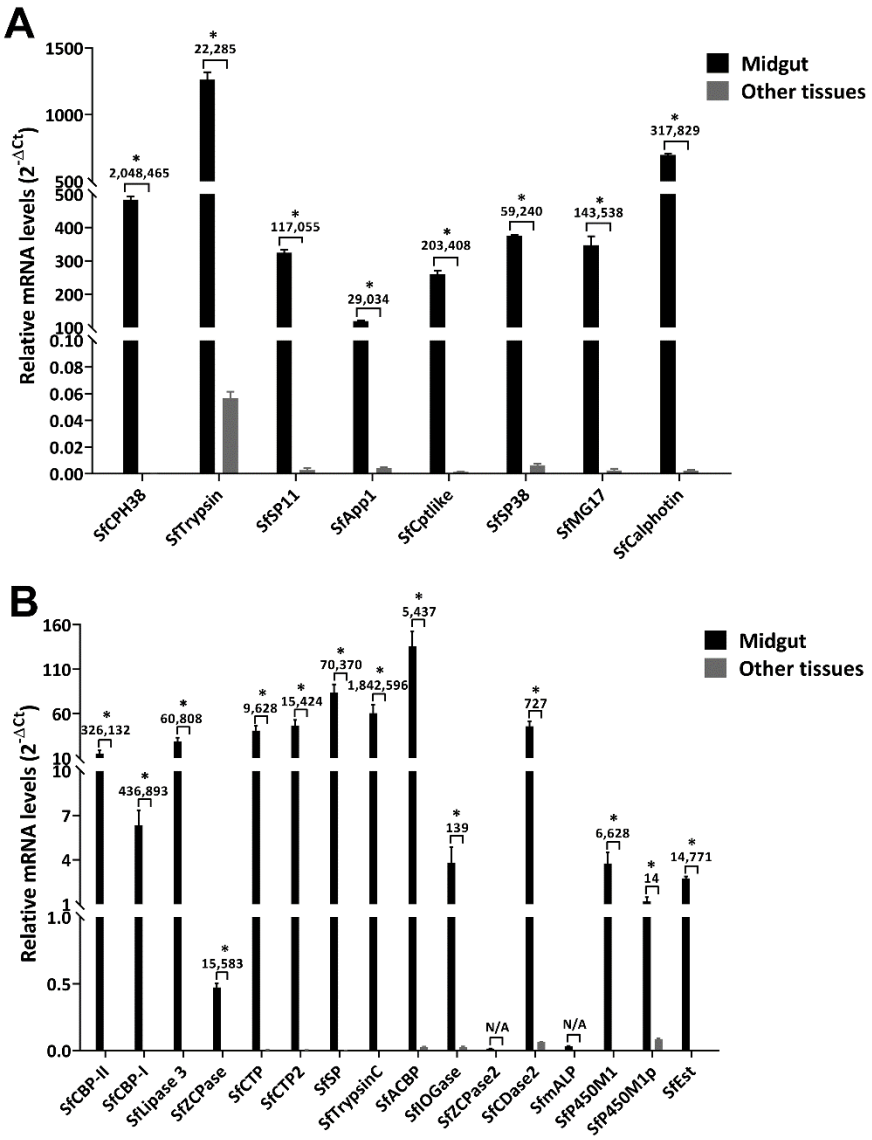



**Figure 1. Relative mRNA levels of genes that are highly expressed in the midgut.**

Midgut-specific genes expressed in the midgut (A) and other tissues (B) of 6th instar FAW larvae. Total RNA was isolated from the midgut and all other tissues and converted to cDNA, and cDNA and gene-specific primers were used in RT-qPCR to determine relative mRNA levels using *28S rRNA* as the reference gene. Means ± SD (n=3) are shown. Data were analyzed using student's t-test. *, $p<0.05$.

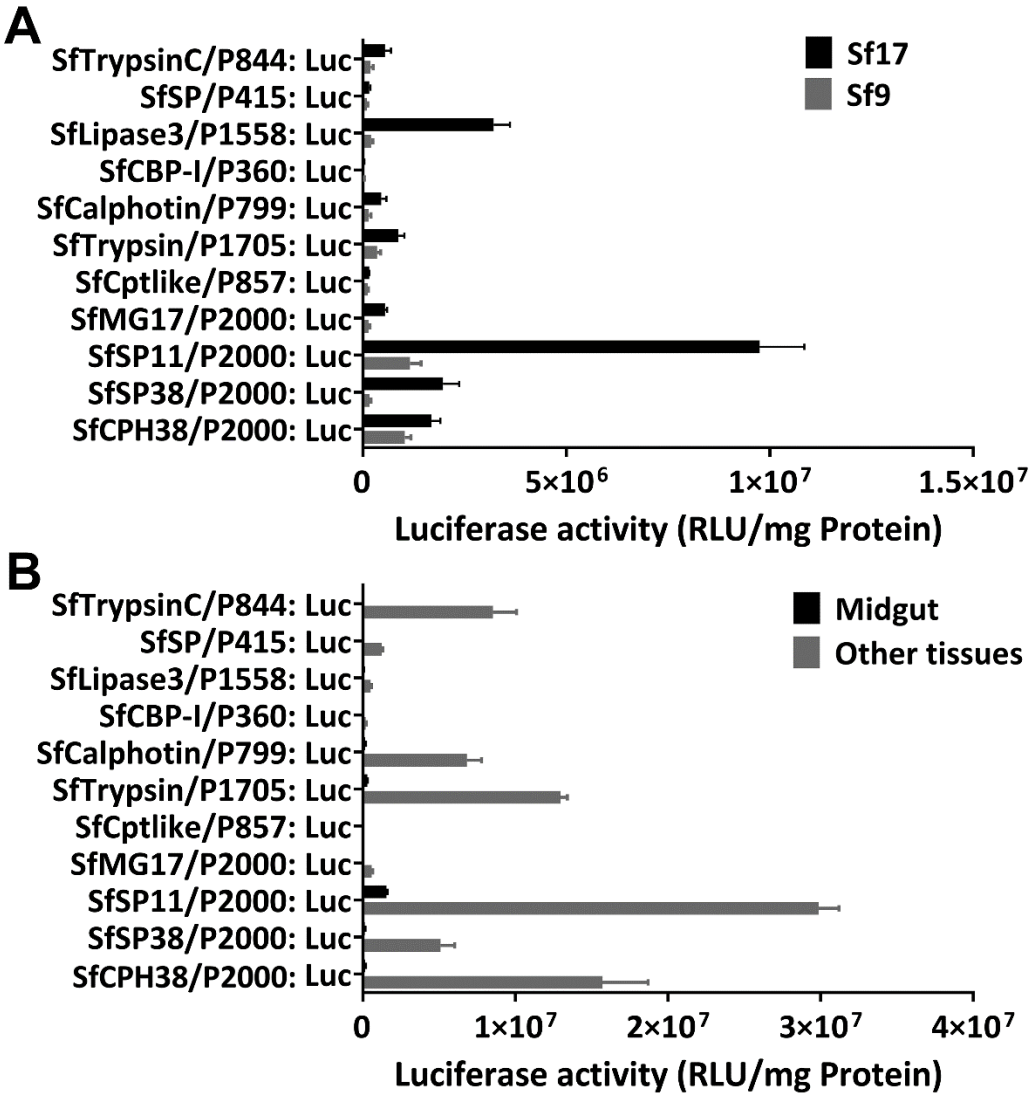



**Figure 2. Luciferase activity of reporter baculoviruses in FAW cell lines (A) and tissues (B).** Baculoviruses harboring a luciferase expression cassette under the control of candidate midgut-

specific promoters were generated. The viruses were added to Sf9 cells and Sf17 cell medium. Infected cells were collected, and luciferase activity was measured at 24 hr after adding the virus. Newly molted 6$^{th}$ instar larvae were injected with each virus. Midgut and other tissues dissected on the 3$^{rd}$ day postinjection were subjected to the luciferase activity assay. Means ± SD (n=4 for cells, n=3 for tissues) are shown.

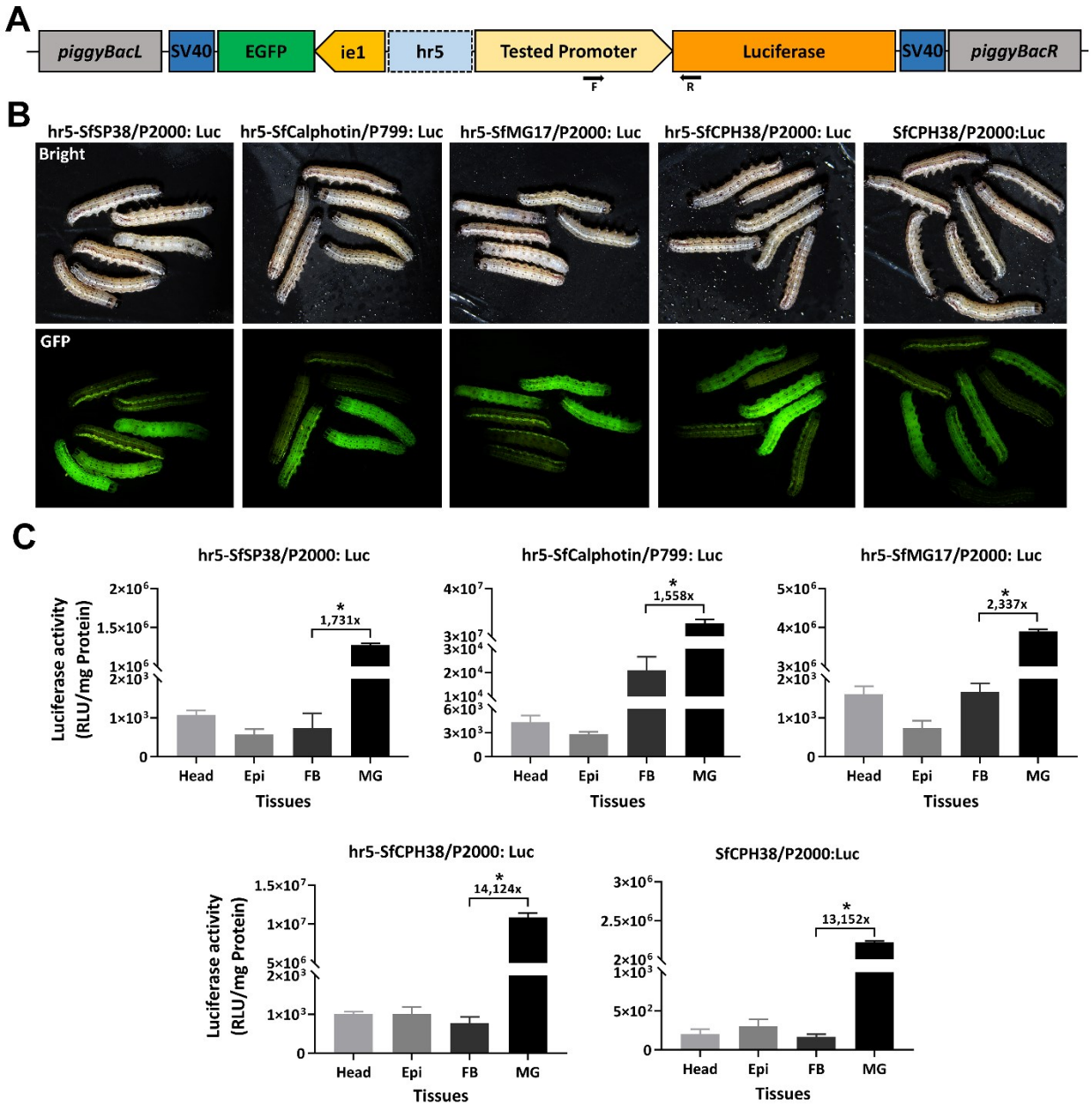
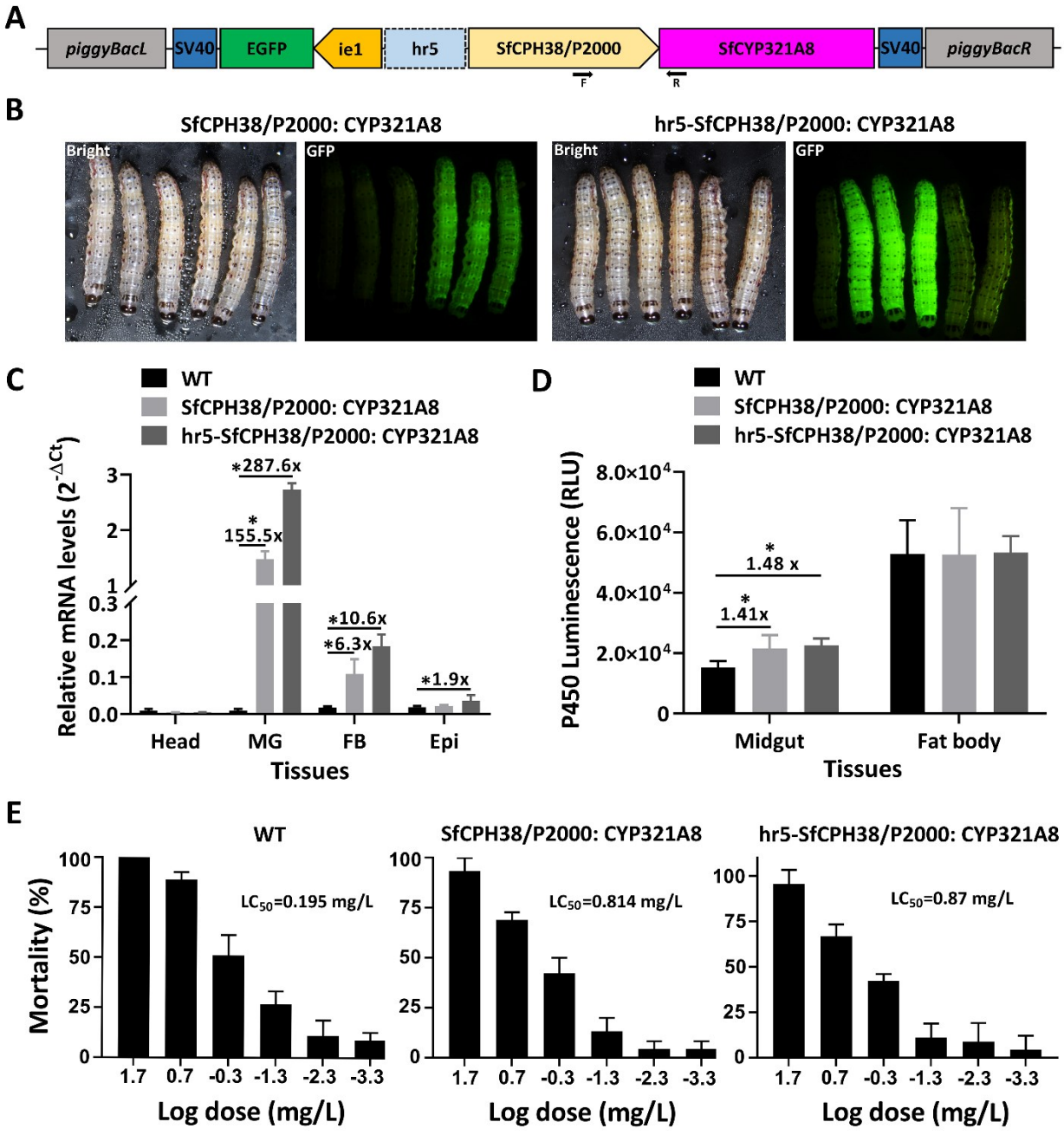


**Figure 3. Characterization of transgenic reporter FAW lines to determine midgut-specific promoter activity *in vivo*.** (A) Schematic representation of *piggyBac*-based reporter constructs. Expression of luciferase driven by four candidate midgut-specific promoters, SfSP38/P2000, SfCalphotin/P799, SfMG17/P2000, and SfCPH38/P2000. Expression of a marker gene, EGFP, is under the control of the ie1 promoter. The hr5 enhancer, highlighted in the dashed quadrangle, was inserted between ie1 and the midgut-specific promoter in some reporter constructs. (B) Fluorescence in GFP-negative and GFP-positive animals. (C) Luciferase activity in the head, epidermis (Epi), fat body (FB), and midgut (MG) of transgenic reporter animals. Tissues were dissected from transgenic $6^{th}$ instar larvae and subjected to luciferase activity assays. Means ± SD (n=3) are shown. Data were analyzed using student's t-test. *, $p<0.05$.




554

555 **Figure 4. Characterization of transgenic FAW overexpressing P450 in the midgut.** (A)
556 Schematic representation of *piggyBac*-based constructs expressing *SfCYP321A8* under the control
557 of a midgut-specific promoter, SfCPH38/P2000. Expression of a marker gene, EGFP, is under the
558 control of the ie1 promoter. The hr5 enhancer, highlighted in the dashed-line rectangle, is located
559 between the ie1 and SfCPH38/P2000 promoters. (B) Fluorescence in GFP-negative and GFP-

positive animals. (C) Relative mRNA levels of SfCYP321A8 in wild-type (WT) and two transgenic lines. Total RNA was isolated from four tissues, including the head, midgut (MG), fat body (FB), and epidermis (Epi). The RNA was converted to cDNA, and cDNA and gene-specific primers were used in RT-qPCR to determine relative mRNA levels using *28S rRNA* as the reference gene. Means ± SD (n=3) are shown. Data were analyzed using student's t-test. *, $p<0.05$. (D) P450-Glo assay of proteins extracted from the midgut and fat body from transgenic animals. The midgut and fat body were dissected from wild-type and transgenic FAW 6$^{th}$ instar larvae, and homogenates were prepared and assayed using Luciferin-Be as substrate. Mean ± SD (n=3) are shown. Data were analyzed using student's t-test. *, $p<0.05$. (E) Bioassay of deltamethrin in wild-type and transgenic neonate larvae. GFP-positive neonates were collected under a fluorescence microscope and fed leaf discs treated with different concentrations of deltamethrin. Mortality was recorded at 48 hr post-treatment. Mean ± SD (n=3) are shown.

**Supplementary information**

# Midgut-specific expression of *CYP321A8* P450 gene increases deltamethrin tolerance in the fall armyworm, *Spodoptera frugiperda*

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**Supplementary Tables**

**Table S1**

## Table S1. Primers used in this study.

| Primer | Sequence (5'-3') |
|---|---|
| SfCPH38-QF | TGACACTCACCCCATCTCTG |
| SfCPH38-QR | ACGTCAGCAGAAGCCTTGTT |
| SfTrysin-QF | TGGATGGGGAACTACCTCTG |
| SfTrysin-QR | CGAAAGAGCAGACACCAACA |
| SfSP11-QF | TCCTCCGCATTAGCTCTGTT |
| SfSP11-QR | GTTGGAGATCACGGGGAGTA |
| SfApp1-QF | TATCAAGCCTGAGCCTGTCC |
| SfApp1-QR | GCCTGGTTGATGTTCAGGAT |
| SfCptlike-QF | TAGCAGAAGCGTGGTTGATG |
| SfCptlike-QR | ATGGATTGACTTCCCCCTTC |
| SfSP38-QF | ACAACCTTGCCCTCATCAAC |
| SfSP38-QR | CGAACTCGGTAGCCTCAAAG |
| SfMG17-QF | AAATCCTTCGTTGCCCTGTT |
| SfMG17-QR | GGATGGCAGGGTACTCATTG |
| SfCalphotin-QF | CCATCATTGAGAGCCCAGAG |
| SfCalphotin-QR | ACGTCAGCAGAAGCCTTGTT |
| SfCBP-II-QF | CCTCTTGTTCCGATGACGAT |
| SfCBP-II-QR | CTGGGGTCATCGAAGTTGTT |
| SfCBP-1-QF | TAAGCGGTGATGGAGGAAAC |
| SfCBP-1-QR | TGACATTTTCAGGCCAATCA |
| SfLipase3-QF | GAACCAGAAGAAGCCTGTCG |
| SfLipase3-QR | TGCCTATTTCATCCCAGGAG |
| SfZCPase-QF | CGGTCTTTTGGAAATGGAGA |
| SfZCPase-QR | CTTCATTCCAGAGCCAGGAG |
| SfCTP-QF | CATCACCGCCTACGGATACT |
| SfCTP-QF | CAAGGAACCACCACACACAC |
| SfCTP2-QF | TCCAGAATTGTTGGTGGTCA |
| SfCTP2-QR | ACCGAGAACAACGGTGAATC |
| SfSP-QF | TCCTTGTCTGCCACTCTCCT |
| SfSP-QR | CCAGGACACGATACCGAACT |
| SfTrypsinC-QF | TCTTCGGAGTCTGGACTGGT |
| SfTrypsinC-QR | CCGGTGTTACGGTAGGAAGA |
| SfACBP-QF | TCCGTCAGGAACTGGAAGAC |
| SfACBP-QR | GCCAGTTTCTCAGCCATCTC |
| SfIOGase-QF | GTGGGCCAAGTCTATCGTGT |

| | |
|---|---|
| SfIOGase-QR | CCTTCTCTGGGATCTTGCTG |
| SfZCPase2-QF | TCACTGACATGGCCGATAAA |
| SfZCPase2-QR | ACCCAGTCAACACTCGTTCC |
| SfCDase2-QF | TCTACCCAGGATTGCGTTGT |
| SfCDase2-QR | GCAGAGATGAAGGCTTCGTG |
| SfAPN-QF | AGAGGCCAATACGCGACTAA |
| SfAPN-QR | AGATGGTCGAGCGTAGATGG |
| SfmALP-QF | GGAATCGTGAGCAGCTGATG |
| SfmALP-QR | CTAGCTCTACCCAGTTGCGA |
| SfP450M1-QF | TGATCTCGGACTTGCACTTG |
| SfP450M1-QR | GCGACCGCTATCTTCATCTC |
| SfP450M1p-QF | TTGGCTACGAGACGGGTTAC |
| SfP450M1p-QR | TCGCAGTTTCACAGATGGAG |
| SfEst-QF | GGAGCCTCTCTGCTGTATGG |
| SfEst-QR | ATGGTAGGACACCGAAGCAC |
| SfCYP321A8-QF | TCCTAACACCTGCGCTCTTT |
| SfCYP321A8-QR | GCCGTAAACAACGGAGTCAT |
| SfAPN1-QF1 | CTATACGCGAGCGTTGAACA |
| SfAPN1-QR1 | TCAGATGCATACGGAGCAAG |
| SfAPN1-QF2 | GCTATGGAAAACTGGGGCCT |
| SfAPN1-QR2 | CTCAGCCTTGTCCGTCATGT |
| SfAPN2-QF1 | TCAGCCCTGACTCTTGGACT |
| SfAPN2-QR1 | GCGACAGCATTGAAAGTTGA |
| SfAPN2-QF2 | TAGCGACTCGTCTCCTGTCA |
| SfAPN2-QR2 | CGCAAAGTGCTCAACAACGA |
| SfAPN3-QF1 | TATTCCCGATTTTGGAGCTG |
| SfAPN3-QR1 | ATAGAACGTCCCACCAGTCG |
| SfAPN3-QF2 | AGCAACTGCAGCTCGTTTTG |
| SfAPN3-QR2 | GAGGTGATGAGGGTTTGGCA |
| SfAPN4-QF1 | CAAAACCGTGGTTCTCCAGT |
| SfAPN4-QR1 | GCTTGGTTGAGCTGAAGTCC |
| SfAPN4-QF2 | GCTCAGACATTACTTGCGCG |
| SfAPN4-QR2 | GGTGGGTCTAACGCCAGAAA |
| SfAPN5-QF1 | TCTGGATCAACGAGGGATTC |
| SfAPN5-QR1 | GGGACTATTGACGGTGTGCT |
| SfAPN5-QF2 | AATCTGGCTACAGGCACCAC |
| SfAPN5-QR2 | CAGGCTCATCGAAGCTAGGG |
| SfAPN6-QF1 | GGGCTTCTGCTGGTACTGAG |
| SfAPN6-QR1 | CCCTCGTTGATCCAAGTGTT |
| SfAPN6-QF2 | GAAGTTCCCGCCTCGAGAAA |
| SfAPN6-QR2 | CTCAGCTTTGGCCTGGATGA |

| | |
|---|---|
| Luciferase-pfF | ATCACCATCACCATCACGGGAATTCATGGAAGACGCCAAAAACATAAAGAAAG |
| Luciferase-pfR | AGTACTTCTCGACAAGCTTGGTACCTTACACGGCGATCTTTCCGCCCT |
| SfCptlike-pfPF | TAGTTCTAGTGGTTGGCTACGTAgtcggcttgtaacaagactatgcc |
| SfCptlike-pfPR | CGGTTTCTTTCATggtggtGGATCCccccttaccctgaacttaactaag |
| SfTrypsin-pfPF | TAGTTCTAGTGGTTGGCTACGTAgaatcgacgtagacatcgcagcagg |
| SfTrypsin-pfPR | CGGTTTCTTTCATggtggtGGATCCtttgttgctacttgtgaacaagtgtac |
| SfCalphotin-pfPF | TAGTTCTAGTGGTTGGCTACGTAGTCCTTTCGGTCGTTGGACACGCACAG |
| SfCalphotin-pfPR | CGGTTTCTTTCATggtggtGGATCCTTCTGGAATAATAAATTATGGATATAC |
| SfCBP-I-pfPF | TAGTTCTAGTGGTTGGCTACGTAGGTTTACCGGGACTCCGGCTCG |
| SfCBP-I-pfPR | CGGTTTCTTTCATggtggtGGATCCACTGACGCGTGTTTTAAACAATTT |
| SfLipase3-pfPF | TAGTTCTAGTGGTTGGCTACGTAAATGTGATCGTGTAACTGATAGGA |
| SfLipase3-pfPR | CGGTTTCTTTCATggtggtGGATCCTGTTCACTCGCAGCCGTTTAGTA |
| SfSP-pfPF | TAGTTCTAGTGGTTGGCTACGTAggtaccgagtcctcttagtagac |
| SfSP-pfPF | CGGTTTCTTTCATggtggtGGATCCgtttaacaacgcccgcagtgagt |
| SfTrypsinC-pfPF | TAGTTCTAGTGGTTGGCTACGTAatgaaatgatccgactgcattacac |
| SfTrypsinC-pfPR | CGGTTTCTTTCATggtggtGGATCCgtttgtgtttgaatcgaagactact |
| SfCPH38-pfPF | TAGTTCTAGTGGTTGGCTACGTAAGGTACTGTTGTACCCTCTATAGG |
| SfCPH38-pfPR | CGGTTTCTTTCATggtggtGGATCCTTTGATAGTATCTGAAAAGTGATG |
| SfSP38-pfPF | TAGTTCTAGTGGTTGGCTACGTACAATCATTTTATTCTTTACTACGG |
| SfSP38-pfPR | CGGTTTCTTTCATggtggtGGATCCCTTCCAACGGCCAATTCACTGAC |
| SfSP11-pfPF | TAGTTCTAGTGGTTGGCTACGTAGGAAAATGTGTAAATGTAATGGT |
| SfSP11-pfPR | CGGTTTCTTTCATggtggtGGATCCTATTTATAGCATAAATTATACCT |
| SfMG17-pfPF | TAGTTCTAGTGGTTGGCTACGTATCCTGCCTTTATGCAGATGACCTA |
| SfMG17-pfPR | CGGTTTCTTTCATggtggtGGATCCTGTGATGGGTTGAGGTATTTACTGC |
| Luciferase-pbF | gacactggcggcgacaagagacgtcATGGAAGACGCCAAAAACATAAAGAAAG |
| Luciferase-pbR | ctgattatgatctagagtcgggcccTTACACGGCGATCTTTCCGCCCT |
| SfCYP321A8-pbF | gacactggcggcgacaagagacgtcATGTTGTTTCTACCTTTGAGTTTGATAG |
| SfCYP321A8-pbR | ctgattatgatctagagtcgggcccCTATTTGATATTTCTCGGAATTAGTTGG |
| SfCPH38-pbFLuc | ctcactatagggcgaattgggtaccAGGTACTGTTGTACCCTCTATAGG |
| SfCPH38-pbRLuc | TATGTTTTTGGCGTCTTCCATgacgtcTTTGATAGTATCTGAAAAGTGATG |
| SfSP38-pbFLuc | ctcactatagggcgaattgggtaccCAATCATTTTATTCTTTACTACGG |
| SfSP38-pbRLuc | TATGTTTTTGGCGTCTTCCATgacgtcCTTCCAACGGCCAATTCACTGAC |
| SfSP11-pbFLuc | ctcactatagggcgaattgggtaccGGAAAATGTGTAAATGTAATGGT |
| SfSP11-pbRLuc | TATGTTTTTGGCGTCTTCCATgacgtcTATTTATAGCATAAATTATACCT |
| SfMG17-pbFLuc | ctcactatagggcgaattgggtaccTCCTGCCTTTATGCAGATGACCTA |
| SfMG17-pbRLuc | TATGTTTTTGGCGTCTTCCATgacgtcTGTGATGGGTTGAGGTATTTACTGC |
| SfCPH38-pbFLuc/P450 | ctcactatagggcgaattgggtaccAGGTACTGTTGTACCCTCTATAGG |
| SfCPH38-pbF2Luc/P450 | cgataatagtgttgtgcaacctcgagAGGTACTGTTGTACCCTCTATAGG |
| SfCPH38-pbRLuc | TATGTTTTTGGCGTCTTCCATgacgtcTTTGATAGTATCTGAAAAGTGATG |
| SfCPH38-pbRP450 | CTCAAAGGTAGAAACAACATgacgtcTTTGATAGTATCTGAAAAGTGATG |
| SfAPN6-pbFP450 | ctcactatagggcgaattgggtacctaagtaggtgcatttaattgact |

| | |
|---|---|
| SfAPN6-pbRP450 | CTCAAAGGTAGAAACAACATgacgtcttctgcaaatattgaaggaacaa |
| SfSP11-pbFP450 | ctcactatagggcgaattgggtaccGGAAAATGTGTAAATGTAATGGTT |
| SfSP11-pbRP450 | CTCAAAGGTAGAAACAACATgacgtcTATTTATAGCATAAATTATACCT |

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608 **Table S2. NCBI accession numbers of genes studied**

| Gene | ID |
|---|---|
| SfCPH38 | LOC118265824 |
| SfTrypsin | LOC118274791 |
| SfSP11 | LOC118267724 |
| SfCptlike | LOC118265666 |
| SfSP38 | LOC118273955 |
| SfMG17 | LOC118266716 |
| SfCBP-II | LOC118274188 |
| SfCBP-I | LOC118274195 |
| SfLipase3 | LOC118272788 |
| SfZCPase | LOC118275823 |
| SfCTP | LOC118281920 |
| SfCTP2 | LOC118281927 |
| SfSP | LOC118267797 |
| SfTrypsinC | LOC118278126 |
| SfACBP | LOC118267206 |
| SfOGase | LOC118274792 |
| SfZCPase2 | LOC118275719 |
| SfCDase2 | LOC118279197 |
| SfmALP | LOC118271569 |
| SfP450M1 | LOC118270597 |
| SfP450M1p | LOC111352929 |
| SfEst | LOC118273652 |
| SfAPN1 | LOC118268536 |
| SfAPN2 | LOC118268089 |
| SfAPN3 | LOC118268537 |
| SfAPN4 | LOC118268536 |
| SfAPN5 | LOC118268345 |
| SfAPN6 | LOC118268367 |

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**Table S2. Germline transformation of two additional transgenic P450 constructs**

| Plasmids | Injected eggs (n) | G0 Larvae (n) | G0 Pupae (n) | G1 Positive (n) |
|---|---|---|---|---|
| SfAPN6/P1949: CYP321A8 | ~2000 | ~800 | ~600 | 32 |
| SfSP11/P2000: CYP321A8 | ~2000 | ~800 | ~500 | 91 |

**Supplementary Figures**

**Figure S1**

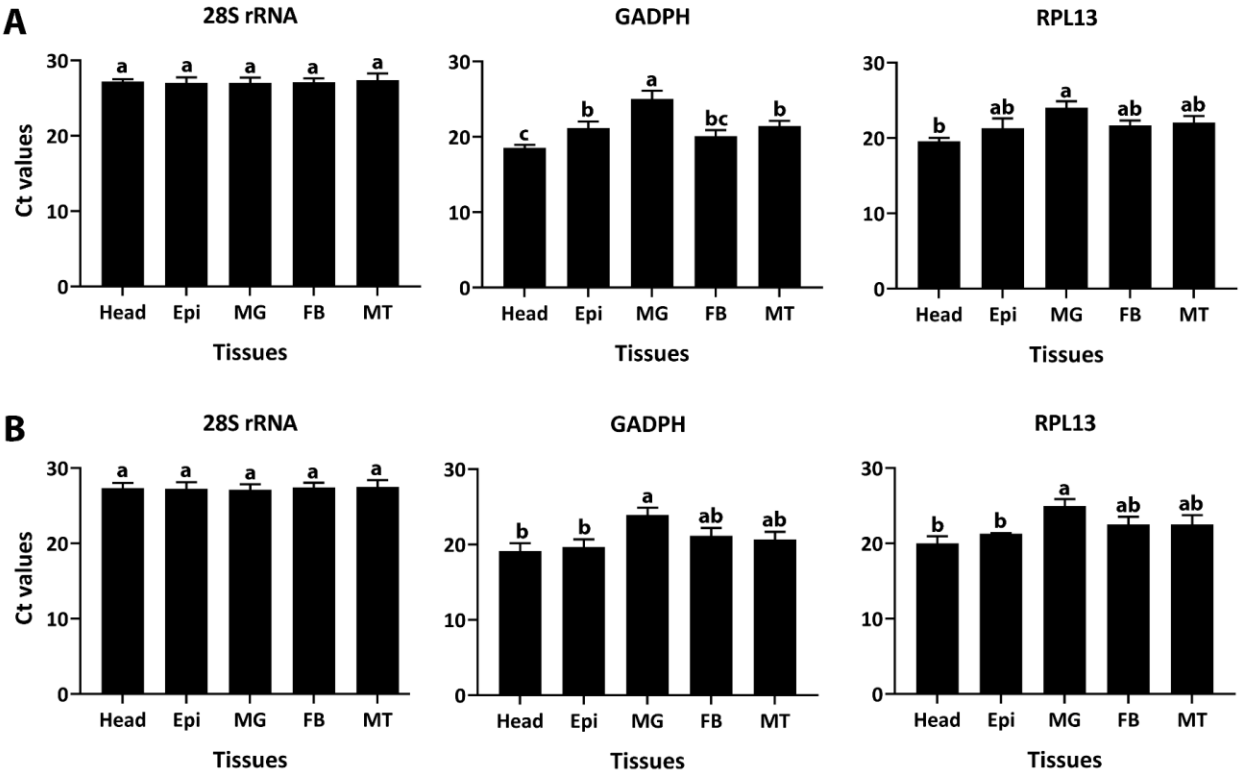


**Figure S1. Stability analysis of three reference genes, *28S rRNA*, *GADPH*, and *RPL13* in the FAW.** Threshold cycle (Ct) values of the reference genes in wild-type (A) and transgenic hr5-SfCPH38/P2000:CYP321A8 FAW (B) tissues, including head, midgut (MG), fat body (FB), Malpighian tubules (MT), and epidermis (Epi), of 6$^{th}$ instar larvae. Mean ± SD (n=3) are shown.

Significant differences among multiple groups were analyzed using one-way ANOVA followed by the Tukey HSD test.

**Figure S2**

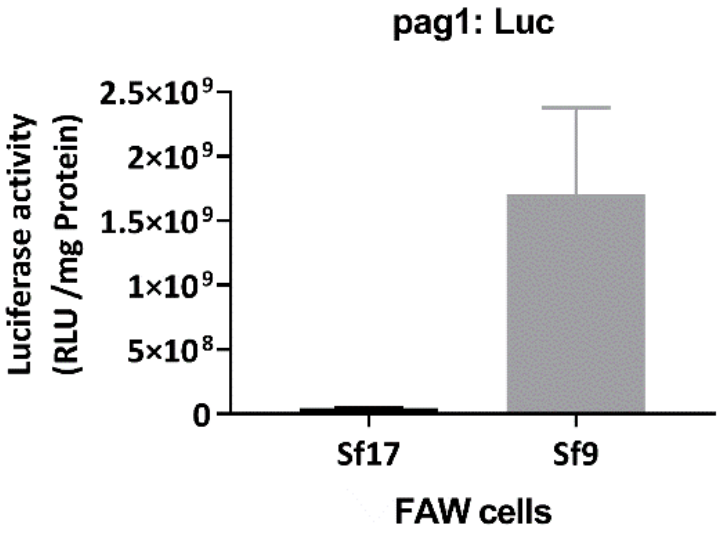


**Figure S2. Luciferase activity of a positive control reporter baculoviruses in cell lines.** The promoter region of an early baculovirus gene, *pag1*, was used to drive the expression of the luciferase gene. The reporter baculovirus, pag1: Luc, was produced and used to infect Sf9 cells and Sf17 cells. The luciferase activity was determined at 24 hr post-infection. Mean ± SD (n=4) are shown.

**Figure S3.**

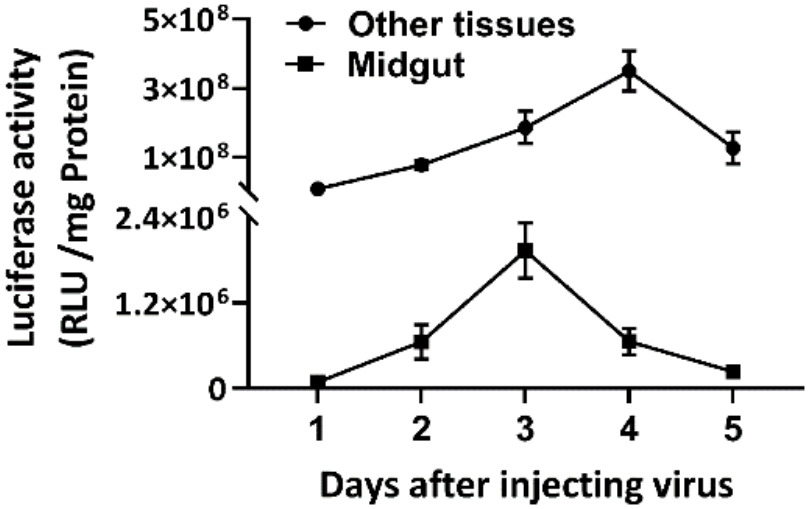


**Figure S3. Time-course of luciferase activity in the midgut and other tissues of 6th instar larvae injected with pag1:Luc reporter baculovirus.** The virus was injected into the newly molted 6th instar larvae. Midgut and other tissues were dissected at 1, 2, 3, 4, and 5 days post-injection. Homogenates were prepared and subjected to the luciferase assay. Mean ± SD (n=3) are shown.

**Figure S4.**

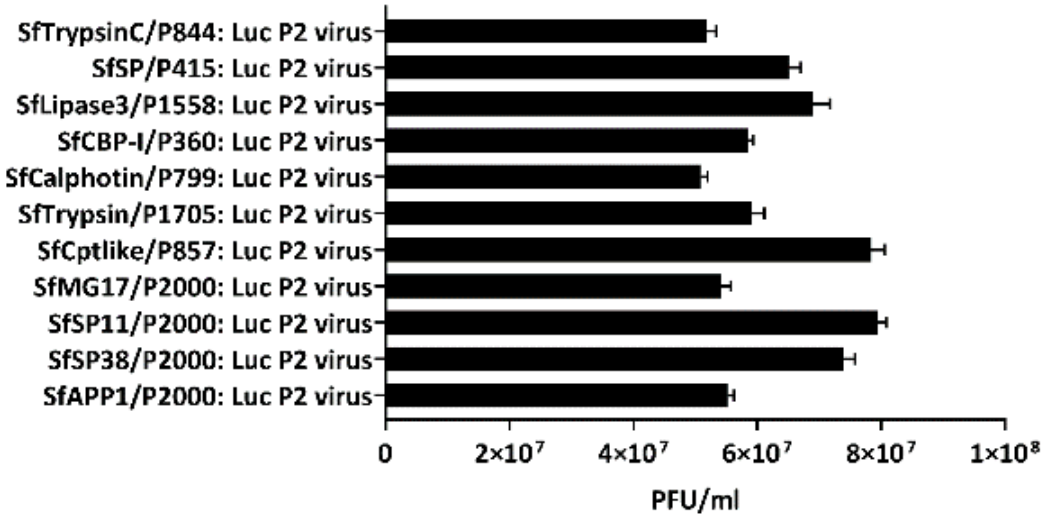

**Figure S4. Determination of the plaque-forming units (PFU) of reporter baculoviruses.** The viral nucleic acids of P2 reporter baculoviruses were purified and subjected to RT-qPCR to measure the PFU of reporter baculoviruses. Mean ± SD (n=4) are shown.

**Figure S5**

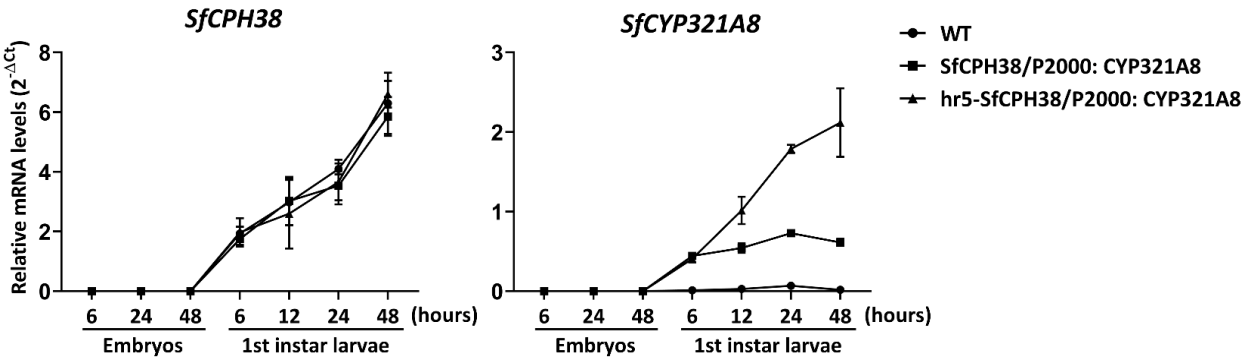


**Figure S5. Relative mRNA levels of *SfCPH38* and *SfCYP321A8* in embryos and 1st instar larvae of wild-type (WT) and two transgenic lines.** Eggs and 1st instar larvae were collected at different time points during development. Total RNA was isolated from the midgut and all other tissues. The RNA was converted to cDNA and the cDNA and gene-specific primers were used in RT-qPCR to determine relative mRNA levels using *28S rRNA* as the reference gene. Means ± SD (n=3) are shown.

**Figure S6**

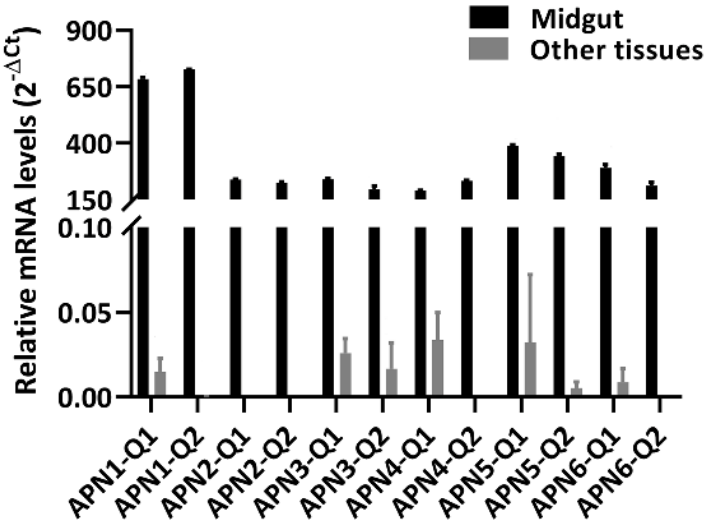


**Figure S6. Relative mRNA levels of six *SfAPN* genes in the midgut and other tissues of 6th instar larvae.** Total RNA was isolated from the midgut and all other tissues and converted to cDNA. Two pairs of gene-specific primers were used in RT-qPCR to determine relative mRNA levels using *28S rRNA* as the reference gene. Means ± SE (n=3) are shown.

684 **Figure S7**

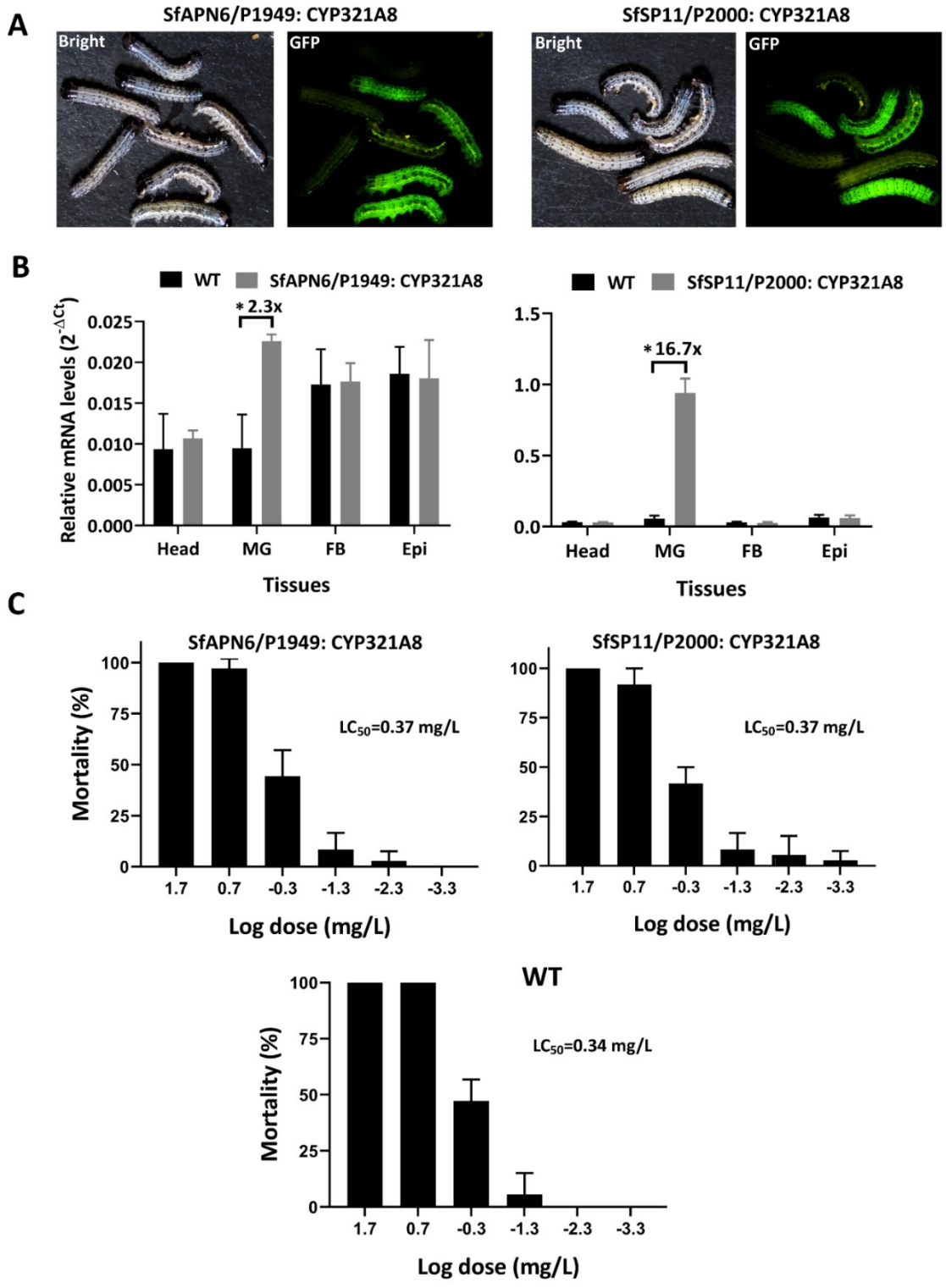


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**Figure S7. Characterization of transgenic FAW lines expressing *SfCYP321A8* under the control of SfAPN6/P1949 and SfSP11/P2000 promoters.** (A) Fluorescence in GFP-negative and –positive animals. (B) Relative mRNA levels of *SfCYP321A8* in wild-type (WT), and two transgenic lines. Total RNA was isolated from four tissues, including head, midgut (MG), fat body (FB), and epidermis (Epi). The RNA was converted to cDNA and the cDNA and gene-specific primers were used in RT-qPCR to determine relative mRNA levels using 28S rRNA as the reference gene. Means ± SD (n=3) are shown. Data were analyzed using student's t-test. *, $p<0.05$. (C) Deltamethrin bioassay in wild-type and transgenic neonate larvae. The GFP-positive neonates were collected under the fluorescent microscope and fed on leaf discs treated with different concentrations of deltamethrin. Mortality was recorded at 48 hr post-treatment. Mean ± SD (n=3) are s