Title:

Sublethal doses of broflanilide prevents molting in the fall armyworm, *Spodoptera frugiperda* via altering molting hormone biosynthesis

Running title: Devolopmental effects of broflanilide on fall armyworm

Full names of authors:

Zhong Qiang Jia [a], En Ling Zhan [a], Su Gui Zhang [a], Andrew K. Jones [b], Lin Zhu [a], Ying Nan Wang [a], Qiu Tang Huang [a], Zhao Jun Han [a], Chun Qing Zhao [a, *]

The authors' institutional affiliations at which the work was carried out:

[a]. Key Laboratory of Integrated Pest Management on Crops in East China, Ministry of Agriculture, Nanjing Agricultural University, Nanjing, 210095, P.R. China;

[b]. Department of Biological and Medical Sciences, Oxford Brookes University, Oxford, UK;

Corresponding author: Chun Qing Zhao;

Tel: +86-025-84399025;

Fax: +86-025-84399063;

E-mail: zcq@njau.edu.cn;

E-mail address lists:

1) Zhong Qiang Jia, zqjia2018@yeah.net;

2) En Ling Zhan, eileenzhan0904@163.com;

3) Su Gui Zhang, 1445819559@qq.com;

4) Andrew K. Jones, a.jones@brookes.ac.uk;

5) Lin Zhu, 2966427932@qq.com;

6) Ying Nan Wang, wyn19990117@163.com;

7) Qiu Tang Huang, Hqiutang@163.com;

8) Zhao Jun Han, zjh@njau.edu.cn;

9) Chun Qing Zhao, zcq@njau.edu.cn;

Abbreviations

cDNA, complementary DNA; FAW, fall armyworm; GABA, γ-aminobutyric acid; GABAR, GABA receptor; JH, juvenile hormone; MH, molting hormone; ORF, open reading frame; CYP, cytochrome P450; RDL, resistance to dieldrin gene; RT-qPCR, real-time quantitative polymerase chain reaction.

ABSTRACT

Broflanilide is a novel insecticide with a unique mode of action on the insect GABA receptor and is registered worldwide for the control of agricultural pests. It shows high efficacy in controlling the fall armyworm (FAW) *Spodoptera frugiperda*, which is a destructive pest to various crops. FAW were exposed to sublethal concentrations of broflanilide to determine its impact on insect development. Sublethal doses (LD_{10} and LD_{30}) caused failure of ecdysis, reduced body length of larvae, malformation of pupae, and vestigial wing formation in adults. Also, broflanilide at LD_{30} significantly reduced the amount of molting hormone (MH) . After exposure to LD_{10} or LD_{30} broflanilide, expression of five Halloween genes, which participate in MH biosynthesis, were found to be altered. Specifically, the transcript levels of *SfrCYP307A1* (Spook), *SfrCYP314A1* (Shade) and *SfrCYP315A1* (Shadow) in 3^{rd} day larvae were significantly decreased as well as *SfrCYP302A1* (Disembodied) and *SfrCYP306A1* (Phantom) in 5^{th} day pupae. In contrast, the transcript levels of *SfrCYP302A1* in 3^{rd} day larvae, *SfrCYP307A1* and *SfrCYP314A1* in 5^{th} day pupae, and *SfrCYP306A1*, *SfrCYP307A1* and *SfrCYP315A1* in 0.5^{th} day adults were significantly increased. Our results demonstrate that broflanilide caused the failure of ecdysis in FAW possibly by influencing the intake of cholesterol through inhibition of feeding and also via altering expression of genes important for MH biosynthesis. (Words count: 214)

Keywords: broflanilide; sublethal effects; molting hormone; Halloween genes; *Spodoptera frugiperda*

1. Introduction

The fall armyworm (FAW), *Spodoptera frugiperda*, is one of the most destructive crop pests worldwide including in China, due to its polyphagous nature and voracity of feeding (Day, et al., 2017; Kebede and Shimalis, 2019). It has invaded Africa and Asia (Goergen, et al., 2016; Li, et al., 2020) from its area of origin in the Americas (Sparks, 1979). To date, FAW has become resistant to many types of chemical and biological insecticides, such as diamides (e.g., chlorantraniliprole) (Bolzan, et al., 2019), pyrethroids (e.g., lambda-cyhalothrin) (Yu, et al., 2003), and *Bacillus thuringiensis* (Bt) toxins (e.g., Cry1F) (Vassallo, et al., 2019). Therefore, introduction of novel insecticides such as broflanilide (Casida, 2015; Nakao and Banba, 2016) is urgent for the continued control of FAW.

Broflanilide is a novel and representative meta-diamide insecticide, which acts on the insect γ-aminobutyric acid (GABA) receptor (GABAR) with a unique mode of action (Casida, 2015; Nakao and Banba, 2016). Previous studies suggested that broflanilide is firstly metabolized to desmethyl-broflanilide, and then acts as a non-competitive antagonist on the RDL (resistant to dieldrin) GABAR (Nakao and Banba, 2016). Broflanilide shows high insecticidal activity on various agricultural pests, such as the cotton bollworm *Helicoverpa armigera* (Hübner), the beet armyworm *Spodoptera exigua* (Hübner), the common cutworm *Spodoptera litura* Fabricius, the diamondback moth *Plutella xylostella* (Linnaeus) and the two-spotted spider mite, *Tetranychus urticae* Koch (Katsuta, et al., 2019; Shen, et al., 2021; Tang, et al., 2021). Broflanilide was registered as an insecticide in Australia and China in 2019 and 2020, respectively, and is expected to be used to protect crops, especially against lepidopteran insect pests (Sun, et al., 2021). A recent study indicated that broflanilide exhibits higher activity on FAW than fipronil, which also targets RDL (Zhan, et al., 2021). In addition to lethal activity, larvae treated with broflanilide showed reduced appetite, shorter body length, weight loss and the inability to molt (Zhan, et al., 2021), which are considered as sublethal effects.

As is well-known, the molting hormone (MH, also known as 20-hydroxyecdysone) participates in many physiological processes of insects including ecdysis, pupariation and reproduction (Xu, et al., 2018). MH works via nuclear hormone receptors to direct spatial and temporal regulation of gene transcription including genes required for cell death as well as the removal of obsolete larval tissues (Xu, et al., 2020). MH is synthesized from cholesterol through a series of biochemical reactions, which are mediated by a set of cytochrome P450 (CYP) enzymes encoded by Halloween genes such as *CYP302A1* (Disembodied), *CYP306A1* (Phantom), *CYP307A1* (Spook), *CYP314A1* (Shade) and *CYP315A1* (Shadow) (Rewitz, et al., 2006). Specifically, cholesterol is converted firstly into: 7-dehydrocholesterol by Neverland oxygenase in the prothymic gland cells, (Yoshiyama, et al., 2006); secondly into 5β-ketodiol by Shroud (a short-chain dehydrogenase/reductase), CYP307A1 and Cyp6t3 (Ono, et al., 2006; Ou and

King-Jones, 2013); thirdly into ecdysone via CYP306A1 in the endoplasmic reticulum, as well as CYP302A1 and CYP315A1 in the mitochondria; and finally into MH via CYP314A1 after being transported to peripheral tissues by hemolymph circulation (Ou and King-Jones, 2013).

However, the potential mechanism of how sublethal doses of broflanilide alter the ecdysis/development of FAW is still unclear. The objective of this study was to assess the sublethal effects of broflanilide on FAW MH levels and Halloween gene expression.

2. Materials and methods

2.1. Insect strain and insecticide

FAW was collected from Guangdong province (113ºE, 23ºN) in April 2019, and reared with a standard artificial diet at a temperature of 26 ± 2 ºC, photoperiod of 16:8 h (light: dark) and relative humidity (RH) of 60% - 70% (Zhan, et al., 2021). Broflanilide with technical grade (purity ≥ 98.67 %) was obtained from BASF Corporation (Florham Park, NJ).

2.2.Treatment of FAW larvae with sublethal doses of broflanilide

Third instar FAW larvae were treated with broflanilide at LD_{10} or LD_{30} as described previously (Jia, et al., 2020; Meng, et al., 2020). Briefly, broflanilide was dissolved in acetone before being diluted in 0.1% Tween-80 solution (1:1, v/v) and mixed with a 100-fold volume of fresh artificial diet (v/v) to obtain a mixture of artificial diet containing broflanilide at a sublethal dose of LD_{10} (0.074 mg kg^{-1}) or LD_{30} (0.132 mg kg^{-1}). The artificial diet containing broflanilide was cut into flakes and placed into 12-well plates. Two hundred newly emerged 3rd instar FAW larvae were selected and transferred into the 12-well plates individually for each treatment. The artificial diet containing only acetone and 0.1% Tween-80 was used as control. Three days later, living FAW were transferred into another 12-well-plate containing the fresh artificial diet without broflanilide, acetone or Tween-80. Six individually treated larvae from each group at the 3rd day (3rd day larvae), pre-pupae, female pupae at 1st day (1st day pupae), 3rd day (3rd day pupae) and 5th day (5th day pupae), and female adults at 0th day (less than half hour of adult), half day (0.5th day adult) and 1st day (1st day adult) were collected in triplicate, immediately frozen in liquid nitrogen then stored at -80 ºC for further assays.

2.3. Detection of MH titer

Frozen FAW samples were ground into powder using pre-cooled pestle and mortar, and 100-200 mg powder from

each sample was used to determine the MH titer using the Insect Ecdysone ELISA Kit (Shanghai Enzyme-linked Biotechnology Co., Ltd, Shanghai, CHN) (Peng, et al., 2019).

2.4. Identification of putative Halloween genes

Extraction of total RNA was performed using TRIzol® Reagent (Invitrogen, Carlsbad, CA) followed by purification with an RNA Clean Kit (TianGen Biotech Co., Ltd, Beijing, CHN). Complementary DNA (cDNA) was generated using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Biomedical Technology Co. Ltd, Beijing, CHN). Putative Halloween genes were identified from the FAW transcriptome (GenBank: GESP00000000.1) using local BLAST with amino acid query sequences from *Bombyx mori* L. [GenBank accession number: BAD99022.1 (*Bm*CYP302A1), NP_001106222.1 (*Bm*CYP306A1), NP_001104833.1 (*Bm*CYP307A1), NP_001106219.1 (*Bm*CYP314A*1*) and BAD23845.1 (*Bm*CYP315A1)]. Open reading frames (ORF) of FAW Halloween genes were amplified by PCR using specific primers (**Table 1**) and 2 × Phanta® Max Master Mix (Vazyme Biotech Co., Ltd, Nanjing, Jiangsu province, CHN). The amplified cDNA products were visualized by 1.5% agarose gel electrophoresis then purified using the *EasyPure*® PCR Purification Kit (TransGen Biotech Co., Ltd, Beijing, CHN) before being ligated into the *pEASY*® - Blunt 3 Cloning Vector (TransGen Biotech Co., Ltd). The combined vectors were transferred into *Trans*1-T1 Phage Resistant Chemically Competent Cells (TransGen Biotech Co., Ltd) and cloned ORFs were sequenced using the Sanger method (BGI Tech Solutions Co., Limited, Beijing, CHN).

The verified nucleotide sequences were translated into amino acids using DNAMAN 7 software (Lynnon Corporation, San Ramon, CA), and the conserved domains were predicted by alignment with other published orthologs from *B. mori* and *H. armigera*. The confirmed amino acid sequences were used to construct phylogenetic trees with 1000 bootstrap replications using MEGA 7 with the neighbor-joining method (Kumar, et al., 2016). The phylogenetic tree was annotated using the EvolView online tool (https://www.evolgenius.info) (He, et al., 2016).

[Table 1 was inserted here]

2.5. Detection of mRNA relative expression levels

The transcript levels of putative Halloween genes in eight developmental stages treated with broflainilide were measured by real-time quantitative polymerase chain reaction (RT-qPCR). For RT-qPCR, 20 μL reactions with TB Green ® *Premix Ex Taq* ™ II (Tli RNaseH Plus) (Takara Biomedical Technology Co., Ltd) (Jia, et al., 2020) were

143 performed using the Quant Studio™ 6 Flex Real-Time PCR System (Life Technologies Corporation, Carlsbad, CA).
144 Specific primers for the putative Halloween genes and the reference gene, EF1α, (**Table 1**) were designed by Beacon
145 Designer 8.13 (Premier Biosoft International, Palo Alto, CA). For each biological sample, three technical replications
146 were conducted, and the relative transcript levels were calculated using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen,
147 2001).
148
149 *2.6. Statistical analysis*
150 The values for relative mRNA levels and the MH titer were shown as mean ± standard error (SE), and figures used
151 in statistical analysis were generated by GraphPad 5 (GraphPad Software, Inc., La Jolla, CA). Significant differences
152 were determined using IBM SPSS Statistics 22 (International Business Machines Corporation, Armonk, NY) by
153 one-way ANOVA with Tukey test, and values were considered statistically significant when $P < 0.05$.
154
155 **3. Results**
156 *3.1. Broflanilide induced ecdysis failure in FAW*
157 The 3rd instar FAW larvae were treated with artificial diet containing sublethal doses of broflanilide. Compared to
158 the control group, broflanilide prevented ecdysis in larvae, pupae and adults (**Fig. 1**), reduced body length in larvae
159 (**Fig. 1A**), resulted in malformation in pupae (**Fig. 1B**) and caused vestigial wing formation in adults (**Fig. 1C**).
160
161 **[Fig. 1 was inserted here]**
162 *3.2. Broflanilide inhibit MH titer in FAW*
163 As shown in **Figure 2**, the MH titer in FAW after exposure to sublethal doses of broflanilide was determined.
164 Compared with the control, the MH titer in all tested stages including 3rd instar larvae, prepupae, 1st day pupae, 3rd
165 day pupae, 5th day pupae, 0th day adults, 0.5th day adults and 1st day adults were significantly decreased after
166 treatment with LD_{30} broflanilide ranging by 63.38%, 62.55%, 47.66%, 33.01%, 43.23%, 38.89%, 41.66% and
167 45.47%, respectively. However, after treatment with LD_{10} broflanilide, the MH titer was significantly decreased only
168 at 3rd day larvae, prepupae and 0th day adults by 21.12%, 6.54% and 18.03%, respectively (**Fig. 2**).
169
170 **[Fig. 2 was inserted here]**
171 *3.3. Identification of Halloween genes from FAW*
172 The ORFs of five putative Halloween genes were cloned from FAW. Sequence analysis revealed that

SfrCYP302A1, *SfrCYP306A1*, *SfrCYP307A1*, *SfrCYP314A1* and *SfrCYP315A1* contain ORFs of 1,524, 1,620, 1,620, 1,554 and 1,458 base pairs, respectively, and shared 66.51%, 77.92%, 74.77%, 79.30% and 55.12% amino acid identities with the homologous proteins in *B. mori* (**Fig. S1**). The deduced amino acid sequences of the five Halloween genes possessed motifs characteristic of insect CYPs, such as Helix-C (a heme-interacting region, WxxxR), Helix-I (a putative oxygen-binding pocket, GxE/DTT/S), Helix-K (a putative hydrogen binding sequence, ExLR), PERF motif (the aromatic region, PxxFxPE/DRF) and the heme-binding domain (PFxxGxRxCxG/A) (**Fig. S1**). Phylogenetic analysis including sequences from other insect species showed that *Sfr*CYP302A1, *Sfr*CYP306A1, *Sfr*CYP307A1, *Sfr*CYP314A1, *Sfr*CYP315A1 clustered into Disembodied, Phantom, Spook, Shade and Shadow subgroups, respectively (**Fig. S2**).

3.4. Halloween genes have a developmental-dependent expression profile

The relative transcript levels of the Halloween genes *CYP302A1*, *CYP306A1*, *CYP307A1*, *CYP314A1* and *CYP315A1* in eight developmental stages of FAW were determined (**Fig. 3**). Five Halloween genes showed the highest transcript levels in adult stages with *CYP302A1*, *CYP307A1* and *CYP315A1* highest in 0.5th day adults, and *CYP306A1* and *CYP314A1* highest in 1st day adults. The transcript levels of *SfrCYP302A1* and *SfrCYP315A1* were relatively low at larvae and pupae stages, but extremely high in adults (**Fig. 3A and 3E**). *SfrCYP306A1* and *SfrCYP314A1* showed gradual increase in transcript levels from the prepupae stage to the 1st day adult stage (**Fig. 3B and 3D**). *SfrCYP307A1* showed relatively low transcript levels in the larvae, pupae stages and 0th day adult stages (**Fig. 3C**).

[Fig. 3 was inserted here]

3.5. Halloween gene expression is influenced by sublethal doses of broflanilide

The sublethal effects of broflanilide on transcript levels of Halloween genes in FAW were studied in three representative stages, 3rd day larvae, 5th day pupae and 0.5th day adult (**Fig. 4**). Compared to the control group, the relative transcript levels of *SfrCYP307A1*, *SfrCYP314A1* and *SfrCYP315A1* were significantly down-regulated 46.78%, 66.41% and 61.96%, respectively, after larvae were treated for three days with LD_{10} broflanilide, and 67.78%, 83.21% and 63.04% with LD_{30} broflanilide (**Fig. 3C, 3D** and **3E**). *SfrCYP302A1* was significantly up-regulated by 101.12% and 207.87% with treatment of LD_{10} and LD_{30} broflanilide, respectively, in 3rd day larvae (**Fig. 3A**). In contrast to the significantly reduced transcript levels of *SfrCYP302A1* and *SfrCYP306A1* in 5th day pupae with 16.67% and 35.48%, respectively, after treatment with LD_{10} broflanilide, and 35.42% and 55.91% after treatment with LD_{30} broflanilide (**Fig. 3A** and **3B**), *SfrCYP307A1* was significantly increased by 90.63% and 55.21%

after treatment with LD_{10} and LD_{30} broflanilide, respectively (**Fig. 3C**). Broflanilide also induced a 186.29% increase in transcript levels of *SfrCYP314A1* with LD_{30} treatment in 5^{th} day pupae (**Fig. 3D**). In 0.5^{th} day adults, the transcript levels of *SfrCYP306A1* and *SfrCYP307A1* were significantly up-regulated by 227.14% and 105.43% after exposure to LD_{10} broflanilide and by 125.71% and 120.65% after exposure to LD_{30} broflanilide (**Fig. 3B** and **3C**), whilst transcript levels of *SfrCYP315A1* was increased by 164.52% after exposure to broflanilide at LD_{30} (**Fig. 3E**).

[Fig. 4 was inserted here]

4. Discussion

In general, introduction and application of novel chemical insecticides is still a crucial strategy in preventing the selection for resistance and the resulting outbreak of agricultural pests (Nakao and Banba, 2016; Sun, et al., 2021). Broflanilide, a newly registered meta-diamide insecticide, shows high insecticidal activity against lepidopteran pests and was launched into the market in 2019 (Nakao and Banba, 2016; Shen, et al., 2021; Sun, et al., 2021; Tang, et al., 2021). The sublethal effects of insecticide has attracted more attention and is being more widely studied (Han, et al., 2011; Lai and Su, 2011; Lutz, et al., 2018; Zhang, et al., 2013). For example, sublethal concentrations of chlorantraniliprole reduced survival and reproduction in *P. xylostella* (Han, et al., 2011), and in *S. exigua* it prolonged larval periods and decreased hatching rate (Lai and Su, 2011). Similarly, broflanilide at sublethal doses caused reduction in appetite, shortened body length and loss of weight, adversely affecting development and growth in FAW (**Fig. 1**) (Zhan, et al., 2021).

In the process of metamorphosis, MH plays an important role in regulating insect reproduction and development, such as in ecdysis (Feyereisen, 2006; Jia, et al., 2015). Decrease in MH titer is associated with abnormal phenotypes. For example, a sublethal concentration (LC_{25}) of methoxyfenozide significantly reduced the MH titer in *H. armigera*, which showed extended larval duration,–loss of pupal weight, ecdysis failure and reduced rates of pupation and emergence (Zhang, et al., 2021). In *Chilo suppressalis* (Walker), chlorantraniliprole significantly reduced the MH titer down to 33.83 and 35.94% at LC_{10} and LC_{30}, respectively, where extension of larval duration, loss of the mean weight of male pupae and shortened male adult longevity were observed (Huang, et al., 2016; Meng, et al., 2020). In the present study, ecdysis of larvae, pupae and adult FAW failed (**Fig. 1**) when the MH titer was significantly decreased after exposure to LD_{10} or LD_{30} broflanilide (**Fig. 2**). Therefore, we speculate that the decrease of MH titer in FAW is a key factor for its retardation in growth and inability of ecdysis.

As is well-known, the MH titer is affected by the intake of cholesterol from food and MH-related synthase activity *in vivo* (Rewitz, et al., 2006). Some insecticides reduce insect feeding (Alexander, et al., 2007; Morita, et al., 2007). For example, flonicamid significantly inhibits food intake by aphids within 0.5 h of treatment causing death (Morita, et al., 2007). Imidacloprid reduces food intake by the mayflies, *Epeorus longimanus* Eaton and oligochaetes *Lumbriculus variegatus* Müller, at 0.5 to 10 μg/L (Alexander, et al., 2007). Food intake by Q-type *Bemisia tabaci* adults was inhibited by LC_{25} imidacloprid, pymetrozine, spirotetramat, and cyantraniliprole (He, et al., 2021). Therefore, we speculate that broflanilide indirectly affects the MH titer in FAW by reducing intake of the artificial food (Zhan, et al., 2021), including cholesterol.

Futhermore, the Halloween genes, which are critical MH-related synthases *in vivo*, could mediate the biosynthesis of MH by virtue of their terminal CYP hydroxylase activity. For example, knockdown of *CYP306A1*, *CYP307A1*, *CYP314A1* and *CYP315A1* reduced the MH titer and subsequently interupted insect development (Jia, et al., 2015; Marchal, et al., 2012; Peng, et al., 2019; Shahzad, et al., 2015). In this study, five Halloween genes, *CYP302A1* (Disembodied), *CYP306A1* (Phantom), *CYP307A1* (Spook), *CYP314A1* (Shade), and *CYP315A1* (Shadow) were identified from FAW, which possessed motifs conserved in insect P450s (**Fig. S1**). Expression levels of *SfrCYP302A1*, *SfrCYP307A1* and *SfrCYP315A1* during different developmental stages were similar to those observed in *P. xylostella* (Peng, et al., 2019), and levels of *SfrCYP306A1* and *SfrCYP314* were similar to those of *C. suppressalis* (Meng, et al., 2020). However, the relationship between the transcript levels of Halloween genes and MH titer is unclear. In the desert locust, *Schistocerca gregaria* (Forskàl), the temporal transcript profiles of *SgCYP307A1* and *SgCYP306A1* in hemolymphs correlated with the MH titer (Marchal, et al., 2011). In contrast, the temporal transcript profiles of the five Halloween genes in FAW did not closely correlate with the MH titer (**Fig. 3**). Similar results were also observed in other lepidopteron, which indicates there may be functional diversity among different species (Iga and Smagghe, 2010; Meng, et al., 2020; Rewitz, et al., 2006; Zhou, et al., 2016).

It is worth noting that malnutrition directly induced by reduction of food intake resulting from exposure to some insecticides could alter the expression of Halloween genes (Cong, et al., 2015; He, et al., 2021; Morita, et al., 2007). Food deprivation significantly increased the transcript levels of *CYP302A1* and *CYP314A1* by 2.14 and 1.99 fold, respectively, in *Bactrocera dorsalis* (Hendel) after 6 h, but significantly decreased the transcript levels of both genes after 24 h. Food deprivation can also lead to reduced transcript levels of *CYP306A1* in *B. dorsalis* after 24 h (Cong, et al., 2015). In addition, the transcript levels of *CYP306A1* in *Culex quinquefasciatus* Say (Gong, et al., 2013),

CYP306A1, *CYP307A1* and *CYP314A1* levels in *C. suppressalis* (Meng, et al., 2020), and *CYP302A1*, *CYP306A1* and *CYP314A1* levels in *B. mori* (Li, et al., 2015) were increased by exposure to permethrin, chlorantraniliprole and phoxim, respectively. In this study, broflanilide also significantly affected the transcript levels of Halloween genes.

It is concluded that broflanilide mainly affected the feeding of FAW, which subsequently lead to malnutrition and altered expression of Halloween genes along with the biosynthesis of MH *in vivo*. Thus, sublethal doses of broflanilide resulted in abnormal phenotypes such as reduced larvae body length and impaired ecdysis.

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Figure legends

Fig. 1 Phenotypes of FAW larvae (A), pupae (B) and adults (C) treated with sublethal doses (LD_{10} or LD_{30}) of broflanilide. Red arrows indicate unsuccessful ecdysis, and blue arrows indicate vestigial wings.

Fig. 2. Change of MH titer after FAW larvae were treated with sublethal doses of broflanilide. Statistically significant differences were shown as different lowercase letters above the bars, when $P < 0.05$.

Fig. 3. Relative transcript levels of Halloween genes in FAW. Statistically significant differences are shown as different lowercase letters above the bars, when $P < 0.05$.

Fig. 4. Change of transcript levels in FAW after larvae were exposed to sublethal doses of broflanilide. The statistically significant differences were shown as different lowercase letters above the bars, when $P < 0.05$.

Supplementary materials

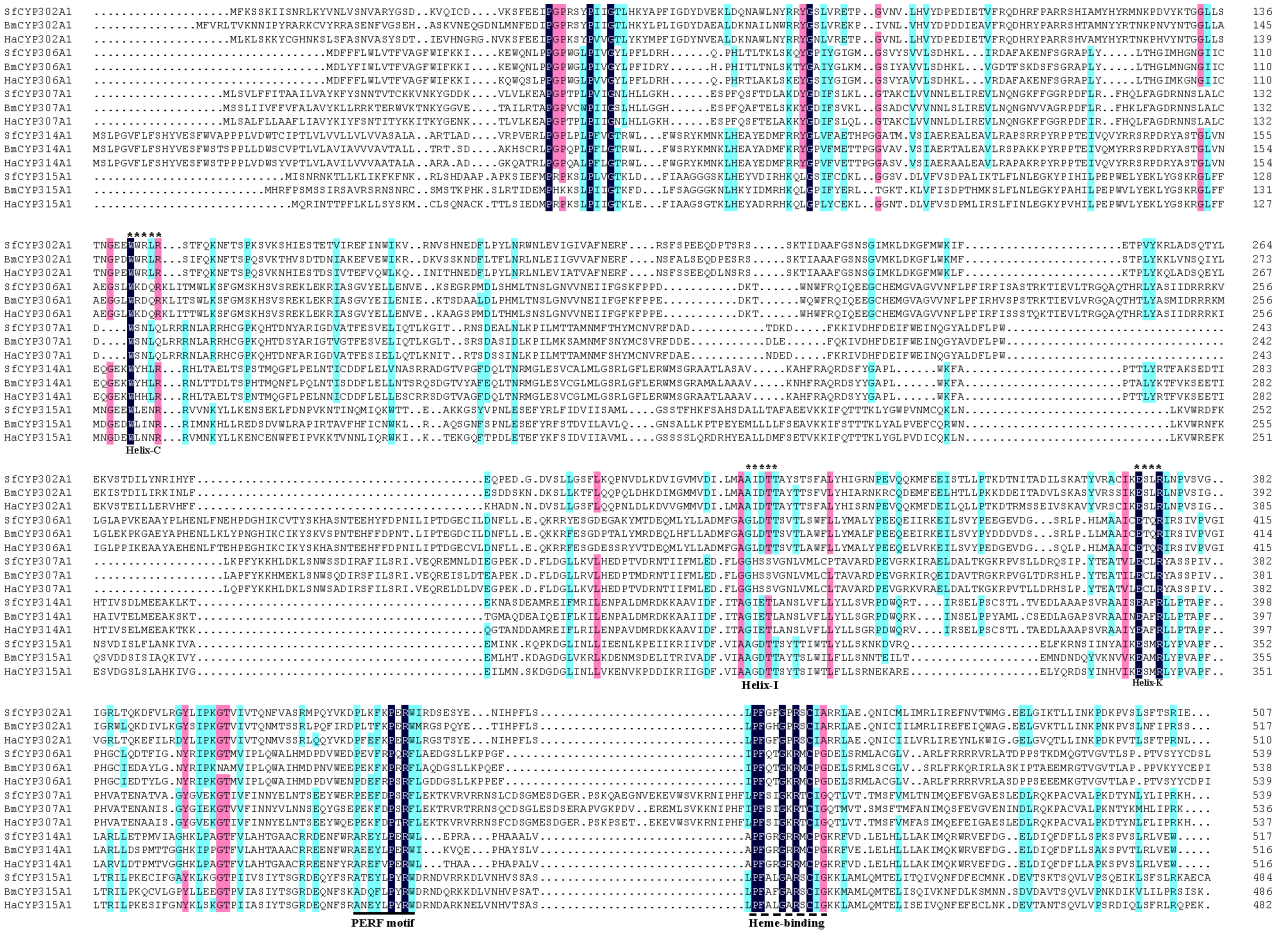

Fig. S1. Alignment comparison of the amino acid sequences of Halloween genes from FAW, *B. mori* and *H. armigera*. The characteristic conserved domains, including Helix-C, Helix-I, Helix-K, PERF motif and Heme-binding domain, are marked. GenBank accession numbers are as follows: *Bm*CYP302A1 (BAD99022.1), *Bm*CYP306A1 (NP_001106222.1), *Bm*CYP307A1 (NP_001104833.1), *Bm*CYP314A1 (NP_001106219.1), *Bm*CYP315A1 (BAD23845.1), *Ha*CYP302A1 (AID54852.1), *Ha*CYP306A1 (AID54855.1), *Ha*CYP307A1 (AID54856.1), *Ha*CYP314A1 (ALJ84054.1), and *Ha*CYP315A1 (ALJ84053.1).

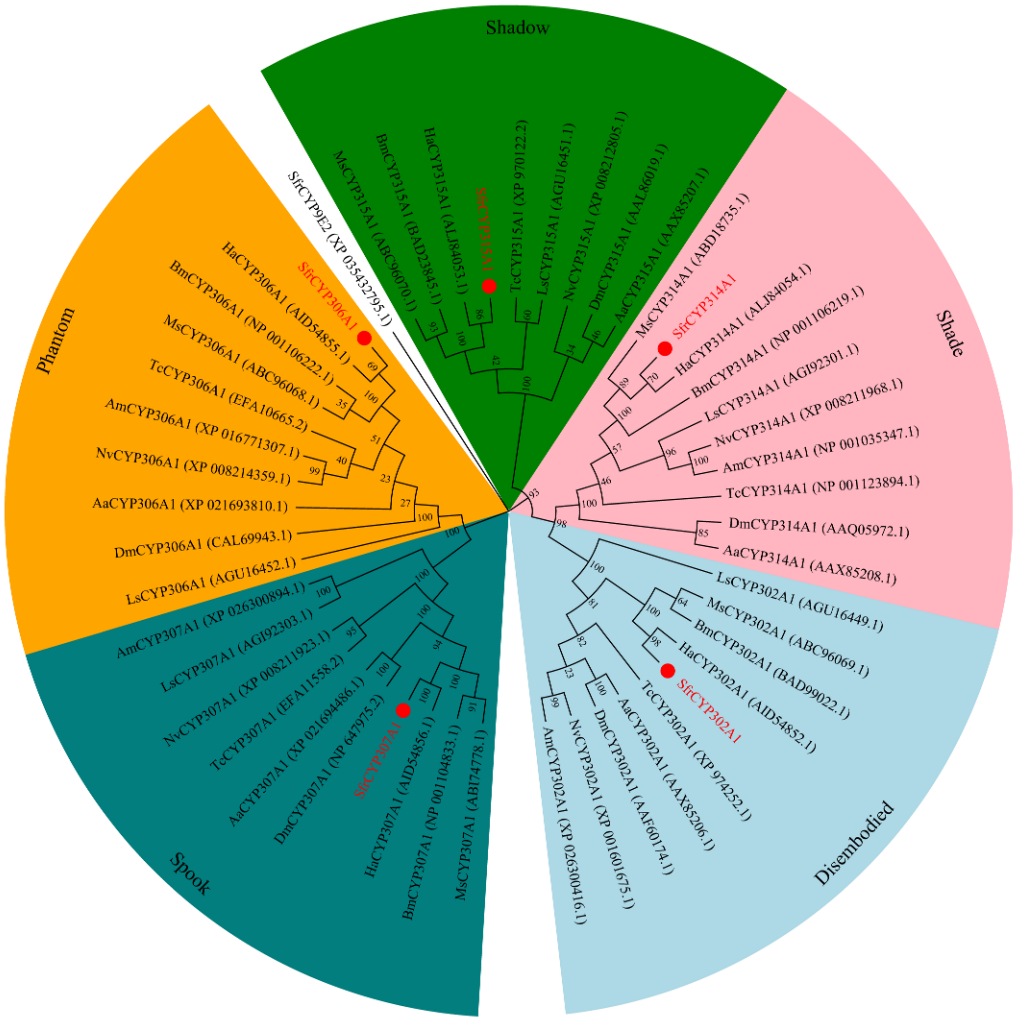

Fig. S2. Phylogenetic analysis of Halloween genes from FAW and other species. The analysis was conducted using the Neighbor-Joining method with confidence of each node estimated by 1000 bootstrap replications. The CYP9E2 sequence of FAW was chose as an out-group.