Original article

Expression and functional analysis of P450 gene induced tolerance/resistance to lambda-cyhalothrin in quercetin fed larvae of beet armyworm *Spodoptera exigua* (Hübner)

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ABSTRACT

Beet armyworm, *Spodoptera exigua* (Hübner) is an agronomical important and most devastating polyphagous pest that damages a variety of crops around the globe including China. Quercetin is one of the abundant dietary flavonoids and the important defense allelochemicals in plants. Therefore, the changes in insect detoxification enzymes activities in response to plants allelochemicals may result increased the sensitivity to insecticides. In this study, we examined the induced effect of quercetin on larval tolerance to lambda-cyhalothrin in *S. exigua*. Application of cytochrome P450 inhibitor piperonyl butoxide (PBO) significantly synergized the lambda-cyhalothrin toxicity in quercetin-fed *S. exigua* larvae. Moreover, larval weight significantly reduced in quercetin, lambda-cyhalothrin, and quercetin + lambda-cyhalothrin treatment. Furthermore, our results showed that the P450 detoxification enzyme effectively increased in all treatments as compared to the control. Quantitative Real-time PCR analysis revealed that expression level of *CYP6AE10* significantly upregulated in larvae treated with quercetin, lambda-cyhalothrin and quercetin + lambda-cyhalothrin in the midgut and fat body respectively. In addition, RNAi mediated knockdown of *CYP6AE10* in *S. exigua* larvae significantly decreased the transcription level of target cytochrome P450 gene followed by the exposure with quercetin, lambda-cyhalothrin, and quercetin + lambda-cyhalothrin. Similarly, the knockdown of *CYP6AE10* by the injection of dsRNA led to increased mortality after the treatment with respective chemicals. Overall, these data showed that P450s might possibly play an important role in the metabolic adaptation of *S. exigua* larvae to its host plant defense

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allelochemicals as well as insecticides. In conclusion, *S. exigua* can take benefit from its host plant's secondary metabolites to elaborate its defense against synthetic insecticides.

1. Introduction

In the ecological interaction of plants and insects, plants evolved the variety of mechanism to defend against phytophagous insects by intervening in the basic metabolic, biochemical, physiological functions and even behavior of herbivorous insects (Duisembecova and Dubovskiyb, 2017; LoPresti et al., 2018; Wink, 1988). In addition, plant secondary metabolites can act as an insect repellent, deterrents, anti-nutrients, and antidigestive compounds which reduce the development and survival rates of herbivorous insects (Hafeez et al., 2019a,b; Lu et al., 2014; Nikooei et al., 2015). On the other side, the herbivorous insect can metabolize potentially toxic phytochemical accumulated by plants to resist or evade herbivorous insects, which often involve the enhanced expression of detoxification enzymes such as, cytochrome P450 monooxygenase (Schuler, 2011; Wittstock et al., 2004). Interestingly, some plant secondary metabolites help the insects to detoxify insecticide by elevating the sophisticated defense mechanism (Chen et al., 2017a,b; Dermauw et al., 2013; Tao et al., 2012).

The Cytochrome P450 monooxygenase (P450 or CYP) family is a large and versatile group of hemoproteins present in all type of organisms (Guo et al., 2012; Nelson et al., 2013; Schuler and Berenbaum, 2013). In phytophagous insects, P450 enzymes are engaged in diverse functions including, the synthesis and regulation of hormones, growth, and development or the metabolism of xenobiotic compounds (Nelson et al., 2013; Zhou et al., 2010). The prominent role of cytochrome P450s in the metabolism of insecticides has often caused in their involvement increasing insecticide resistance in insect population (Guo et al., 2012; Nelson et al., 2013). In addition, elevated expression of specific detoxifying P450 enzymes within an insect pest is one of the most common occurrence mechanism, which causes resistance to certain phytochemicals and synthetic insecticides (Bautista et al., 2009; Niu et al., 2011; Pentzold et al., 2014). Therefore, the changes in insect detoxification enzymes activities in response to plant allelochemicals may result in deviations in insecticides toxicity. It is well known that pre-treatment with plant secondary metabolites could affect the sensitivity of insect to insecticide. For examples, the overexpression of P450s leading to an increase insecticides resistance and allelochemicals tolerance have been documented in many insects orders like, Lepidoptera, Diptera, Coleoptera, Hemiptera and Hymenoptera (Bass et al. 2011; Johnson et al. 2012; Liang et al. 2015; Chen et al., 2017a,b; Wang et al., 2018a,b). In addition, the insecticidal sensitivity of acaricide reduced when two-spotted spider mite (*Tetranychus urticae*) transferred from their common host plant to less accepted host plant due to the different characteristics of the secondary metabolites of the two host plants (Dermauw et al., 2013). Induced resistance and fitness gain to deltamethrin after the gossypol ingestion for multiple generations was reported in *Spodoptera exigau* (Hafeez et al., 2018). Cross-resistance to alpha-cypermethrin and reduces larval sensitivity to lambda-cyhalothrin after xanthotoxin or quercetin ingestion larvae were also observed in *Helicoverpa armigera* and *Helicoverpa zae* (Li et al., 2000a,b; Chen et al., 2017a,b). Quercetin flavonol is an important plant secondary metabolite in terrestrial plants. Moreover, the harmful effects of quercetin on development survival and reproduction of crops insect have been documented (Li et al., 2016; Liu et al., 2015). However, the induced effects of quercetin on herbivores insects to insecticide susceptibility and associated P450 genes expression are rarely documented.

Beet armyworm *S. exigua* (Hübner) is an agronomically important and most destructive polyphagous pest which damages more than 138 host species representing 35 different plant families around the globe (Zheng et al., 2011). At present, Deltamethrin, cypermethrin, and fenvalerate are widely applied in agriculture to effectively control the *S. exigua* (Ahmad and Arif, 2010; Ishtiaq et al., 2012a; Lai et al., 2011). Hence, *S. exigua* has developed a high level of resistance to pyrethroid insecticides due to an excessive and frequent application in the field crops (Ahmad and Arif, 2010; Ishtiaq et al., 2012a; Lai et al., 2011). The overuse of synthetic chemical pesticides lead to the development of insecticide resistance and cause environmental pollution and health problems. Hence, it is necessary to develop environmentally safe techniques and examine the factors that contribute to the development of insecticides resistance.

RNA interference (RNAi) as an effective gene-silencing technique, which has been developed in a variety of organisms (Hannon, 2002). Double standard RNA (dsRNA) mediated inference has become one of the most powerful approaches for rapid analysis of gene function and has considerable perspective in the application of pest control (Ai et al., 2018; Mao et al., 2011; Tao et al., 2012). Previous studies show that, many P450s and other important genes from different insects have been knocked down, rather than knocked out (Hafeez et al., 2019a,b; Mao et al., 2011; Poreddy et al., 2017; Rodrigues et al., 2018; Taning et al., 2016; Tao et al., 2012; Wang et al., 2018a,b) by droplet-feeding of dsRNA (Wang et al., 2018a,b), the microinjection of dsRNA (Jan et al., 2017), and feeding of exogenous dsRNA from an artificial diet (Ai et al., 2018). These outcomes strongly advocate that RNAi mediated genes silencing is an effective technique for controlling insect pests. Studies have also shown that genes that encode proteins with basic functions in insects are the best targets for RNA interference to increase morbidity and mortality.

In this study, we first investigated the lambda-cyhalothrin tolerance to quercetin fed larvae of *S. exigua*. Secondly, we examined the potential roles played of P450 genes conferring resistance to insecticides in *S. exigua* by quantifying the analysis of P450 detoxification enzyme. RT-PCR (qRT-PCR) was performed to investigate the tissues specific expression pattern of three P450 genes and their potential roles in detoxification of lambda-cyhalothrin and quercetin were tested using RNAi followed by droplet-feeding bioassay. Additionally, we evaluated the mortality rate after ingestion of dsRNA in *S. exigua* larvae.

2. Materials and methods

2.1. Insects

Laboratory-reared susceptible colony of Beet armyworm, (*S. exigua*) was collected from Jingzhou, Hubei province of China in 2003. The larvae were reared on the semisynthetic artificial diet without exposure to any insecticide in the College of Plant Science and Technology Huazhong Agriculture University Wuhan, China at the laboratory condition (25 ± 2 °C, 65–75% R.H) and a photoperiod of 14 h:10 h (L:D). The eggs were sterilized with a 0.1% sodium hypochlorite and the adult moths were fed with a 10% honey solution as a food source.

2.2. Chemicals

Quercetin, piperonyl butoxide (PBO), phenylmethylsulfonyl fluoride (PMSF) and NADPH were bought from Sigma-Aldrich (St. Louis, MO, USA). The commercial formulation of lambda-cyhalothrin was obtained from Jiangsu yangnong chemical group co, LTD. Dithiothreitol (DTT), glycerol, and Tris were bought from Beijing Solarbio Scientific and Technology Company. 7-ethoxycoumarin, 7-hydroxycoumarin, EDTA and bovine serum albumin were from Beijing Biotopped Scientific and Technology Company. All chemicals and solvents used were reagent grade.

2.3. Preparation of chemical-supplemented diets

First wheat germ base artificial diet was prepared according to the method explained by (Elvira et al., 2010) with slight modification and chemical-supplemented diet was prepared according to the method described by (Chen et al., 2017a,b) with slight modification; the quercetin to be tested was first dissolved in 1% dimethyl sulfoxide (DMSO). The control diet was prepared by adding the same volume of DMSO to artificial diet. While a stock solution of lambda-cyhalothrin insecticide was first prepared by diluting it in distilled water. Five serial dilutions were then prepared in distilled water containing 0.1% (v/v) Triton X-100 and 1% DMSO. For bioassays, A required amount of insecticide from the stock solution was pipetted into 15-ml small sterilized transparent plastic cups size (4.0 cm diameter × 3.0 cm height) still containing liquid wheat bran based artificial diet and then incorporated by stirring for 2 min before solidification of agar (40–45 °C). The control diet was prepared with the same method but without any chemical and stored at 4 °C prior to use for bioassays.

2.4. Toxicity bioassay

Effects of quercetin uptake tolerance to lambda-cyhalothrin on newly moulted third instar feeding stage *S. exigua* larvae were used for all treatments, as this growth stage is more amenable to monitoring weight gain and mortality. Early third instar larvae of *S. exigua* were first fed on 0.2% quercetin-supplemented artificial diet (g/g artificial diet) for 48 h before bioassay, while the control (CK) larvae fed on the artificial diet without quercetin. A diet incorporation method was used to assess the toxicity of lambda-cyhalothrin to the third-instar larvae of *S. exigua* (Wang et al., 2015a). In brief, a stock solution (3000 mg/L) of lambda-cyhalothrin was dissolved in distilled water firstly and then further diluted in five serial concentrations with distilled water containing 0.1% (v/v) Triton X-100 and 1% DMSO for bioassays, (140, 170, 200, 230 and 260 of lambda-cyhalothrin mg/L for 0.2% quercetin pretreated group and 60, 85, 110, 135 and 160 mg of lambda-cyhalothrin/L without quercetin). A required amount of insecticide from above mention solutions was pipetted into 20-ml small-sterilized transparent plastic cups (4.0 cm diameter × 3.0 cm height) still containing liquid wheat bran based artificial diet and then incorporated by stirring for 2 min. An equal amount of 0.1% (v/v) Triton X-100 and 1% DMSO was added into the diet for the control and covered all cups with lid containing small holes for ventilation. For each bioassay series, ninety larvae from 0.2% quercetin pretreated or without quercetin-exposed group were transferred on each concentration of insecticide, control larvae reared on diet without any chemical as described above (30 for each concentration, three replicates for each concentration) and mortality was recorded at 72 h of post-treatment. Each experiment was conducted in triplicate.

2.5. Synergistic effect of PBO on the toxicity of the insecticide

PBO was used as the synergist in this study. A synergistic experiment in the presence or absence of synergist Piperonyl Butoxide (PBO) was evaluated using the bioassay method described above after *S. exigua* larvae fed on an artificial diet with 0.2% quercetin or without quercetin for 48 h. PBO was dissolved in acetone to the concentration of 25 mg/L, and (10 μg/larvae) of PBO solution was topically delivered onto the topically delivered onto the dorsal prothorax of individual larvae of *S. exigua* using Micro4™ Micro-Syring Pump Controller, USA. After 2 h, the PBO-treated *S. exigua* larvae were placed in sterilized small plastic cups containing different concentrations of insecticide solutions for evaluating the toxicity of lambda-cyhalothrin (140, 170, 200, 230 and 260 of lambda-cyhalothrin mg/L for 0.2% quercetin-pretreated and PBO-treated group;) and covered the top. The larvae were fed with quercetin diet for 48 h but without PBO pre-treatment (60, 85, 110, 135 and 160 of lambda-cyhalothrin mg/and covered all cups with lid containing small holes for ventilation. Mortality was assessed 72 h after lambda-cyhalothrin application and LC_{50} value was calculated (Chen et al., 2018). Synergism in this study was defined as mortality exceeding the combined baseline mortality of the toxicant and the synergist (Niu et al., 2012). Each bioassay was repeated at least three times.

2.6. The effect of 0.1% quercetin diet on bodyweight

To evaluate the effect of quercetin on the larval growth of *S. exigua*, 120 third-instar larvae with uniform size were starved for 2 h and transferred to small sterile transparent plastic cups (3 cm diameter, 3.5 cm height) containing artificial diet supplemented with 0.2% quercetin (g/g artificial diet) and control (CK) diet for 1 day. After 24 h, they were weighed and transferred to a diet containing 96.93 mg/L lambda-cyhalothrin (a sublethal concentration) for another 24 h. After 2 days of exposure, the net weight increased or decreased was recorded after they were weighted 72 h, respectively.

2.7. P450 activity assay

2.7.1. Sample collection

The detoxification enzyme P450 activity in the early fourth instar of *S. exigua* larvae midgut homogenates was assayed. Measurements were taken after they were reared on a diet containing 2.0 mg/g quercetin or no quercetin (control) for one day. After 24 h the exposed larvae were placed into new sterilized plastic cups containing artificial diets supplemented with 0.2% quercetin, LC_{50} concentration of lambda-cyhalothrin 96.93 mg/L for 48 and 72 h, or 0.2% quercetin for 24 h followed by lambda-cyhalothrin for 48 and 72 h. The midgut was removed after 48 or 72 h for further analysis. The midguts from all treatments were extracted by dissection on ice. The dissected midguts were gently shaken to free its contents and washed in a cold aqueous solution containing 1.15% (w/v) potassium chloride. The crude homogenates of treated and control *S. exigua* midguts were prepared as previously described by (Liu et al., 2006) with some modification for enzymes activity assay. All experiments were performed in triplicate.

2.7.2. Measurement of P450 activity

The 7-ethoxycoumarin-O-deethylase (ECOD) activity of cytochrome P450 enzyme in the midguts of *S. exigua* larvae using 7-ethoxycoumarin (7-EC) was measured as the substrate according to the method described by (Chen et al., 2017a,b). Approximately, fifteen midguts third-instar larvae of *S. exigua* were homogenized on ice with 2 mL of homogenization buffer 0.1 M PBS at pH 7.5 containing 1.0 mM EDTA, 1.0 mM phenylmethylsulfonyl fluoride

(PMSF), 1.0 mM PTU, 0.1 mM DTT, and 15% glycerol, followed by the centrifugation at 12,000g for 12 min at 4 °C. The supernatant from centrifuged 2-mL tubes was collected and further used for P450s activity assay. The reaction solutions containing a total of 20 μL of NADPH (10 mM stock solution) and 25 μL of 7-EC (10 mM stock solution) in 685 μL solution of 0.1 M Tris–HCl buffer (pH 7.8) and 250 μL of the enzyme homogenate was added to start the enzyme reaction. The incubation was conducted on a shaker for 15 min at 30 °C, and a 300-μL solution of 15% (w/v) trichloroacetic acid (TCA) was added to terminate the reactions. The mixture in 2-mL tubes was centrifuged at 10,800g at 4 °C for 2 min, with around 800 μL of supernatant from tubes being transferred to new 2-mL tubes, and a 450-μL solution containing 1.6 M Gly-NaOH buffer (pH 10.5) was added to adjust the pH 10 of resulting extract. The content of 7-hydroxycoumarin in the reaction mixture was measured immediately by using a SPECTRA max GEMINI XS spectrofluorometer (Molecular Devices, USA) with adjusting 356 nm excitation and 465 nm emission filters. A series of different concentrations of 7-hydroxycoumarin were prepared, and standard substance fluorescence values were measured to draw the standard curves. All biochemical assays were conducted at least three replicates with different preparations of enzymes. Each of the three replicates consisted of five midguts. Protein concentration was determined using the method described by (Bradford, 1976) and with bovine serum albumin as the standard protein.

2.8. Sample preparation

To determine tissue-specific expression patterns for the target genes, the third instar larvae were transferred into new sterilized plastic cups containing artificial diets supplemented with 0.2% quercetin, LC_{50} concentration of lambda-cyhalothrin 96.93 mg/L for 72 h or 0.2% quercetin for 24 h followed by the lambda-cyhalothrin for 72 h and 1.0 mg/g DMSO for the control group. After 72 h of chemical induction, the midguts and fat bodies tissues were taken from all treatments including control and stored at −80 °C for RNA extraction. Each treatment had three biological replicates.

2.8.1. Total RNA isolation and cDNA synthesis

All the samples (midguts and fat bodies) were put in ceramic tiles crushed into powder by liquid nitrogen and 1 mL RNAiso plus (Takara, Japan) was added for total RNA isolation following the experimental procedures. Samples were centrifuged 12,000 RMP at 4 °C for 5 min. After centrifugation, separate the supernatant in new tubes and 200 μL Trichloromethane was added in each tube, shake it hard with hand and keep it for 5 min. After centrifuged with 12,000 rpm for 10 min at 4 °C, 500 μL supernatant was separated and mixed with 500 μL isopropanol, kept the tubes on ice for 10 min, then centrifuged with 12,000 rpm for 10 min at 4 °C. Removed the supernatant and washed the white pellets with 75% ethanol. After centrifuged, the 75% ethanol was removed and the white RNA pellets were dried at room temperature for 5 min, and the required amount of DEPC treated water was added to dissolve the white RNA pellets. The concentration and purity of total RNA were measured by a NanoDrop® spectrophotometer (Thermo Fisher, MA, USA).

2.8.2. cDNA synthesis

First-strand cDNA was synthesized by using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix in 20 μL reactions containing 1 μg of total RNA (500 ng), 1 μL Anchored Oligo (dT)18 Prime (0.5 μg/μL), 10 μL 2 × TS Reaction mixture, TransScript® RT/RI Enzyme Mix and gDNA Remover at 42 °C for 30 min. Three independent RNA preparations representing three biological replicates were used for cDNA synthesis.

2.8.3. Quantitative real-time PCR

The expression levels of cytochrome P450 genes were quantified by quantitative real-time PCR (qRT-PCR) using an MYiQ' RT-PCR system Bio-Rad, California, USA) and Real Master Mix 2xSYBR Green qPCR mix (Aidlab Biotechnologies Co., Ltd, China). QRT-PCR of each cDNA sample and template-free was performed in triplicate. Specific primers of *CYP6AE10*, CYP9A11, and *CYP321A8* were designed for qPCR (Table 2). Reaction volume of 20 μL was used (0.5 μL of each primer 10 μM, 1 μL cDNA, 8 ul ddH20 and 10 μL 2x cyber master mix for quantification using the following cycling parameters: 94 °C for 3 min, followed by 40 cycles of 94 °C for 15 sec, 57–60 °C for 30 s and 70 °C for 30 s. For each gene, a serial dilution from 10- to 1000-fold of each cDNA template was performed in order to assess the efficiency of PCR. The relative expression values were calculated using the $2^{-\Delta\Delta CT}$ methods as previously described by Livak and Schmittgen (2001). Results were expressed as the mean expression ratio (±S.E.) of three biological replicates between chemical treatments and controls. One-way analysis of variance (ANOVA) and the Tukey HSD test for the significant difference was performed to determine the statistical difference between means (SPSS, version 19).

2.8.4. dsRNA synthesis

For double-stranded RNA (dsRNA) synthesis, a 423 bp fragment of the target gene (*CYP6AE10*) was selected from the open reading frame (ORF), a 688 bp fragment from green fluorescent protein (*GFP*) (GenBank accession ACY56286). Both target and control gene was first amplified by PCR. The primers used for the *CYP6AE10* amplifications were designed to add the T7 polymerase promoter sequence at the 5 ends. Two pairs of primers (*CYP6AE10-F* and *T7CYP6AE10-R*, *T7CYP6AE10-F* and *CYP6AE10-R*) were used to amplify CYP6AE10 (Table 2). As a control, dsGFP was synthesized using the same method by two pairs of primers (GFP-F and T7GFP-R, T7GFP-F and GFP-R) (Table 2). PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA, USA), and DNA concentrations were determined using a Nano-Drop® spectrophotometer (Thermo Fisher, MA, USA). The dsRNA corresponding to *CYP6AE10* was prepared from the purified PCR-generated templates according to the instructions provided by the commercially available kit (T7 RiboMAXTM Express RNAi System (Promega). After preparation dsRNA product was purified by MEGA clear (Ambion) and the resulted dsRNAs integrity were quantified by NanoDrop® spectrophotometer (Thermo Fisher, MA, USA) and stored at − 80 °C prior to use.

2.8.5. Knock-down of CYP6AE10 by RNAi

The final concentration of dsRNA for injection was adjusted to 1.5 μg/μL using diethylpyrocarbonate (DEPC) treated water. The fourth instar larvae were placed individually in 12-orifice tissue culture plates and starved for 6 h. The fourth-instar larvae (pre-exposed with quercetin for 24 h) were injected with 2 μL (3.0 μg) of dsRNA from the side of each thorax of *S. exigua* using a (Micro4™ MicroSyring Pump Controller, USA) and the injection points were sealed immediately with paraffin (Wang et al., 2015b). Midguts and fat bodies of surviving larvae were collected from *S. exigua* 48 and 72 h after injection with *dsCYP6AE10* and *dsGFP* larvae. For each treatment, 60 fourth-instar larvae were used to examine the transcript levels of *CYP6AE10*. Each treatment was replicated three times. The total RNA extraction and Reverse transcription quantitative real time RT-PCR procedures used were described above.

To assess the role of *CYP6AE10* in the detoxification of lambda-cyhalothrin to quercetin-fed *S. exigua* larvae. For toxicity analysis, after 24 h of dsRNA post-injection, 60 *S. exigua* larvae for each independent treatment (Each of three replicate consisted of 20 larvae) were transferred individually into 12-orifice tissue culture plate

containing artificial diets supplemented with 0.2% quercetin, LC_{50} concentration of lambda-cyhalothrin 96.93 mg/L for 72 h, and 0.2% quercetin for 24 h followed by lambda-cyhalothrin for 72 h and standard diet. A non-supplemented diet was used as a control group. The mortality data were recorded at 48 and 72 h after injection of dsRNA on different treatments including control. (Wang et al., 2018a,b). All experiments were performed in triplicate.

2.9. Data analysis

LC_{50} values were calculated by standard probit analysis (Abbott, 1925). The data of enzymatic activity, body weight gain and relative expression level of P450s genes were analyzed using the SPSS 20.0 Software Package (SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by the Tukey HSD was employed to analyze differences among different tissues. The Student's *t*-test was used to analyze data from the RNAi and feeding experiments with different chemicals. Statistical differences were considered as significant at $p < 0.05$.

3. Results

3.1. Effect of PBO synergist on lambda-cyhalothrin toxicity in quercetin fed S. exigua larvae

The induced effect of dietary quercetin to lambda-cyhalothrin tolerance and the sensitivity of *S. exigua* larvae are listed in Table 1. In present results, quercetin-pretreated *S. exigua* larvae showed higher tolerance to a lambda-cyhalothrin insecticide with respect to the control treatment. The LC_{50} value of lambda-cyhalothrin in a quercetin-pretreated population of *S. exigua* larvae was 196.0 mg/L, while the LC_{50} value of lambda-cyhalothrin for the control group (larvae without exposure to quercetin) was 96.93 mg/L, which was lowered than the treatment group. Furthermore, synergism bioassay was conducted on treatment and control group to determine the PBO effect on the P450 activity and the toxicity of lambda-cyhalothrin. Results indicate that the addition of PBO lowered the LC_{50} values of lambda-cyhalothrin in both treatments respectively. Thus, a marked synergism of lambda-cyhalothrin was observed with PBO in quercetin fed *S. exigua* larvae, with synergism ratio of 2.02 (Table 1).

3.2. Induced effect of dietary quercetin on the activity of P450 detoxification enzyme in S. exigua

It is well known that many cases overexpression of P450 enzyme causes the metabolic resistance to plants allelochemicals and insecticide. In the present works, the effect of quercetin, lambda-cyhalothrin and quercetin + lambda-cyhalothrin diet on ECOD activity of cytochrome P450 in late third-stage *S. exigua* larvae after exposure 48 and 72 h are shown in Fig. 1. The ECOD activity of P450 detoxification enzyme was significantly elevated in the midgut of third-stage stage *S. exigua* larvae after fed on quercetin (1. 0.85), lambda-cyhalothrin (0.691) and quercetin + lambda-cyhalothrin (1.33) at 48 has compared to control (0.58) treatment

respectively. As well, the increasing trend of ECOD activity of P450 detoxification enzyme when *S. exigua* larvae after fed on quercetin (1.73), lambda-cyhalothrin (1.49) and quercetin + lambda-cyhalothrin (2.15) at 72 h as compared to control (0.85) treatment respectively (Fig. 1).

3.3. The effect of dietary quercetin on the bodyweight of S. exigua

To find out the effect of quercetin on the body weight gain of *S. exigua* larvae. We first examined the effect of 0.2% quercetin treatment prior to lambda-cyhalothrin exposure. The late third-instar larvae of *S. exigua* were divided into two groups, one group was fed on 0.2% quercetin-supplemented diet and other on quercetin free diet (control) for one day, respectively, before transferring to a sublethal concentration of lambda-cyhalothrin for next day. We found that quercetin-fed larvae exhibited slower weight increase than the control after transferring to lambda-cyhalothrin supplemented diet 72 h (Fig. 2).

3.4. Expression levels of P450 genes to quercetin and lambda-cyhalothrin exposure

Quantitative real-time -PCR experiment was performed to determine the relative expression of three P450 genes (*CYP6AE10*, *CYP9A11*, and *CYP321A8*) in the midgut and fat body of *S. exigua* larvae after exposure to quercetin, lambda-cyhalothrin and quercetin + lambda-cyhalothrin for 72 h (Fig. 3). Present results indicate that steady-state transcript level of *CYP6AE10*, in the midgut tissues were significantly increased in the treatment group (quercetin 6.38, lambda-cyhalothrin 9.04 and quercetin + lambda-cyhalothrin 12.04-fold) relative to control treatment after 72 h. Similarly, expression levels *CYP9A11* and *CYP321A8* in the midgut tissues were also significantly increased in the treatment group relative to control treatment, but higher transcript level of *CYP6AE10* was observed in midgut (Fig. 3). Moreover, the steady-state transcript level of *CYP6AE10* in fat body tissue was markedly increased in the midgut tissues when larvae exposed to (quercetin 3.49, lambda-cyhalothrin 5.19 and quercetin + lambda-cyhalothrin 7.33-fold) relative to control treatment after 72 h. As we also observed transcript level of *CYP9A11* and *CYP321A8* in the case of fat body samples, e.g. quercetin, lambda-cyhalothrin, and quercetin + lambda-cyhalothrin respectively, much smaller increased relative to control treatment for 72 h. While no significant difference in the transcription level of *CYP321A8* between the quercetin and control treatment respectively (Fig. 3).

3.5. Effect of dsCYP6AE10 on the toxicity of quercetin and lambda-cyhalothrin

Silencing effect of *dsCYP9A10* on the mortality of the fourth-instar larvae of *S. exigua* droplet feeding with DEPC, *dsGFP* or ds *CYP6AE10* and exposed to quercetin, lambda-cyhalothrin, and quercetin + lambda-cyhalothrin are shown in Fig. 4. Present results indicate that injection of dsRNA-*CYP6AE10* to larvae significantly enhanced the insecticidal activity of quercetin and insecticide,

Table 1
Induced effect of quercetin ingestion and synergism effect of piperonyl butoxide (PBO) on the lambda-cyhalothrin toxicity to third-instar larvae of *Spodoptera exigua*.

Treatment	LC_{50} (mg a.i./L)	95% CL	Slope ± SE	df	$\chi 2$	SR
Control	96.93	89.20 ± 106.36	3.323 ± 0.46	3	0.635	
Control + PBO	83.16	73.18 ± 91.61	2.89 ± 0.44	3	1.37	0.857
Quercetin	196.00	185.50 ± 202.33	5.68 ± 0.73	3	0.83	2.02
Quercetin + PBO	103.17	94.07 ± 111.85	3.81 ± 0.53	3	0.65	1.06

Where, LC_{50} = lethal concentration to kill 50% of the population; a.i. = active ingredient; CL = confidence limits; SE = standard error; df = degree of freedom; $\chi 2$ = Chi-square value; SR = resistance ratio.

Table 2
Primers used in this study for quantitative real time polymerase chain reaction (qRT-PCR).

| Function	Primer name	Primer sequence (5′-3′)
Real-Time PCR		
CYP6AE10	CYP6AE10-sense	GGACAATGGTGAAGACTGGC
	CYP6AE10-anti-sense	TGCGACAAACTTGAGTGCTC
GADPH	GADPH- sense	CTGAGGAACAGGTCGTGTCATC
	GADPH-anti-sense	GATCGATAACGCGGTTGGAGTA
dsRNA synthesis		
CYP6AB14	T7 CYP6AE10-sense1	ggatcctaatacgactcactataggATCACGCTGTATCTGTTGCTC
	CYP6AE10-anti-sense1	TTGGCTGATGAGAATAGAGGC
	CYP6AE10- sense2	ATCACGCTGTATCTGTTGCTC
	T7 CYP6AE10-anti-sense2	TTGGCTGATGAGAATAGAGGCggatcctaatacgactcactatagg
dsRED	T7dsRED- sense1	ggatcctaatacgactcactataggGCAAGCTATGCATCCAACGCGTTGGG
	dsRED-anti-sense2	CAAGCTATGCATCCAACGCGTTGGGAG
	dsRED-sense2	GCAAGCTATGCATCCAACGCGTTGGG
	T7dsRED-anti-sense2	CAAGCTATGCATCCAACGCGTTGGGAGggatcctaatacgactcactatagg
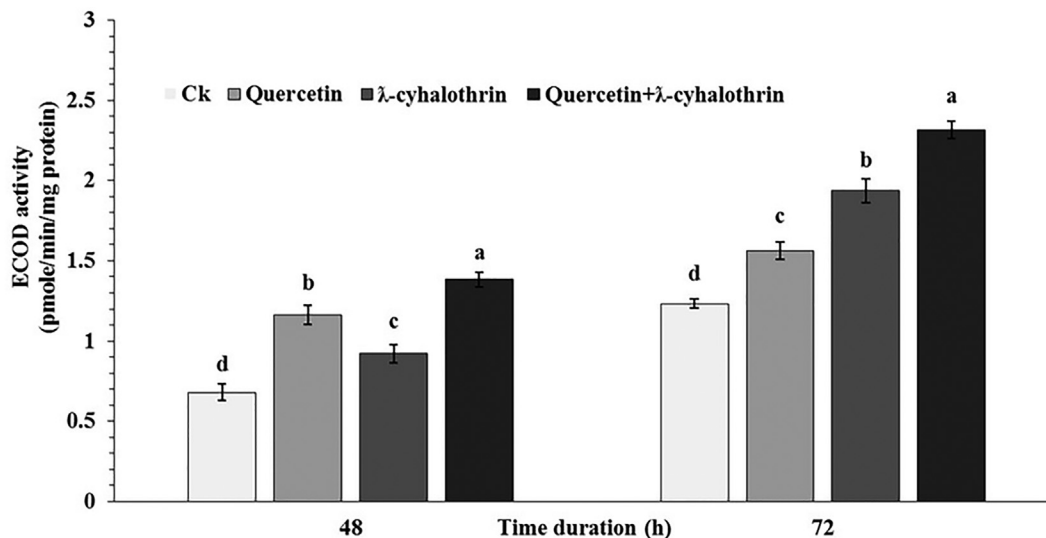
Fig. 1. Effects of quercetin on *Spodoptera exigua* larvae tolerance to deltamethrin and O-deethylase activity of P450s after 48 and 72 h. Data in the figure are means ± SE. Different letters above bars indicate significant differences (p < 0.05) according to the Tukey HSD test.

while delivering *dsGFP* did not (Fig. 4). Following exposure to quercetin (2 mg), lambda-cyhalothrin and quercetin + lambda-cyhalothrin at concentrations, larvae exposed via droplet feeding with dsRNA-*CYP6AE10* significantly enhanced the larval mortality caused by quercetin (16.33%), lambda-cyhalothrin (33.66%) and quercetin + lambda-cyhalothrin (41.33%) compared to the dsGFP (8.33, 23.33 and 33.33) after 48 h. Similarly, fourth-instar larvae of *S. exigua* fed on quercetin, lambda-cyhalothrin and quercetin + lambda-cyhalothrin experienced 28.33%, 45%, and 60% mortality after 72 h of exposure respectively. Furthermore, results indicated that the highest mortality level approximately 60% was observed in the combined effect of quercetin + lambda-cyhalothrin. Taken together, these results strongly suggest that *CYP6AE10* might play a significant role in the induced effect of quercetin to the toxicity of lambda-cyhalothrin insecticide in *S. exigua* (Fig. 4).

3.6. Functional analysis of CYP6AE10 by RNAi

The potential role-played by of *CYP6AE10* in the midgut and fat body of *S. exigua* larvae in the detoxification of was further analyzed using RNAi via its corresponding dsRNA to specifically inhibit the expression levels of *CYP6AE10*. In the midgut tissue, the steady-state transcript levels were significantly reduced following by droplet feeding of dsRNA-*CYP6AE10* demonstrating the efficiency of this approach in *S. exigua* (Fig. 1). Results showed that following

exposure to quercetin (0.2%), lambda-cyhalothrin (96.93 mg/L) and quercetin + lambda-cyhalothrin (0.2% + 96.93 mg/L) at concentrations, larvae exposed via injection with dsRNA-*CYP6AE10*. The expression level of *CYP6AE10* gene in midgut significantly reduced by (quercetin: 52.11, lambda-cyhalothrin: 73 and quercetin + lambda-cyhalothrin 67.49) compared to control group (quercetin: 82.57, lambda-cyhalothrin: 89.06 and quercetin + lambda-cyhalothrin 88.65) after 48 h respectively. Furthermore, QRT-PCR results show that expression level of *CYP6AE10* was significantly reduced in the midgut of *S. exigua* larvae after exposure to (quercetin: 47.75, lambda-cyhalothrin: 36.42 and quercetin + lambda-cyhalothrin 22.37) in the midgut after 72 h (Fig. 5). Similarly, the expression of *CYP6AE10* in fat body *S. exigua* larvae was significantly reduced by diet supplemented with (lambda-cyhalothrin: (68.91) and quercetin + lambda-cyhalothrin: (53.81) compared to control (dsGFP) (78.84, 79.34 and 70.76) after 48 h respectively whereas, no significantly, changes were measured between quercetin and control after 48 h. Further results show that expression level of CYP6AE10 in fat body was reduced by following exposure of *S. exigua* larvae to (quercetin: 76.99, lambda-cyhalothrin: 47.61 and quercetin + lambda-cyhalothrin: 38.68) compared to dsGFP (quercetin: 83.92, lambda-cyhalothrin: 70.19 and quercetin + lambda-cyhalothrin: 68.27) as control after 72 h respectively (Fig. 5). Present results demonstrate the efficacy of RNAi approach in *S. exigua*. Plants are constantly under the threat by a wide array
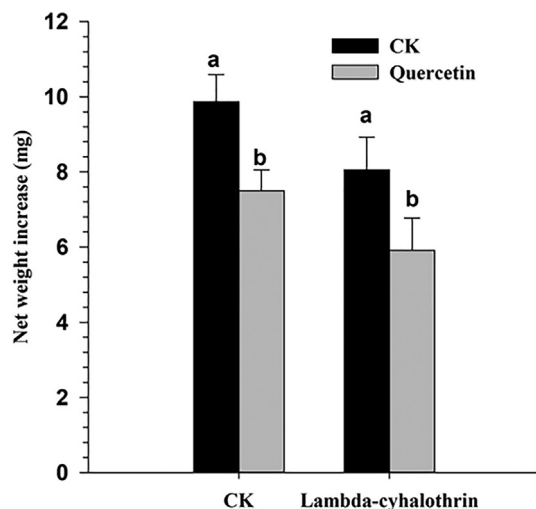
Fig. 2. Net weight increase in quercetin-pretreated larvae on lambda-cyhalothrin-supplemented diet. The early third instar *Spodoptera exigua* larvae had previously fed on control (CK) or 1 mg/g quercetin-supplemented (quercetin) diet for 1 day; after recording the initial weight, two independent groups of each treatments were transferred to 0.887/L lambda-cyhalothrin-supplemented (lambda-cyhalothrin) and CK dietary, respectively, weight increases were recorded 1 day later. Error bars represent standard deviation. Different letters above bars indicate significant differences ($p < 0.05$) according to the Student's *t*-test.

of biotic and abiotic stresses, in which biotic stress due to insect herbivorous is an important constraint in crop production.

4. Discussion

Plants produce a wide variety of secondary compounds, or allelochemicals, that serve as defensive agents against herbivores and pathogens, some of these allelochemicals attract the natural predators of herbivores (Takabayashi et al., 1991; War et al., 2011a). On the other hand, the adaptation of insect herbivores to their host plants is assumed to be intimately associated with the pervasive development of pesticides resistance in agriculture environment (Li et al., 2007; Zhu et al., 2016). Beet armyworm (*S. exigua*) is a highly polyphagous and major agricultural pest that causes considerable losses in economically important crops (Zheng et al., 2011). Chemical insecticides have been extensively applied on almost all crops for the control of this pest. However, overuse of insecticides

has led to the development of resistance of this pest to different synthetic insecticides (Ahmad et al., 2018; Ishtiaq et al., 2012b; Lai et al., 2011). Previous studies show that herbivorous insects heavily rely on their detoxification enzymes to cope the potential toxicity of plant secondary metabolites and others xenobiotics (Hafeez et al., 2018; Tao et al., 2012; War et al., 2011b). Here, we investigated the effect of diet incorporation quercetin on the sensitivity of *S. exigua* larvae to commonly used insecticide lambda-cyhalothrin, and examined the impact of quercetin on the enhanced activity of P450 detoxification enzymes, the transcription level of cytochrome genes *CYP6AE10*, *CYP9A11* and *CYP321A8*.

In the present study, diet incorporated-quercetin ingestion significantly increased the larval insensitivity to lambda-cyhalothrin insecticide in *S. exigua*, which is consistent with the previous studies. For example, quercetin ingestion significantly increased the larval tolerance to lambda-cyhalothrin in *H. armigera* and increased tolerance to cypermethrin in *H. zea* larvae after xanthotoxin exposure (Li et al., 2000a,b; Chen et al., 2018). In addition, gossypol exposure increased resistance to deltamethrin after several generations in *S. exigua* and after long-term induction of host plants enhanced insecticides insensitivity in *B. tabaci* (Hafeez et al., 2018; Xie et al., 2011). The effects of plant allelochemicals on feeding behavior, physiology or growth and development of various insects have been widely reported (Chen et al., 2018; Guo et al., 2013; Kessler et al., 2006; Lee et al., 2015). In this study, quercetin ingestion exhibits the suppressive effects on the growth of *S. exigua* larvae. Similar studies have also been reported, in which insect growth could be affected by the exposure of plant secondary metabolites. For example, the quercetin significantly inhibited the growth and body weight of silkworm and *H. armigera* (Chen et al., 2018; War et al., 2013; Zhang et al., 2012). Predominantly, one of the mechanism in insect herbivores underlying the impacts of plant secondary metabolites on the susceptibility to insecticides is the role of activity enhancement of the detoxification system that lead to an increase in metabolic defense in insects (Francis et al., 2005; Terriere and Max-Planck, 1984; Zhu et al., 2016). In the present study, significantly enhanced in the activity of P450s enzyme was observed in quercetin, lambda-cyhalothrin, and quercetin + lambda-cyhalothrin-fed *S. exigua* larvae from 48 to 72 h. Our present results are consistent with the previous studies (Hafeez et al., 2019a,b; Arain et al., 2018; Chen et al., 2018; Hafeez et al., 2018; Tao et al., 2012). Furthermore, similar to our study the application of PBO as synergist can inhibit the activity of P450s in herbivores insect (Badawy et al., 2015). Present results strongly suggest that these P450s play critical role in metabolizing
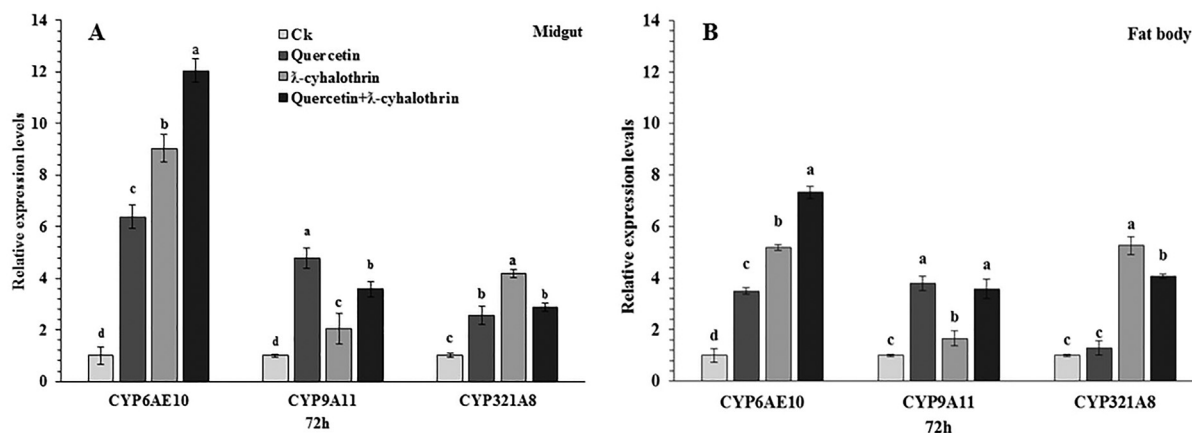

Fig. 3. Effect of 0.2% quercetin on *Spodoptera exigua* larvae tolerance to lambda-cyhalothrin and relative expression levels of three P450s genes in midgut (A) and fat body (B) after 72 h. The transcription levels of three P450s genes determined by quantitative real-time PCR, normalized to three reference genes. Each bar indicates the mean of three biological samples (±SE), each implemented in replicates. Different letters above bars indicate significant differences ($p < 0.05$) according to Tukey HSD test.
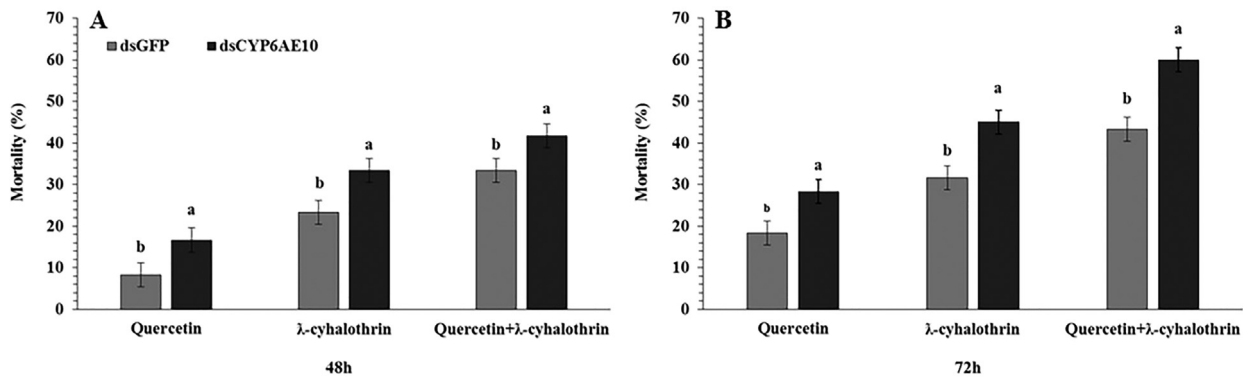
Fig. 4. Mortality of *Spodoptera exigua* larvae (pre-exposed with 0.2 quercetin for 24 h) followed by dsCY6AE10 and dsCYPA98 plus LC_{50} lambda-cyhalothrin treated diet for (A) 48 and (B) 72 h. Data shown are means ± SE derived from three biological replicates. Different letters above bars indicate significant differences (p < 0.05) according to the Student's *t*-test.
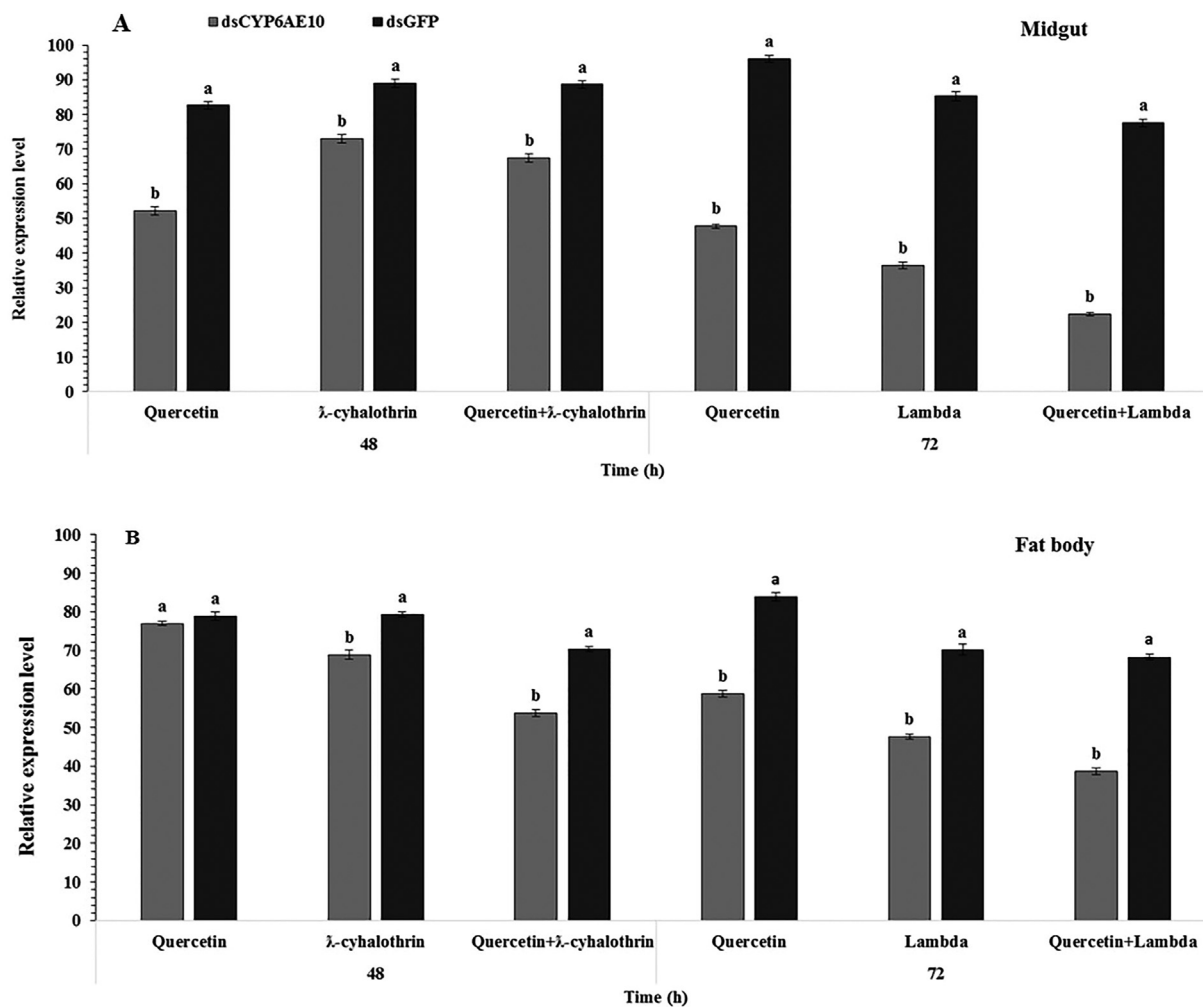

Fig. 5. Effects of 0.2% quercetin pre-exposed *Spodoptera exigua* larvae tolerance to lambda-cyhalothrin and relative expression changes of CY6AE10 and dsRED in the midgut (A) and fat body (B) after dsRNA injection. Data shown are mean ± SE derived from three biological replicates. Different letters above bars indicate significant differences (p < 0.05) according to the Tukey HSD test.

the natural compounds as well as synthetic pesticides fed by this pest.

Frequently, increased inactivity of P450 detoxification enzyme in the individual due to the up-regulation of one or more than one P40 genes seems to be a general molecular mechanism (Elzaki et al., 2016). In this study, qRT-PCR results revealed the

significantly increased overexpression level of three P450 genes *CYP6AE10*, *CYP9A11* and *CYP321A8* in quercetin, lambda-cyhalothrin and quercetin + lambda-cyhalothrin-fed *S. exigua* larvae midgut and fat body compared to the control group. In previous studies, induced effects of plant secondary metabolites to insecticides on multiple P450 genes have been extensively

reported in many insect species. For example, the expression level of three P450 genes *CYP6B6, CYP6B8* and *CYP321A1* was increased in quercetin and xanthotoxin-fed *S. exigua* larvae (Chen et al., 2018; Li et al., 2000a,b; Liu et al., 2006). Further studies showed that elevated transcription level of *CYP6B1* and *CYP6B3* was induced by xanthotoxin in *P. polyxenes* (Petersen et al., 2001), and four plant secondary metabolites including gossypol increased the larval tolerance to methomyl and deltamethrin in *H. armigera* by induced expression of P450, *CYP6B1, CYP6B6, CYP6B7, CYP9A14* and *CYP9A12* (Mao et al. 2007; Tao et al. 2012; Chen et al. 2018). These results strongly, suggest that increased tolerance to insecticides may result from the ability of its host plants allelochemicals induced over-expression of multiple P540 genes in polyphagous insect herbivores.

RNA interference (RNAi) is a universal gene-silencing technology has been widely used to knock down the genes in insect herbivores for functional analysis of protein involved in growth, development and resistance to toxin compounds (Hu et al., 2014; Kim et al., 2015a,b; Mao et al., 2007; Tao et al., 2012;. Wang et al., 2018a,b). Double-strand RNA is highly effective at triggering RNAi, and can possibly be delivered by different methods e.g. microinjection or by feeding as a dietary component (Kim et al., 2015a,b; Mao et al., 2007; Wang et al., 2018a,b; Wang et al., 2017). In the present study, the *CYP6AE10* knockdown in *S. exigua* larvae followed by the exposure with quercetin, lambda-cyhalothrin and quercetin + lambda-cyhalothrin caused significantly decreased in the transcription level of target cytochrome P450 gene. Similarly, the knockdown of *CYP6AE10* by the injection of dsRNA led to increased mortality after the treatment of quercetin, lambda-cyhalothrin, and quercetin + lambda-cyhalothrin. Our speculation is consistent with many previous works in which silencing effect of dsRNA on many P450 genes increased the mortality in different species of herbivores insects (Mao et al., 2011; Tao et al., 2012; Turner et al., 2006; Wang et al., 2018a,b; Wang et al., 2017, 2015b). Similar results have been documented the silencing effect of dsRNA on *CYP6BG*1 and *CYP321E1 P. xylostella* (Bautista et al., 2009; Hu et al., 2014). The present results strongly suggest that *CYP6AE10* might play a significant role in induced resistance to pesticides by the ingestion of plant secondary metabolites in *S. exigua*.

5. Conclusion

In conclusion, the induced effect of plant secondary metabolites could increase the sensitivity to pesticides by elevating the detoxification enzymes activity in herbivores insects. However, insect cytochrome P450 detoxification system undoubtedly play an important role in the adoption to plant defense chemicals (Scott et al., 1998). We further investigated the transcription level of induced cytochrome P450 genes responsible for the metabolism of quercetin and lambda-cyhalothrin in *S. exigua* larvae. The present study suggested that CYP6AE10 RNAi treatment followed by the exposure with quercetin and lambda-cyhalothrin increased the mortality of *S. exigua*. Further should be conducted for the identification of genes responding specifically to plant toxins and insecticides to better understand the detoxification mechanism in insects and the results of these will influence future strategies for insect pest management.

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