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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A B S T R A C T

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 treatment. 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.
1. Introduction

In the ecological interaction of plants and insects, plants evolved the variety of mechanisms to defend against phytophagous insects by intervening in the basic metabolic, biochemical, physiological functions and even behavior of herbivorous insects (Duisembecova and Dubovskiy, 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; Ifti 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; Ifti 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 interference 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 (4.0 cm diameter, 3.5 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 mg of lambda-cyhalothrin mg/L for 0.2% quercetin pretreated group and 60, 85, 110, 135 and 160 mg 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 LC₅₀ 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.

2.5. 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/mL, and (10 mg/larvae) of PBO solution was topically delivered onto the dorsally protracted 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 mg of lambda-cyhalothrin mg/L for 0.2% quercetin-pretreated and PBO-treated group; and) 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 mg of lambda-cyhalothrin mg/L and covered all cups with lid containing small holes for ventilation. Mortality was assessed 72 h after lambda-cyhalothrin application and LC₅₀ 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, LC₅₀ 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.
(PMSE), 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 10,800 g 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 mea-
sured immediately by using a SPECTRA max GEMINI XS spectru-
fluorometer (Molecular Devices, USA) with adjusting 356 nm
excitation and 465 nm emission filters. A series of different con-
centrations of 7-hydroxycoumarin were prepared, and standard
substance fluorescence values were measured to draw the stan-
dard 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, LC50 concentration of lambda-cyhalothrin 96.93 µg/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
0 °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 RNAase 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 cen-
trifuged 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 dis-
solve 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 rea-
tions 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, Trans-
Script® RT/RNaseH 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 quanti-
fied by quantitative real-time PCR (qRT-PCR) using an Mitech™ 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 tripli-
cate. 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 ddH2O and 10 µL 2
x syber 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 dilu-
tion 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−ΔΔCT 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 differ-
ence 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 GPF-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 RibobMAXTM Express RNAi Sys-
tem (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
(Micro4TM 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 transcr-
ption 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 inde-
dependent 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, LC50 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

LC50 values were calculated by standard probit analysis (Abbott, 1925). The data of enzymatic activity, body weight gain and relative expression level of P450 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 LC50 value of lambda-cyhalothrin in a quercetin-pretreated population of S. exigua larvae was 196.0 mg/L, while the LC50 value of lambda-cyhalothrin for the control group (larvae without exposure to quercetin) was 96.93 mg/L, which was lower 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 LC50 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 S. exigua larvae after fed on quercetin (1.08), lambda-cyhalothrin (0.691) and quercetin + lambda-cyhalothrin (1.33) at 48 hours 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 dsCYP6AE10 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

| Treatment                  | LC50 (mg a.i./L) | 95% CL          | Slope ± SE      | df   | χ²  | 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 ± 0.23     | 3    | 0.65 | 1.06   |

Where, LC50 = lethal concentration to kill 50% of the population; a.i. = active ingredient; CL = confidence limits; SE = standard error; df = degree of freedom; χ² = Chi-square value; SR = resistance ratio.
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

### Table 2

| Function          | Primer name                  | Primer sequence (5′-3′)                           |
|-------------------|-------------------------------|---------------------------------------------------|
| Real-Time PCR     | CYP6AE10-sense                | GGACAATGTCGAGACTGGC                                |
|                   | CYP6AE10-anti-sense           | TGGCACAACCTGAGTGCCT                                 |
|                   | GADPH-sense                   | CTGGAGAACAGTTGTGTCATC                               |
|                   | GADPH-anti-sense              | GATCGATAAACCCTGGTGGAGTA                             |
| dsRNA synthesis   | CYP6AE10-sense1               | ggatcttaatagcactactataggATCACGCTGTATCTGGCTC       |
|                   | CYP6AE10-anti-sense1          | TTGGCTGATGAGAATAAGGGA                              |
|                   | CYP6AE10-sense2               | ATCCACGTGATCTCGTTGCTC                              |
|                   | dsRED-sense1                  | TTGGCTGATGAGAATAAGGGA                              |
|                   | dsRED-anti-sense2             | ATCCACGTGATCTCGTTGCTC                              |
|                   | dsRED-sense2                  | GCAAGCTATGCATCCACCGTTGGGCC                        |
|                   | T7dsRED-anti-sense2           | CAAGCTATGCATCCACCGTTGGGCC                        |

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.
of biotic and abiotic stresses, in which biotic stress due to insect herbivores 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...
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

Fig. 4. Mortality of Spodoptera exigua larvae (pre-exposed with 0.2 quercetin for 24 h) followed by dsCY6AE10 and dsCYP9A11 plus LC50 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.
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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