Bulletin of Entomological Research (2014) **104**, 801–808
doi:10.1017/S0007485314000698

Identification of the 2-tridecanone responsive region in the promoter of cytochrome P450 *CYP6B6* of the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae)

F. Li[1], X.N. Liu[1]*, Y. Zhu[1], J. Ma[1], N. Liu[2] and J.H. Yang[3]

[1]Xinjiang Key Laboratory of Biological Resources and Genetic Engineering, College of Life Science and Technology, Xinjiang University, Urumqi 830046, China: [2]National Cotton Engineering &Technology Research Center, Urumqi 830091, China: [3]Department of Pediatrics, Texas Children's Cancer Center, Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, TX 77030, USA

Abstract

Eukaryote transcription is controlled by regulatory DNA sequences and transcription factors, so transcriptional control of gene plays a pivotal role in gene expression. In this study, we identified the region of the *CYP6B6* gene promoter of *Helicoverpa armigera* which responds to the plant secondary toxicant 2-tridecanone. Transient transfection assay results from five of stepwise deletion fragments linked to the luciferase reporter gene revealed that the promoter activity of each *CYP6B6* fragment was significantly higher than that of their basal activity after the Sf9 cells were treated with 2-tridecanone. Among all, the fragment spanning −373 to +405 bp of the *CYP6B6* promoter showed an obviously 2-tridecanone inducibility ($P < 0.0001$), which might have the 2-tridecanone responsive element based on promoter activity. Electrophoretic mobility shift assays revealed that the nuclear protein extracted from midgut of the 6th instar larva of *H. armigera*, reared on 10 mg 2-tridecanone per gram artificial diet for 48 h, could specifically bind to the active region from −373 to 21 bp of the *CYP6B6* promoter. The combination feature also appeared when using a shorter fragment from −292 to −154 bp of the *CYP6B6* promoter. Taken together, we found a 2-tridecanone core responsive region between −292 and −154 bp of the *CYP6B6* promoter. This may lead us to a better understanding of transcriptional mechanism of P450 gene and provide very useful information for the pest control.

Keywords: *Helicoverpa armigera*, cytochrome P450 CYP6B6, promoter activity, 2-tridecanone responsive region

(Accepted 29 August 2014; First published online 2 October 2014)

Introduction

In insects, enzymatic detoxification of plant toxins and insecticides is well documented to be one of the common and major mechanisms of host plant adaptability and insecticide resistance (Wu & Gong, 1997; Li *et al*., 2002; Yang *et al*., 2004; Chen *et al*., 2005). Cytochrome P450s play a crucial role in

*Author for correspondence
Phone: 13579817922
E-mail: liuxn0103@sina.com

insect adaptation to their host plants and in insecticide resistance through metabolic detoxification. Survival of insects in the presence of toxic secondary metabolites depends on their metabolism by more limited groups of P450s (Schuler, 2011). Cytochrome P450s belong to a large enzyme family involved in a wide variety of metabolic functions, including insecticide detoxification, and increased cytochrome P450-mediated insecticide metabolism is a common resistance mechanism (Feyereisen, 1999; Vontas *et al*., 2005). Resistance results from genetic changes leading to either altered gene expression or altered function (Willoughby *et al*., 2006), inducibility is a general characteristic of cytochrome P450 (Harrison *et al*., 2001). In insects, insecticide resistance-associated overexpression of one or more CYP genes has been observed in many insect species (Scott, 1999; Zhou *et al*., 2010). Insects exhibit induction responses to other xenobiotics when they come into contact with toxic plant compounds. For example, the black swallowtail butterfly *Papilio polyxenes* induces the P450 genes *Cyp6B1* and *Cyp6B3* in response to the toxic furanocoumarin and xanthotoxin produced by plant families (Cohen *et al*., 1992; Hung *et al*., 1995).

In recent study, we have known that the efficacy of P450-mediated detoxification depends on the levels of P450 transcript, which may change after exposure to xenobiotics. Transcriptional control of gene expression plays an important role in many areas of biology. Gene regulation in eukaryotes occurs primarily at the level of transcription. Eukaryote transcription is controlled by *cis*-acting DNA elements and *trans*-acting factors (Harshman, 1998; Mantovani, 1998). Aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, mediates xenobiotic signaling to enhance the expression of target genes, including drug-metabolizing cytochrome P450s (Fujii-Kuriyama & Mimura, 2005; Kawajiri & Fujii-Kuriyama, 2007). Research have reported that both of the *CYP6B1* and *CYP6B4* promoters contain XRE–AhR elements (xenobiotic response element to the aryl hydrocarbon receptor) that are activated by binding to activated AhR–ARNT complexes (Schmidt & Bradfield, 1996; Whitlock & Jr, 1999; McDonnell *et al*., 2004) .

In China, cotton is an important economic crop; however, it is the target of a variety of pests. *Helicoverpa armigera* (Lepidoptera: Noctuidae) is a major insect pest in a wide range of agricultural and economic crops in many parts of the world including China (Liu *et al*., 2006). Among the bollworm, the damage caused by *H. armigera* is the major constraint in increasing the cotton productivity. Biologically, it is one of the most successful pests due to its high fecundity, great migration potential, wide host range, and diapause behavior to overcome unfavorable environmental conditions. It is very difficult to control pest because of its ability to develop resistance against almost all the conventional insecticide chemicals applied for its control (Ahmad *et al*., 2001).

Helicoverpa armigera is a polyphagous insect, which can encounter secondary toxicant in its host plants, such as cotton, corn, solanaceous vegetable, and so on. 2-tridecanone, a commonly plant allelochemicals is found naturally in the trichomes of wild tomato plants, It is important in resistance to herbivory (Williams *et al*., 1980; Goncalves *et al*., 1998; Kimps *et al*., 2011). In previous experiments, in order to convince the induction of CYP6B6 expression which was linked to the insects' tolerance to the 2-tridecanone, we had checked the survival rate of 2-tridecanone treated or non-treated on *H. armigera*. The cytochrome P450s gene can be up-regulated by 2-tridecanone. For example, the larvae of *Heliothis virescens* were more tolerant to diazinon when fed on leaves of wild tomato or treatment of the larvae with 2-tridecanone (Riskallah *et al*., 1986). In this study, using real-time polymerase chain reaction (RT-PCR), we detected that the *CYP6B6* gene was over-expressed after the 2-tridecanone induced. In order to illuminate the regulatory over-expression mechanism of the *CYP6B6* promoter when *H. armigera* is exposed to the 2-tridecanone, We cloned the 5′ flanking of the *CYP6B6* and constructed the deletion fragment, then found the 2-tridecanone core responsive region using two detection technique. By luciferase reporter gene assay, a 202 bp region localized at positions −373 to −172 bp of the *CYP6B6* gene promoter is crucial for transcriptional activation, and an inhibitory region is located at positions −577 to −374 bp. Initial characterization of the factors regulating the promoter was performed using electrophoretic mobility shift assays (EMSA). We further identified a fragment at positions −292 to −154 bp that can specifically bind to a nuclear factor and modulate the activity of the activator domain. These results may help us to determine the region of *CYP6B6* which responds to the plant secondary material and provide some useful clue for insect controlling.

Materials and methods

Insects

Larvae of *H. armigera* were reared on an artificial diet at 28 ± 1°C and photoperiod according to previously described in Liu *et al*. (2006).

Chemicals and reagents

Thiazolyl blue (MTT) and 2-tridecanone were purchased from Sigma (St. Louis, USA). TRIzol reagent, real master mix SYBR green PCR kit, Cellfectin II Reagent from Invitrogen (Carlsbad, USA), DNase I, oligo(dT) 18 primer (50 mM), RNase M-MLV(RNase H-), RNase inhibitor, dNTP mixture (2.5 mM), Taq polymerase from Takara (Dalian, China). Other reagents are commonly used for laboratory analytical reagents.

Cell culture

Sf9 cells derived from *Spodoptera frugiperda*, a lepidopteran distantly related to *H. armigera*. Sf9 insect cells were kindly provided by Academy of military science and cultured in Sf-900 II SFM liquid media (Invitrogen) supplemented with 10% fetal bovine serum (Evergreen, Hangzhou, China) and 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin. Cells were maintained at 27°C without CO_2 condition.

Reporter plasmids

Throughout this paper, we report nucleotide positions in the proximal promoter relative to the transcriptional start site indicated by +1, with upstream or sequences 5′ of it preceded by '−' and downstream or 3′ sequences preceded by '+'. To construct the pGL3-CYP6B6-promoter plasmid pBK-1412, the region of *CYP6B6* (−1007 to +405 bp) was PCR-amplified using *H. armigera* genomic DNA as template and a pair of primers listed in supplemental table 1. The PCR product was digested by *Bgl* II and *Kpn* I restriction enzymes and subcloned into promoterless pGL3-basic vector (Promega, Madison, USA) digested by the same enzymes, yielding pGL3-CYP6B6-promoter. A series of stepwise deletion

https://doi.org/10.1017/S0007485314000698 Published online by Cambridge University Press

Table 1. The PCR primers for deletion fragment.

Primer name	Primer sequence	Products length (bp)	Position
Downstream	5′CAGATCTCCCATCAGCGTGGAACAAGTTTGA3′		
Upstream	5′GGTACCTACTTGTAAATTACCTGCCAAAG3′	1412	−1007/+405
	5′GGTACCAAGCAATTAAAAGACATACAG3′	1234	−829/+405
	5′GGTACCGGTACGACACACCCGATATAG3′	982	−577/+405
	5′GGTACCTATTCTTGACATACATATAAGGGA3′	778	−373/+405
	5′GGTACCAGTAACAAACTACTCATGCCT3′	576	−171/+405

The *Bgl* II and *Kpn* I sites engineered are underlined.

fragments of the *CYP6B6* promoter starting at position +405 and extending to −171, −373, −577, −829 bp were generated by PCR using pBK-1412 as a template, a forward primer and a common reverse primer as indicated in table 1. All sequences were confirmed by sequencing (Sangon Biotech, Shanghai, China) and termed respectively as pBK-576, pBK-778, pBk-982 and pBK-1234.

MTT assay

The MTT assay previously described to measure cytotoxicity and cell proliferation was further explored to extend its application to the measurement of cell activation (Gerlier & Thomasset, 1986). Firstly we added healthy Sf9 cells at a 5.0×10^4 cells ml^{-1} density to each well of a 96-well microtiter plate with 90 µl of Sf-900 medium (without serum and antibiotics), and cultured until cells reached 90% viability then added 10 µl of medium mixed with drug dilutions. Nine different concentrations of 2-tridecanone were made in serial dilutions from 0 to 1×10^7 ng ml^{-1}. 100 µl of medium only was used as a blank and negative controls without drug were interspersed throughout the plate. Each test was set up in triplicate. The plate was then incubated for 24 h at 27°C without CO_2. Cells were continuously exposed to the 2-tridecanone throughout this period. After drug exposure, the plate was inverted followed by a rapid flick to remove the medium plus any drug and added 20 µl of 5 mg ml^{-1} MTT to each well and incubated for 4 h. The formazan crystals formed were dissolved in 100 µl of DMSO for 10 min. The plate was then read on a BIO-RAD microplate spectrophotometer at 540/655 nm. The cell growth inhibition rate of live cells per well was calculated as following formula:

The cell growth inhibition rate (%) = [1-(Test $OD_{540/655}$ − Blank $OD_{540/655}$)/(Negative $OD_{540/655}$ − Blank $OD_{540/655}$)] × 100%

Dual luciferase reporter assay

Cationic lipofectin transfection was performed according to Cellfectin II following its protocols. For transient transfections, 9.0×10^5 cells ml^{-1} density were added to each well of a 6-well microtiter plate and cultured until cells reached 70–80% viability. 210 µl of combine transfection mixtures containing 8 µl of lipofectin, 3.2 µg of reporter plasmid DNA and 0.32 µg of internal control Renilla luciferase plasmid pRL-TK (Promega) was added to each well. After 6 h, the serum-free medium was replaced by the medium with 10% fetal bovine serum and 2-tridecanone continued to incubate until harvested. After 2-tridecanone induced, Luciferase activity was determined using a Dual-Luciferase Reporter Assay Reagent Kit (Promega). Luciferase reporter assays were performed in Sf9 cells according to the manufacturer's instructions. Briefly, cells were pelleted and 200 µl of 1 × passive lysis buffer was added to each well, at room temperature to lyse cells for 30 min. The cells were then centrifuged at 1500 rpm for 5 min at 4°C. The clear supernatant was collected from each well and assayed immediately for F-luc and R-luc activities by FLUOROSKAN ASCENTFL (Thermo, Massachusetts, USA). R-luc activity was used to normalize the data, which were expressed as the ratio of F-luc to R-luc activity.

cDNA synthesis and real-time quantitative PCR analysis

To obtain the relevant transcription factors in the nuclear protein from *H. armigera* as much as possible, we should determine the expression level of the P450 *CYP6B6* mRNA of the cotton bollworm treated with 2-tridecanone. The levels of the *CYP6B6* gene expression in 2-tridecanone induced of *H. armigera* were quantified using a real-time quantitative PCR (qRT-PCR), a 7500 Real Time PCR System (Applied Biosystems, USA) and a real-time master mix SYBR Green PCR kit.

Sixth instar larvae of *H. armigera* were exposed to the different concentrations of 2-tridecanone via the food source for different hours. At least three larvae were treated in each group. The midgut was isolated from the experimental larvae on ice and then used for extraction of total RNA according to the instruction of TRIzol Reagent kit. RNA was digested by DNase I in order to eliminate genomic DNA contamination. In addition, cDNA was synthesized by reverse transcription in 20 µl of reaction system according to the manufacture's instructions. The elongation β-actin was used as a reference gene to normalize the target gene expression levels among samples. qRT-PCR of each cDNA sample and template-free was performed in triplicate. All the primer sets used in this study were listed as follows. The relative expression levels of target genes were calculated by the critical threshold (CT) cycle method then further processed using Microsoft Excel. Primers of *CYP6B6* (Gene bank: AY950636) and β-actin (Gene bank: EU527017.1) (5′ − 3′):

CYP6B6-F:TTCAAACTTATACCATGTCCACAATT,-R:CCAATTGACGGAGCTCTAGAATCA;

β-actin-F:ATCATCGACGCTCCCGGACA,-R:TAGCTGCTTGACTCCGAGGGTG.

Electrophoretic mobility shift assay

Nuclear proteins were prepared from the midgut of 6th instar larvae, treated with 2-tridecanone, using the Nuclear Extraction Kit (Thermo) following the manufacturer's protocol, and the nuclear protein from untreated larvae were used as a control. Nuclear proteins were quantified using Bradford Protein Assay Kit (BIO TEKE, Beijing, China). DNA probe labeling and subsequent color detection were performed by DIG High Prime DNA Labeling and Detection Starter Kit I

https://doi.org/10.1017/S0007485314000698 Published online by Cambridge University Press

Table 2. Primers for probe HE-1 and HE-2 generation used for EMSA.

Primer name		Primer sequence	Products length (bp)	Position
E-1	Downstream	5′CCTCGCTGTTCACAATG3′	394	−373/+21
	Upstream	5′TATTCTTGACATACATATAAGGGA3′		
E-2	Downstream	5′AAGCATGAGTTGTTACT3′	138	−292/−154
	Upstream	5″CAGTTCTGAATATAATGTGG3′		

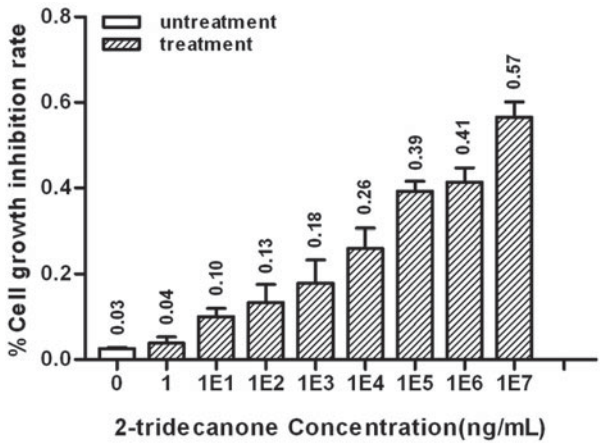

Fig. 1. The effect of 2-tridecanone on Sf9 cell using MTT assay. The cells were exposed to different 2-tridecanone concentrations for 24 h. The cell growth inhibition rate was determined by MTT assay. The cytotoxicity of 2-tridecanone in descending order was as follows (ng ml^{-1}):1E7 > 1E6 > 1E5 > 1E4 > 1E3 > 1E2 > 1E1 > 1 > 0.

(Roche, Basel, Switzerland) following manufacturer's protocol. For specific competition experiments, a 120-fold excess of unlabeled DNA probe was added. For nonspecific competition experiments, a 400 bp non-correlation sequence from *Drosophila melanogaster* was added, termed as DE-1, and the negative control was in the absence of nuclear proteins. Protein-bound probes were separated from free probes on 5% (w/v) nondenaturing polyacrylamide gel in 1× TBE buffer and detected using ImageQuant LAS 4000 (Fijifilm, Tokyo, Japan). The 1× binding buffer composition as follows: 20 mM Tris-HCl (PH 7.6), 30 mM KCl, 0.2% (w/v) Tween20, 1 mM EDTA, 1M DTT, 10 mM $(NH4)_2SO_4$. Probe HE-1 and HE-2 of the *CYP6B6* gene promoter was produced by PCR using primers E-1 and E-2, respectively (table 2).

Statistical analysis

Data are expressed as Mean ± SD deviation from triplicate experiments. The data were analyzed using One-way analysis of variance (ANOVA). In the dual luciferase reporter assay experiment, a different construct of a transfection series as a factor, and the 2-tridecanone-induced time or dose as factors in the qRT-PCR experiment, followed by T-test to compare the differences of two samples. All analysis was conducted by GraphPad Prism 5 software (San Diego, CA, USA).

Results

Determination of 2-tridecanone toxicity to Sf9 cells

The survival of Sf9 cells treated with 2-tridecanone was measured by the MTT assay. After Sf9 cells were continuous exposed to different concentrations of drugs for 24 h, we measured the growth inhibition rate of Sf9 cells. Figure 1 showed the effect of increasing concentration of 2-tridecanone on the cells. With the increasing concentration of the 2-tridecanone, the degree of cell growth inhibition also increased. When the concentration of 2-tridecanone reached to 1×10^6 ng ml^{-1}, more than 50% cells were repressed, so 1×10^6 ng ml^{-1} of 2-tridecanone was made as the suitable induction concentration according to the cell growth inhibition rate under 50%.

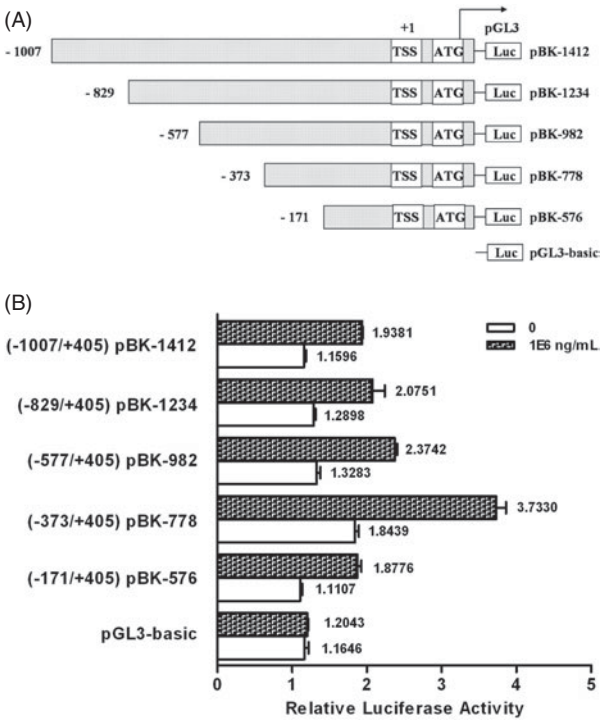

Fig. 2. Deletion analysis of the *CYP6B6* gene promoter activity induced by 2-tridecanone. (A) Diagram of a series of deletion constructs containing different lengths of *CYP6B6* promoters fused to luciferase reporter gene. (B) 2-tridecanone-induced luciferase activity in Sf9 cell transfected with the *CYP6B6* promoter/luciferase reporter gene constructs. 3.2 μg of each reporter plasmid was transfected into Sf9cell line. After 6 h of transfection, the cells were incubated with 1×10^6 ng ml^{-1} of 2-tridecanone for 24 h. After incubation, the luciferase activity in the cell lysates was measured. All experiments were repeated at least three times to confirm the reproducibility of the observations and represented by Mean ± SD.

Analysis of the promoter activity by dual luciferase reporter system in Sf9 cell

The activity of the *CYP6B6* promoter of *H. armigera* and potential consensus sequences for regulatory region and the

https://doi.org/10.1017/S0007485314000698 Published online by Cambridge University Press

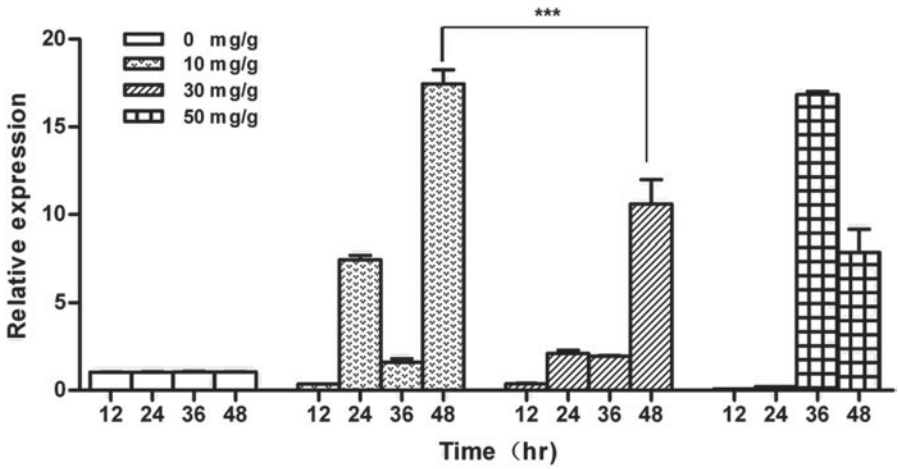

Fig. 3. The effect of 2-tridecanone on *CYP6B6* gene expression. *Helicoverpa armigera* were treated with 2-tridecanone for different time, and mRNA relative expression level of *CYP6B6* was determined. Asterisk stands for significant difference of relative expression ($P = 0.0003$, T-test).

luciferase detection conditions were previously described in Li & Liu, 2012; Li *et al*., 2012. The promoter sequence pBK-1412 has been submitted to GenBank (JN900393). To determine whether 5′UTR plays an important role in 2-tridecanone-mediated *CYP6B6* expression, we generated a set of five deletion constructs and cloned them into pGL3-basic luciferase reporter vectors (fig. 2A). On the basis of the MTT assay, we chose 1×10^6 ng ml^{-1} of 2-tridecanone to treat Sf9 cells for 24 h, The luciferase activities of the deletion constructs were assessed by dual luciferase reporter system in Sf9 cells. All the five deletion constructs had significantly basal and 2-tridecanone-induced luciferase activity compared with the promoterless pGL3-basic construct (basal: $F_{5,12} = 132.3$, $P < 0.0001$, $R^2 = 0.9822$; 2-tridecanone-induced: $F_{5,12} = 268.1$, $P < 0.0001$, $R^2 = 0.9911$). When transfected Sf9 cells were treated with 1×10^6 ng ml^{-1} of 2-tridecanone, compared to basal luciferase activity, the constructs pBK-576 (−171/+405) did not show much 2-tridecanone-induced promoter activity, just had a 1.7-fold higher than that of the basal one ($t_4 = 24$, $P < 0.0001$). And the 2-tridecanone-induced promoter activity of the construct pBK-778(−373 to +405 bp) showed a significant increase ($t_4 = 23.62$, $P < 0.0001$), but for the construct pBK-982 (−577/+405) and pBK-1234 (−829/+405) there was a significant decrease in the 2-tridecanone-induced promoter activity (pBK-982: $t_4 = 30.09$, $P < 0.0001$; pBK-1234: $t_4 = 8.148$, $P < 0.001$), and the activity of the construct pBK-1412 (−1007/+405) was not significantly different from that of the pBK-576 ($t_4 = 2.098$, $P = 0.1039$). The results suggested that the *CYP6B6* gene promoter was capable of regulating the basal and 2-tridecanone-induced expression of this gene, since it contained potential 2-tridecanone responsive region between −373 and −172 bp, in addition, it also included repressed region between −577 and −374 bp, which was able to response to 2-tridecanone.

2-tridecanone regulates expression of the CYP6B6 from midgut of H. armigera

We detected the *CYP6B6* expression level of the 6th instar larvae by qRT-PCR after treated with 2-tridecanone. RNA was

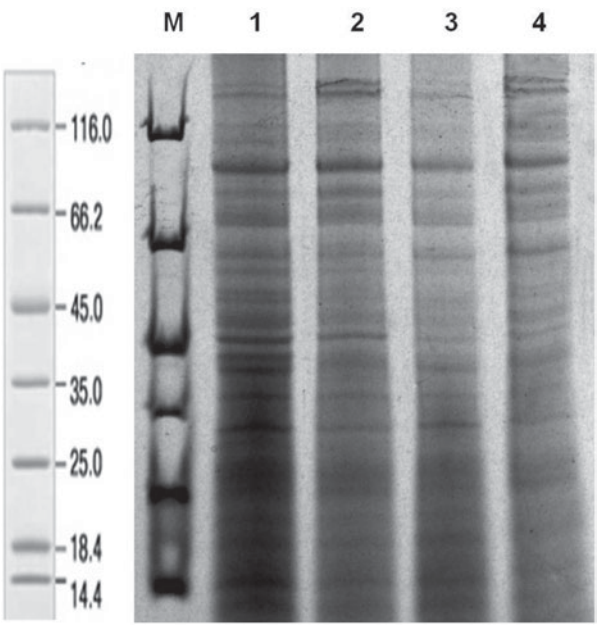

Fig. 4. Quantification of the nuclear protein from midgut of the 6th instar larvae by a 10% Tricine-SDS-PAGE. Lanes 1 and 2: The cytoplasmic and nuclear protein from *H. armigera* after the 2-tridecanone treatment for 48 h; lanes 3 and 4: The cytoplasmic and nuclear protein from *H. armigera* fed on normal diet. M: unstained protein molecular weight marker (Fermentas, Canada).

extracted from the midgut of the 6th instar larvae treated with different concentrations of 2-tridecanone for different time. Using reverse transcribed cDNA as a template, β-actin as a reference gene we calculated the relative level of expression of *CYP6B6* gene using $2^{-\Delta\Delta CT}$. The non-2-tridecanone treatment was negative for all tested hours in all of the experiments ($F_{3,8} = 0.1761$, $P < 0.9096$, $R^2 = 0.06195$). Using treatment of 2-tridecanone, the relative expression of *CYP6B6* was significant for dose by One-way ANOVA (10 mg g^{-1}: $F_{3,8} = 965.4$,

https://doi.org/10.1017/S0007485314000698 Published online by Cambridge University Press

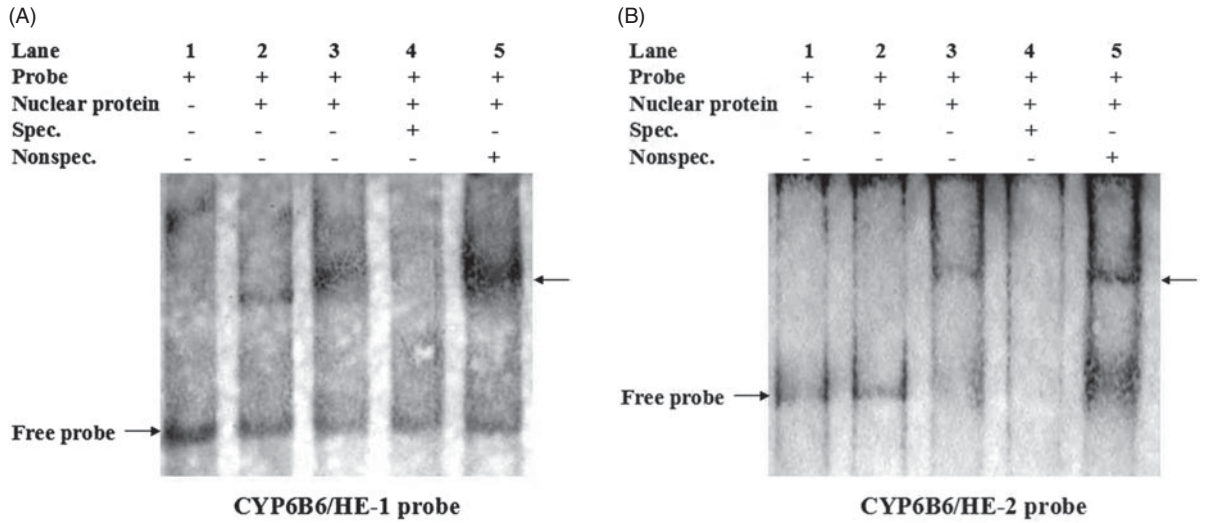

Fig. 5. Electrophoresis mobility shift assay of the activating region. (A) 10 μg of nuclear protein extracts from midgut was used in each reaction (except lane 1). Probes HE-1 was incubated with nuclear protein extracts and the nuclear protein factor was detected by labeled probe HE-1(lanes 2, 3 and 5). HE-1 was incubated with nuclear protein extracts with competitor, 120-fold unlabeled HE-1 (lane 4) or unrelated oligonucleotides DE-1 (lane 5). (B) Probes HE-2 was incubated with nuclear protein extracts, set as above. The shift bands are indicated by arrows.

$P<0.0001$, $R^2=0.9972$; 30 mg g^{-1}: $F_{3,8}=273.0$, $P<0.0001$, $R^2=0.9903$; 50 mg g^{-1}: $F_{3,8}=459.6$, $P<0.0001$, $R^2=0.9942$). The results showed that after treatment with 10 mg g^{-1} and 30 mg g^{-1} 2-tridecanone, there was a minimum level of expression of *CYP6B6* at 12 h. However, the level was increased from 12 to 24 h, then decreased at 36 h and reached the highest level at 48 h. When the 6th instar larvae treated with 50 mg g^{-1} 2-tridecanone, the level of expression of *CYP6B6* was the lowest at 12 and 24 h, the level had a sharp increase between 24 and 36 h, and then decreased a little at 48 h (fig. 3). The relative expression of *CYP6B6* gene had a significant difference at 48 h under 2-tridecanone different concentrations ($F_{3,8}=181.3$, $P<0.0001$, $R^2=0.9855$). The *CYP6B6* expression level induced by 10 mg g^{-1} 2-tridecanone was significantly higher than that induced by 30 mg g^{-1} 2-tridecanone at 48 h ($t_4=11.33$, $P=0.0003$), so it is a suitable induction conditions for our following experiments.

EMSA analysis of the 2-tridecanone responsive region

To further examine the role of the identified 2-tridecanone responsive region, we performed EMSAs. Through the qRT-PCR, we knew the level of *CYP6B6* mRNA was higher after treated with 10 mg g^{-1} 2-tridecanone for 48 h, so the nuclear protein was extracted from midgut of the 6th instar larvae fed on artificial food with 10 mg g^{-1} 2-tridecanone for 48 h and on the artificial diet, respectively, and quantified by a 10% Tricine-SDS-PAGE (fig. 4). 4 ng of two probes HE-1 and HE-2 spanning the active region (−373 to +21 bp) and (−292 to −154 bp) of the *CYP6B6* promoter, were incubated with 10 μg nuclear protein extracts of *H. armigera* midgut, respectively. When incubated the labeled probe HE-1 with nuclear extracts (from the midgut of the 6th instar larvae treated and untreated with 2-tridecanone) they generated one distinct binding complex (fig. 5A, lanes 2 and 3), efficiently removed by excessive unlabeled homologous HE-1, but not by the heterologous DE-1 oligonucleotide (fig. 5A, lanes 4 and 5). The labeled probe HE-2 also produced one distinct binding complex (fig. 5B, lane 3), and the competitive reaction showed the same results as HE-1 (fig. 5B, lanes 4 and 5). No shift band, however, was observed when HE-1 and HE-2 were used as the probes, respectively (fig. 5A, lane 1 and fig. 5B, lane 1). Strangely, there was no shift band when the labeled probe HE-2 mixed with nuclear extracts from the untreated larvae. We considered that the shift band of probe HE-1 (fig. 5A, lane 2) may be a nonspecific binding because this probe is a little long and nearly 400 bp. Therefore, nuclear protein factors from *H. armigera* midgut can specifically interact with the region (−292/−154), and might be the 2-tridecanone responsive region in the promoter of cytochrome P450 *CYP6B6* gene and play a role for transcriptional activation of the gene.

Discussion

Detoxification pathways have evolved to aid in the metabolism of potentially toxic chemical compounds that an organism may encounter in its environment. In many biological systems, substrates of these pathways induce the expression of the metabolic enzymes involved in their metabolism (Denison & Nagy, 2003). Insect monooxygenases can be detected in a wide range of tissues. The highest monooxygenase activities are usually associated with the midgut, fat bodies and Malpighian tubules (Scott, 2008). It is tempting to find the xenobiotics response element to other toxins that do induce the expression of insect metabolic genes. In particular, a range of insecticidal plant secondary metabolites induce the transcription of P450 genes in *H. armigera*. These induction responses have possibly evolved to cope with the challenge posed by these metabolites. In managing field resistance to insecticides the transcriptional regulation needs to be further understood (Willoughby *et al.*, 2006).

https://doi.org/10.1017/S0007485314000698 Published online by Cambridge University Press

qRT-PCR is a sensitive and reliable technique to measure gene expression but requires a reproducible and well-defined methodology for RNA extraction and purification for accurate determination of mRNA levels (Bjarnadottir & Jonsson, 2005; Cury & Koo, 2007). The $2^{-\Delta\Delta Ct}$ method is a convenient way to analyze the relative changes in gene expression from qRT-PCR experiments. In this study, we have examined the effects of the 2-tridecanone on *CYP6B6* gene expression and the results showed that 2-tridecanone enhanced *CYP6B6* expression, which subsequently lead to increased enzyme activity. We mixed the different concentrations of 2-tridecanone into the artificial diet and feed on the 6th instar larvae for different time. We conjecture initially, the larvae require a period of time to adjust the exogenous material, so the expression level of *CYP6B6* was very low during 12 h, especially the high concentration of 2-tridecanone (50 mg g^{-1}), It continued to be low up to 24 h. subsequently, the larvae may detoxificate the toxic using background levels until can not tolerate. In this period, the expression of *CYP6B6* was increased firstly and then decreased. After that, large quantity of enzyme was able to be synthesized in order to survival, so we could detect the highest expression level of *CYP6B6* at 48 h. The above results indicate that the 2-tridecanone affect the level of expression of the *CYP6B6* gene.

Our results from luciferase assays and EMSA assays suggest that the promoter of *CYP6B6* gene has the 2-tridecanone responsible region. Luciferase reporter plasmids carrying different lengths of *CYP6B6* upstream DNA were used to transfect Sf9 cells. These data suggest that the basal and 2-tridecanone-induced promoter activity of the construct pBK-778 (−373 to +405 bp) showed a significant increase, this high activity may be due to the presence of a putative XRE element in these DNA fragments. In the case of *CYP6B6* gene, increasing the length of the upstream DNA from −1007 to −374 bp did not elevate the 2-tridecanone-induced promoter activity further. Nevertheless, the activity was still higher than that of the basal one. But the pGL3-basic construct did not show any 2-tridecanone inducibility. In summary, the fragment between −373 and −172 bp of the *CYP6B6* promoter may have a 2-tridecanone responsive region.

Mobility shift assays are based on decreased mobility of a DNA fragment migrating through a gel when interacting transcription factor protein is associated (Harshman, 1998). According to the expression level of *CYP6B6* mRNA, we obtained the nuclear proteins that may be having the maximum amount of transcription factor that could bind to the promoter of *CYP6B6*. From the luciferase assays, we found a positive fragment from −373 to −172 bp upstream of *CYP6B6*. So we used a probe from −373 to +21 bp and a shorter probe from −292 to −154 bp to confirmation the results. Our results suggest that two probes both can bind to the nuclear protein. The sequence from −292 to −154 bp is analyzed by bioinformatics to find 5′-CATGACACCTG-3′, which is homology with a conserved sequence 5′-CACGCNA-3′ named XRE (Watson & Hankins, 1992). The XRE sequence is also found in the promoter of *Papilio glaucus CYP6B4v2* (5′-CACGCAAGCA-3′), *CYP6B5v1* (5′-CACCCAAGCA-3′) and the *P. polyxenes CYP6B3v2* (5′-CTCGCAAGGCA-3′) (Prapaipong *et al*., 1994; Hung *et al*., 1996), and is activated by binding to activated AhR–ARNT complexes (Schmidt & Bradfield, 1996; Whitlock & Jr, 1999; McDonnell *et al*., 2004). Our observations suggest that sequences present in the −292 to −154 bp fragment upstream of *CYP6B6* are critical for AhR-mediated activation of the *CYP6B6* promoter by 2-tridecanone.

In conclusion, we demonstrate that 2-tridecanone enhances expression of *CYP6B6* by XRE and AhR interactions. These findings should have an important impact on pest control as well as on better understanding of the P450 *CYP6B6* detoxification mechanism.

ACKNOWLEDGEMENTS

This work was supported by the Natural Science Foundation of China (31471781 & 30960220) and open foundation of Xinjiang Key Laboratory of Biological Resources and Genetic Engineering (XJDX0201-2010-3).

References

Ahmad, M., Arif, M.I. & Ahmad, Z. (2001) Resistance to carbamate insecticides in *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Pakistan. *Crop Protection* **20**, 427–432.

Bjarnadottir, H. & Jonsson, J.J. (2005) A rapid real-time qRT-PCR assay for ovine β-actin mRNA. Journal of Biotechnology **117**, 173–182.

Chen, S., Yang, Y.H. & Wu, Y.D. (2005) Correlation between fenvalerate resistance and cytochrome P450-mediated *O*-demethylation activity in *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Journal of Economic Entomology* **98**, 943–946.

Cohen, M.B., Schuler, M.A. & Berenbaum, M.R. (1992) A host-inducible cytochrome P450 from a host-specific caterpillar: molecular cloning and evolution. *Proceedings of the National Academy of Sciences* **89**, 10920–10924.

Cury, J.A. & Koo, H. (2007) Extraction and purification of total RNA from *Sreptococcus mutans* biofilms. *Analytical Biochemistry* **365**, 208–214.

Denison, M.S. & Nagy, S.R. (2003) Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annual Review of Pharmacology and Toxicology* **43**, 309–334.

Feyereisen, R. (1999) Insect P450 enzymes. *Annual Review of Entomology* **44**, 507–533.

Fujii-Kuriyama, Y. & Mimura, J. (2005) Molecular mechanisms of AhR functions in the regulation of *cytochrome P450* genes. *Biochemical and Biophysical Research Communications* **338**, 311–317.

Gerlier, D. & Thomasset, N. (1986) Use of MTT colorimetric assay to measure cell activation. *Journal of Immunological Methods* **94**, 57–63.

Goncalves, M.I.F., Maluf, W.R., Gomes, L.A.A. & Barbosa, L.V. (1998) Variation of 2-tridecanone level in tomato plant leaflets and resistance to two mite species (*Tetranychus* sp.). *Euphytica* **104**, 33–38.

Harrison, T.L., Zanger, A.R., Schuler, M.A. & Berenbaum, M.R. (2001) Developmental variation in cytochrome P450 expression in *Papilio polyxenes* in response to xanthotoxin, a hostplant allelochemicals. *Archives of Insect Biochemistry and Physiology* **48**, 179–189.

Harshman, L.G. (1998) Differential gene expression in insects: transcriptional control. *Annual Review of Entomology* **43**, 671–700.

Hung, C.F., Harrison, T.L., Berenbaum, M.R. & Schuler, M.A. (1995) *CYP6B3*: a second furanocoumarin-inducible cytochrome P450 expressed in *Papilio polyxenes*. *Insect Molecular Biology* **4**, 149–160.

Hung, C.F., Holzmacher, R., Connolly, E., Berenbaum, M.R. & Schuler, M.A. (1996) Conserved promoter elements in the *CYP6B* gene family suggest common ancestry for

https://doi.org/10.1017/S0007485314000698 Published online by Cambridge University Press

cytochrome P450 monooxygenases mediating furanocoumarin detoxification. *Proceedings of the National Academy of Sciences* **93**, 12200–12205.

Kawajiri, K. & Fujii-Kuriyama, Y. (2007) Cytochrome P450 gene regulation and physiological functions mediated by the aryl hydrocarbon receptor. *Archives of Biochemistry and Biophysics* **464**, 207–212.

Kimps, N.W., Bissnger, B.W., Apperson, C.S., Sonenshine, D.E. & Roe, R.M. (2011) First report of the repellency of 2-tridecanone against ticks. *Medical and Veterinary Entomology* **25**, 202–208.

Li, F. & Liu, X.N. (2012) Optimization of the detection conditions of the promoter activity of Cytochrome P450 *CYP6B6* gene from *Helicoverpa armigera*. *Biotechnology bulletin* **5**, 167–172.

Li, F., Ma, J. & Liu, X.N. (2012) Activity analysis of the promoter region of *Helicoverpa armigera* P450 *CYP6B6* Gene. *Journal of Xinjiang University (Natural Science Edition)* **29**, 13–18.

Li, X.C., Berenbaum, M.R. & Schuler, M.A. (2002) Plant allelochemicals differentially regulate *Helicoverpa zea* cytochrome P450 genes. *Insect Molecular Biology* **11**, 343–351.

Liu, X.N., Liang, P., Gao, X.W. & Shi, X.Y. (2006) Induction of the cytochrome P450 activity by plant allelochemicals in the cotton bollworm, *Helicoverpa armigera* (Hübner). *Pesticide Biochemistry and Physiology* **84**, 127–134.

Mantovani, R. (1998) A survey of 178 NF-Y binding CCAAT boxes. *Nucleic Acids Research* **26**, 1135–1143.

McDonnell, C.M., Brown, R.P., Berenbaum, M.R. & Schuler, M. A. (2004) Conserved regulatory elements in the promoters of two allelochemical-inducible cytochrome P450 genes differentially regulate transcription. *Insect Biochemistry and Molecular Biology* **34**, 1129–1139.

Prapaipong, H., Berenbaum, M.R. & Schuler, M.A. (1994) Transcriptional regulation of the *Papilio polyxenes CYP6B1* gene. *Nucleic Acids Research* **22**, 3210–3217.

Riskallah, M.R., Dauterman, W.C. & Hodgson, E. (1986) Host plant induction of microsomal monooxygenase activity in relation to Diazinon metabolism and toxicity in larvae of the tobacco budworm *Heliothis Virscens* (F.). *Pesticide Biochemistry and Physiology* **25**, 233–247.

Schmidt, J.V. & Bradfield, C.A. (1996) Ah receptor signaling pathways. *Annual Review of Cell and Developmental Biology* **12**, 55–89.

Schuler, M.A. (2011) P450s in plant–insect interactions. *Biochimica et Biophysica Acta* **1814**, 36–45.

Scott, J.G. (1999) Cytochrome P450 and insecticide resistance. *Insect Biochemistry and Molecular Biology* **29**, 757–777.

Scott, J.G. (2008) Insect cytochrome P450s: thinking beyond detoxification. *Recent Advances in Insect Physiology, Toxicology and Molecular Biology*, 117–124.

Vontas, J., Blass, C., Koutsos, A.C., David, J.-P., Kafatos, F.C., Louis, C., Hemingway, J., Christophides, G.K. & Ranson, H. (2005) Gene expression in insecticide resistant and susceptible *Anopheles gambiae* strains constitutively or after insecticide exposure. *Insect Molecular Biology* **14**, 509–521.

Watson, A.J. & Hankins, O. (1992) Dioxin- and *Ah* receptor-dependent protein binding to xenobiotic responsive elements and G-rich DNA studied by *in Vivo* Footprinting. *The Journal of Biological Chemistry* **266**, 6874–6878.

Whitlock, J.P. Jr. (1999) Induction of cytochrome *P4501A1*. *Annual Review of Pharmacology and Toxicology* **39**, 103–125.

Williams, W.G., Kennedy, G.G., Yamamoto, R.T., Thacker, J.D. & Bordner, J. (1980) 2-Tridecanone: a naturally occurring insecticide from the wild tomato *Lycopersicon hirsutum* f. *glabratum*. *Science* **207**, 888–889.

Willoughby, L., Chung, H., Lumb, C., Robin, C., Batterham, P. & Daborn, P.J. (2006) A comparison of *Drosophila melanogaster* detoxification gene induction responses for six insecticides, caffeine and Phenobarbital. *Insect Biochemistry and Molecular Biology* **36**, 934–942.

Wu, K.J. & Gong, P.Y. (1997) A new and practical artificial diet for the cotton bollworm. *Entomologia sinica* **4**, 277–282.

Yang, Y., Wu, Y., Chen, S., Devine, G.J., Denholm, I., Jewess, P. & Moores, G.D. (2004) The involvement of microsomal oxidases in pyrethroid resistance in *Helicoverpa armigera* from Asia. *Insect Biochemistry and Molecular Biology* **34**, 763–773.

Zhou, X.J., Sheng, C.F., Li, M., Wana, H., Liu, D. & Qiu, X.H. (2010) Expression responses of nine cytochrome P450 genes to xenobiotics in the cotton bollworm *Helicoverpa armigera*. *Pesticide Biochemistry and Physiology* **97**, 209–213.