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Identification of a novel cytochrome P450 gene, *CYP321E1* from the diamondback moth, *Plutella xylostella* (L.) and RNA interference to evaluate its role in chlorantraniliprole resistance

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Abstract

Insect cytochrome P450 monooxygenases (P450s) play an important role in catalysis of many reactions leading to insecticides resistance. Our previous studies on transcriptome analysis of chlorantraniliprole-resistant development in the diamondback moth, *Plutella xylostella* revealed that up-regulation of cytochrome P450s are one of the main factors leading to the development of chlorantraniliprole resistance. Here, we report for the first time a novel cytochrome P450 gene *CYP321E1*, which belongs to the cytochrome P450 gene family CYP321. Real-time quantitative PCR (RT–qPCR) analyses indicated that *CYP321E1* was expressed at all developmental stages of *P. xylostella* but was highest in the fourth-instar larvae; furthermore, the relatively high expression was observed in the midgut of the fourth-instar larvae, followed by fat bodies and epidermis. The expression of *CYP321E1* in *P. xylostella* was differentially affected by three representative insecticides, including alphamethrin, abamectin and chlorantraniliprole. Among them, the exposure to chlorantraniliprole resulted in the largest transcript level of this cytochrome P450 gene. The findings suggested potential involvement of *CYP321E1* in chlorantraniliprole resistance of *P. xylostella*. To assess the functional link of *CYP321E1* to chlorantraniliprole resistance, RNA interference (RNAi)-mediated gene silencing by double stranded RNA (dsRNA) injecting was used. Results revealed that injection delivery of dsRNA can greatly reduce gene expression after 24 h. As a consequence of RNAi, a significant increment in mortality of larvae injected *CYP321E1* dsRNA was observed after 24 h of exposure to chlorantraniliprole. These results strongly support our notion that this novel cytochrome P450 gene plays an important role in chlorantraniliprole detoxification in the diamondback moth and is partly responsible for its resistance.

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Introduction

Cytochrome P450 monooxygenases (P450s), is a superfamily of heme-containing proteins, found in almost all living organisms from bacteria to human (Feyereisen, 2006). Cytochrome P450s constitute an important metabolic system and this system plays a central role in the oxidative metabolism of both xenobiotic and endogenous compounds (Feyereisen, 1999). In insects, they are best known for their roles in the metabolism of insecticides, which often results in the development of insecticide resistance (Zhou *et al*., 2010). To date, more than 1000 cytochrome P450 genes in insects have been identified (Nelson, 2009), most of them are distributed to the families of microsomal CYP4, CYP6, CYP9, CYP28, CYP321 and mitochondrial CYP12 (Feyereisen, 2005). Furthermore, many cytochrome P450 genes, especially belonging to the families of CYP4, CYP6, CYP9 and CYP12, are frequently involved in insecticide metabolism and resistance (Feyereisen, 2006; Li *et al*., 2007).

It has often been mentioned that insecticide metabolism is enhanced by overexpression of cytochrome P450s and it appears to be a frequent type of resistance mechanism in insects (Liu & Scott, 1998; Li *et al*., 2006). Overexpression of cytochrome P450s has been reported in many insect species. For example, increased transcription of *CYP6D1* (Liu & Scott, 1998) in *Musca domestica* pyrethroid-resistant strain; *CYP6BG1* and *CYP6BG2* in permethrin-resistant strain of *Plutella xylostella* (Bautista *et al*., 2007); *CYP4H1, CYP4H22v1, CYP4J6v1* and *CYP4J6v2* in deltamethrin-resistant strain of *Culex pipiens pallens* (Shen *et al*., 2003). Expression profiles of cytochrome P450 genes are highly diverse in insects (Scott & Wen, 2001). And different developmental stage or tissue-specific expression patterns may imply their specific functions, such as the metabolism of signal molecules, adaptation to host plants or insecticide resistance (Chung *et al*., 2009; Feyereisen, 2012). In insects, higher cytochrome P450s activities are usually associated with the midgut, fat bodies and Malpighian tubules (Brun *et al*., 1996). Inducibility is a general characteristic of cytochrome P450s, and it has been well documented in a number of insect species (Fuchs *et al*., 1994; Harrison *et al*., 2001). Several cytochrome P450 genes induced by xenobiotic, such as insecticides have been identified (Ranasinghe & Hobbs, 1998; Brandt *et al*., 2002; Poupardin *et al*., 2008; Baek *et al*., 2010). Xenobiotic inducible cytochrome P450 genes may be more likely to be involved in resistance (Le Goff et al., 2006). Therefore, it is important to study the expression profiles of cytochrome P450 genes and examine their inducibility by the particular insecticides, the findings may help researchers better understand functions of the genes and their interactions with insecticides at molecular levels and provide useful genetic information to assess potential consequences of insecticide exposures in insects.

The diamondback moth (DBM), *P. xylostella*, is a notorious insect and one of the most destructive cruciferous vegetables pests in the world due to its extreme ability to develop resistance (Talekar & Shelton, 1993). Worldwide cost for chemical control of *P. xylostella* is about $4–5 billion (US dollar) annually (Furlong *et al*., 2013). Chlorantraniliprole, as an alternative to traditional insecticides, is a reduced-risk insecticide being introduced for global control of *P. xylostella* and has been shown to be a highly efficacious insecticide in pest control (Wang *et al*., 2010). However, due to intensive use of chlorantraniliprole, high levels of resistance in some field populations of *P. xylostella* have been identified in Guangdong province, China (Wang & Wu, 2012). As an important detoxification enzyme family, cytochrome P450s play an important role in the resistance of *P. xylostella* to various insecticides, including chlorantraniliprole (Ninsin & Tanaka, 2005; Wang *et al*., 2013). To date, ten cytochrome P450 genes have been identified from *P. xylostella*, among them, *CYP6BG1* and *CYP6BG2* are observed to be constitutively overexpressed in a permethrin-resistant strain (Bautista *et al*., 2007), and the *CYP6BG1* gene has been evaluated for its role in resistance by the RNA interference (RNAi) method (Bautista *et al*., 2009). However, no P450 genes have been identified or reported to be responsible for chlorantraniliprole resistance in *P. xylostella*.

The previous studies on the transcriptome analysis of chlorantraniliprole resistance development in *P. xylostella* showed that the up-regulation of detoxification genes (P450, GSTs and prophenoloxidase) and the down-regulation of ryanodine receptors (the chlorantraniliprole-blinding target) are the main factors leading to the development of chlorantraniliprole resistance (Lin *et al*., 2013). Here, based on one of the up-regulation cytochrome P450 genes cDNA fragments, a novel cytochrome P450 gene *CYP321E1* was identified and reported for the first time. Furthermore, the gene expression profile, inducibility by three representative insecticides and its specific detoxification function using RNAi were also observed. Our findings will help elucidate its possible role in chlorantraniliprole resistance of *P. xylostella* and shed on new light on functional importance of this gene in detoxification of insecticides.

Materials and methods

Insects

The studies were carried out on a chlorantraniliprole-resistant strain of *P. xylostella* (GXA), as reported by Lin *et al*. (2013), which was collected from a cabbage field in Guangxi Province. The strain was maintained on cabbage in the laboratory for more than 20 generations, and selected using chlorantraniliprole every 2 to 3 generations to mitigate the influence of sensitive individuals and other field factors. The laboratory conditions were maintained at 25 ± 2°C, 70–80% relative humidity (RH) and 16:8 h light: dark photoperiod. Adults were fed on 10% honey solution and allowed to lay eggs on aluminum-foil paper with cabbage juice.

cDNA cloning of CYP321E1

Total RNA was extracted using Easy Spin Total RNA Extraction Kit (Biomed, China) and the first-strand cDNA was synthesized from 2 μg RNA with an oligo (dT) primer using

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Reverse Transcritase M-MLV (Takara, China) according to the manufacturer's instructions. The resulting cDNA was used as a PCR template with the following primers: 5′-GCT ACA ACC GGC TTT ATT CG-3′ and 5′-AAC AGG CAG GAT GGA TTC AC-3′. The primer designs were based on up-regulation cytochrome P450 gene cDNA fragments: CL6103.contig 1, which was obtained from *P. xylostella* EST database (Lin *et al*., 2013). PCR was performed for 1 cycle at 94°C for 5 min, then for 35 cycles at 94°C for 35 s, 42°C for 35 s and 72°C for 1 min, followed by 1 cycle at 72°C for 10 min. The amplified fragment was purified using a Gel Extraction Kit (Omega, China), then cloned into the pMD18-T vector (Takara, China) and sequenced. To get the full-length cDNA of *CYP321E1*, the 5′- and 3′-end specific primers were designed according to the manufacturer's protocol of SMART RACE cDNA Amplification Kit (Clontech, USA) based on this partial amplified fragment. For 5′-end amplification, a forward primer and a nested primer were: CYP-GSP1 (5′-CCA CCG ACT GCC GTT CAG GAA AGG-3′) and CYP-NGSP1 (5′-GGA TGT TTG ACG AAT AAA GCC GGT TG-3′), respectively. For 3′-end amplification, a forward primer and a nested primer were: CYP-GSP2 (5′-GGC TAA GCG CCC TGA CAT ACT AAA AC-3′) and CYP-NGSP2 (5′-CGT TTA CAT CCA CCG ATT TCG CTG CT-3′), respectively. The amplified PCR fragments from each reaction were purified, cloned and sequenced as described above. The resulting overlapping sequences were assembled to obtain the full-length *CYP321E1* cDNA sequence.

Finally, to confirm the assembled cDNA sequence from overlapping PCR products, the entire coding region of *CYP321E1* was amplified by PCR reaction with the primer pair CYP-F (5′-ATG ATT TTA GCA ATA ATA TTG TTA T-3′) and CYP-R (5′-TCA CTC TTT ATT CCT AGG TAC AAA T-3′). The following cycling parameters were used: 94°C for 5 min followed by 35 cycles of 94°C for 40 s, 48°C for 30 s and 72°C for 1 min, followed by 1 cycle at 72°C for 10 min. PCR product was cloned and then sequenced in both directions.

Sequence analysis

The prediction of the open reading frame (ORF) was administered using ORF Finder tool at the NCBI website (http://www.ncbi.nlm.nih.gov/guide/all/#tools_). The translation of the cDNA sequence into amino acid sequence was performed using the translate tool at the ExPASy proteomic website (http://www.expasy.org/tools). The protein isoelectric point (*pI*) and molecular mass were predicted based on the amino acid sequence. Multiple sequence alignments of the amino sequences were made using Multiple Alignment software (http://www.phylogeny.fr/).

Expression profile of CYP321E1

The total RNA was extracted from various developmental stages and tissues of the fourth-instar larvae of *P. xylostella* using the same method as described above. Total RNA was collected from six developmental stages, including eggs, first-, second-, third-, fourth-larvae and pupas. Tissues used for RNA extraction were hemocytes, fat bodies, epidermis, Malpighian tubule and midgut of the fourth-instar larvae (one day after molting). Subsequently, the first-strand cDNA was synthesized from 2 µg RNA using iScript™ cDNA Synthesis Kit (Bio-rad, USA).

The pair of primers used for real-time quantitative PCR (RT–qPCR) analysis were designed and sequenced as follows: CYP-qPCR-F forward primer, 5′-TCG CCG AAA GTC TTC CAA AT-3′ and CYP-qPCR-R reverse primer, 5′-CGC TGG ATT CGT CTT TCA TC-3′ (178 bp product). RT–qPCR was performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-rad, USA) using IQ SYBR Green supermix (Bio-rad, USA). The thermal cycling profile consisted of an initial step at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 45°C for 30 s and 72°C for 20 s. The PCR mixture (20 µl) contained 10 µl SYBR Green supermix, 0.5 µl of each primer, 1 µl of 1:20 diluted cDNA templates and sterilized water to reach the final volume. The samples were analyzed in triplicate. β-*Actin* (Lin *et al*., 2013) was used as a reference gene to normalize the target gene expression levels among the samples.

Insecticide exposures

Although the cytochrome P450 gene *CYP321E1* was one of the up-regulation detoxification genes in chlorantraniliprole resistance strain of *P. xylostella*, it is still necessary to evaluate the effect of different insecticides, including chlorantraniliprole and other commonly used insecticides on the expression of *CYP321E1* in *P. xylostella*. In this study, three insecticides were used, alphamethrin, abamectin and chlorantraniliprole, each insecticide with a dose of LC_{15} (250, 20 and 25 µg ml^{-1}, respectively). The leaf dip method described by Tabashnik *et al*. (1987) was adapted. In each treatment, about 20 third-instar larvae and a treated cabbage leaf were placed into the plastic Petri dish (9 cm in diameter) and kept at 25 ± 2°C, 65 ± 5% RH. Each treatment was repeated three times and larvae treated with sterilized water were used as controls. After 24 h, surviving larvae were quickly frozen in liquid nitrogen. Subsequent experiments, including total RNA extraction, synthesis of the first-strand cDNA and RT–qPCR analyses were conducted as described above.

Functional analysis of CYP321E1 by RNAi

In order to obtain specific RNAi effect for the target gene, a primer pair dsCYP-F (5′-TAA TAC GAC TCA CTA TAG GG GGC TCC TAC AAT GAG TCT TA-3′) and dsCYP-R (5′-TAA TAC GAC TCA CTA TAG GG TCA AAG TCG GTT TCG TGG CA-3′) were designed for double stranded RNA (dsRNA) syntheses based on the entire coding sequence of *CYP321E1*. At the same time, dsGFP was synthesized as a negative control based on the sequence of pEGFP-N1 plasmid (TIANGEN, China).The primer sequences were: dsGFP-F 5′-TAA TAC GAC TCA CTA TAG GG AAG GGC GAG GAG CTG TTC ACC G-3′ and dsGFP-R 5′-TAA TAC GAC TCA CTA TAG GG CAG CAG GAC CAT GTG ATC GCG C-3′. The bold font portion of sequence was a T7 promoter sequence. Template for *in vitro* transcription reaction was prepared by PCR amplification using the primer pairs and the PCR products (dsCYP 580 bp and dsGFP 657 bp, respectively) and were obtained using the following cycling parameters: 95°C for 3 min, 30 cycles of 95°C for 40 s, 56°C for 30 s and 72°C for 40 s, followed by a final extension step of 72°C for 8 min. Aliquots of 2 µl PCR products were analyzed by 1% agarose gel and the PCR product was cloned and sequenced to confirm its identity. Then the expected fragment (45 µl) was examined by 1% agarose gel and purified by Gel Extraction Kit (Omega, China). dsRNA was synthesized using T7 RiboMAX™ Express RNAi System

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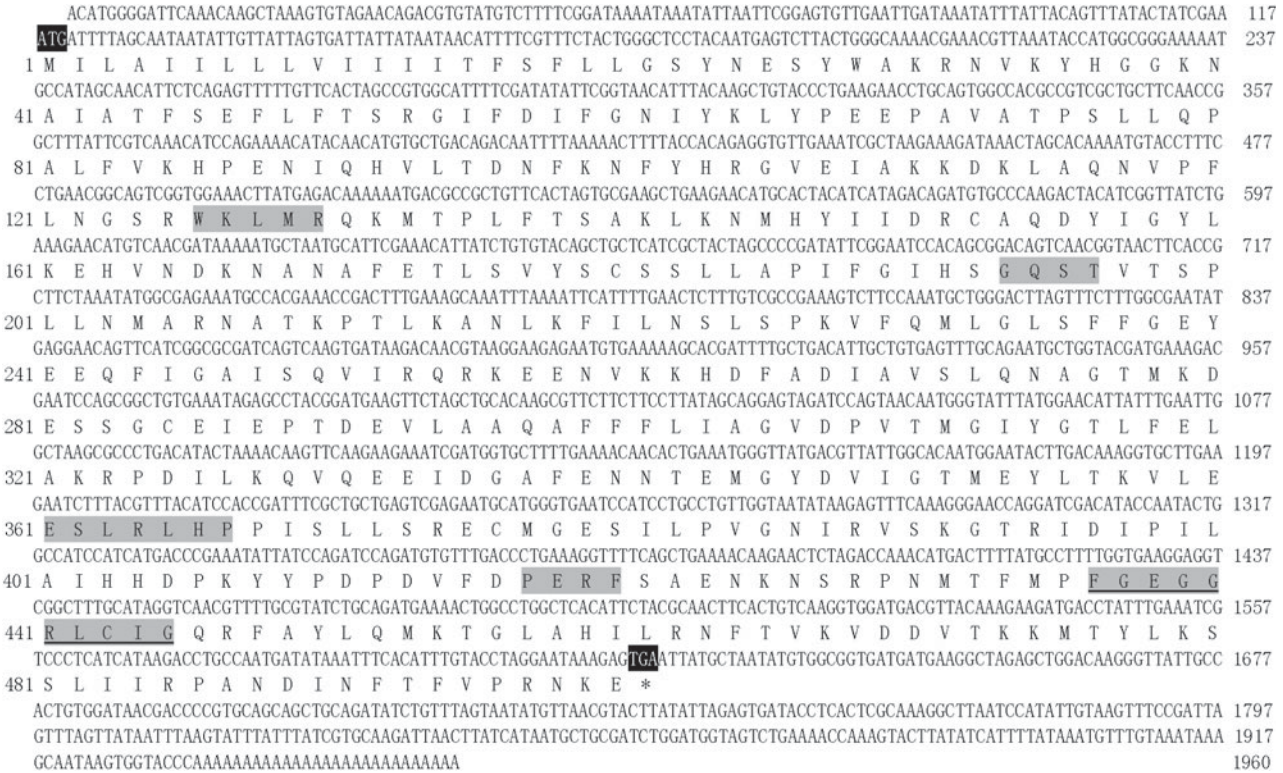

Fig. 1. Complete nucleotide and deduced amino acid sequence of *CYP321E1* cDNA of *P. xylo*stella. The start codon (ATG) and stop codon (TGA) are highlighted in black. The P450s signature motifs (GXXT, EXXRXXP, PXRF and WXXXR) are highlighted in gray and the heme-binding sequence motif of FXXGXXXCXG is also highlight in gray and underline in black. The sequence was deposited in the GenBank (Assession No: KC 626090).

(Promega) following the manufacturer's instructions and dissolved in nuclease-free water and examined by 1.5% agarose gel. After the concentration of dsRNA was determined by Thermo Scientific NANODROP 2000C Spectrophotometer (Thermo, USA), the final concentration of dsRNA was adjusted to 4 μg μl^{-1}.

The fourth-instar larvae (one day after molting) were used for dsRNA injection experiments. In each treatment, ten larvae were injected with 0.5 μl dsRNA (2 μg insect^{-1}) into the abdomen between third and fourth abdominal segments using Micro 4™ MicroSyringe Pump Controller (Sarasota, USA). The larvae injected with dsGFP were used as the negative control. Each treatment or control was repeated three times. In order to assess the expression level of each treatment, the whole body of the larvae was used for total RNA extraction. For each group, five larvae were picked out for RNAi efficiency test at two times (12 and 24 h) after injection by the RT–qPCR method.

For insecticide bioassay after RNAi, 60 larvae from the dsRNA-injected or negative control group after the 24 h injection time (the time point showed relatively high RNAi efficiency, and allowed them to feed with non-toxic leaves prior to mortality assessment) were separated into four replicates, 15 larvae per replicate and the cabbage leaf dip method described by Tabashnik *et al*. (1987) was adapted to assay the efficiency of RNAi. Each insecticide with a dose of LC_{50} and the procedure was conducted similarly as above described. The mortalities of the treated larvae were assessed at 48 h after insecticide treatment.

Results

cDNA cloning and analysis of CYP321E1

In the present study, a 922 bp cDNA fragment from *P. xylostella* was amplified and cloned by reverse-transcription PCR (RT–PCR) with specific primers. Based on this sequence, 5′- and 3′-ends RACE reactions were performed, and the full-length cDNA was obtained by overlapping the RACE cDNA fragments. The full-length cDNA sequence of *CYP321E1* is 1960 bp long with an ORF of 1503 bp, which encodes a protein of 500 amino acid residues. The ORF sequence began with the first ATG codon at position 118 and ended with a TGA termination codon at 1617. The 5′-UTR and 3′-UTR were 117 and 340 bp, respectively. Based on the translated amino acid sequence of ORF, *CYP321E1* has a predicted mass of 56.62 kDa and a theoretical PI value of 8.35. The heme-binding sequence motif of FGEGGRLCIG (FXXGXXXCXG) was identified at amino acid residues 436–445. The putative 'meander'-binding sequence of ESLRLHP (EXXRXXP) and PERF (PXRF) in helix – K, the sequence motif of GQST (GXXT) in helix – I and WKLMR (WXXXR) in helix – C were also identified (fig. 1). The putative amino acid sequence of *CYP321E1* has less than 50% similarity to all other amino acid sequences that already existed in GenBank, only shared optimal homology to three cDNA clones: *Helicoverpa zea CYP321A1* (48% identity), *Spodoptera littoralis CYP321A11* (47% identity) and *Spodoptera litura CYP321B1* (44% identity) (fig. 2). Finally, the cytochrome P450 gene was named *CYP321E1* (GenBank Accession No: KC 626090) by the P450 Nomenclature

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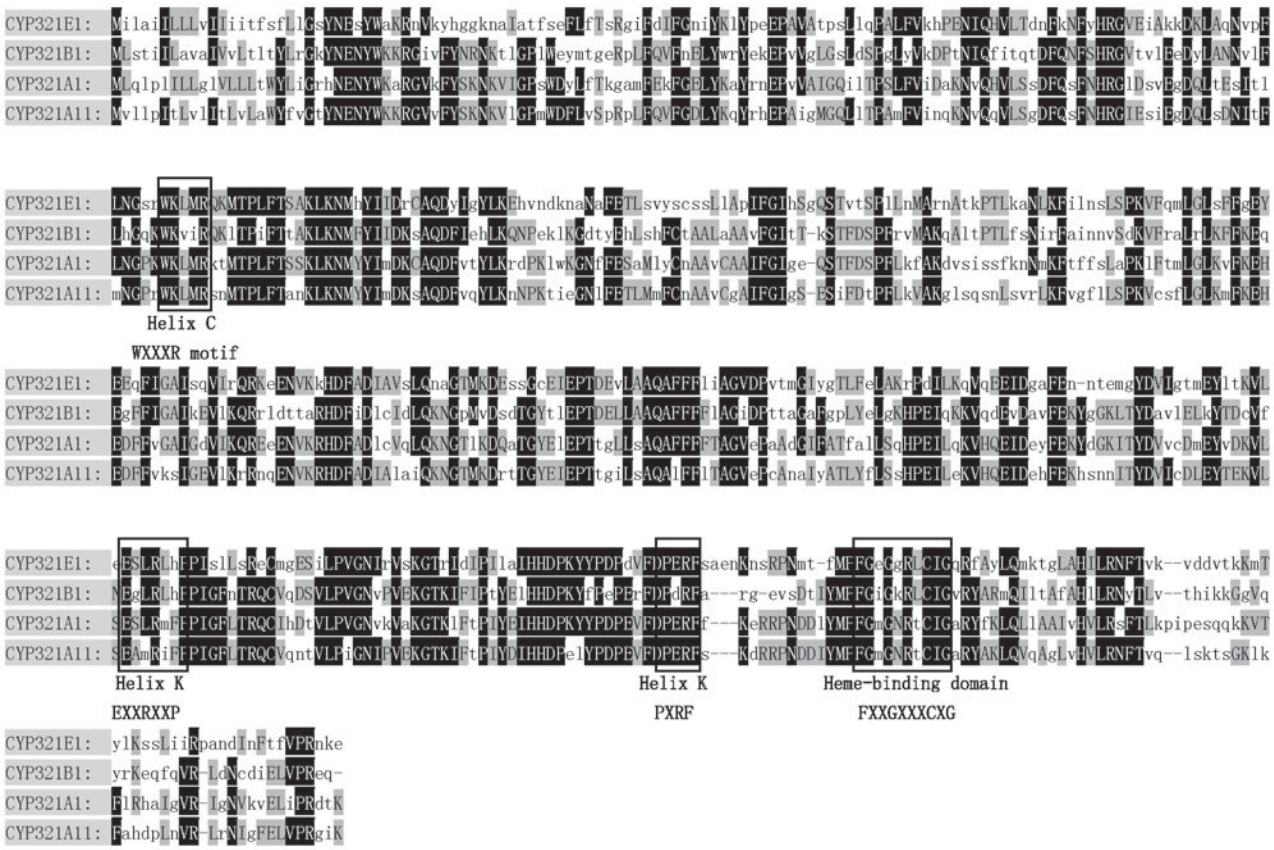

Fig. 2. Comparison of amino acid sequences of *CYP321E1* (KC626090) from *P. xylos*tella, *CYP321A1* (AAM54724) from *Helicoverpa zea*, *CYP321A11* (AFP20596) from *S. littoralis* and *CYP321B1* (ADA68175) from *Spodoptera litura*, several conserved motifs of these cytochrome P450 proteins are boxed.

Committee (Dr D. Nelson). It is a novel member of CYP321 family in *P. xylostella*.

Expression profile of CYP321E1

The expression profiles of *CYPA321E1* at different developmental stages and tissues of the fourth-instar larvae of *P. xylostella* were examined using RT–qPCR. β-*Actin* was used as an internal reference gene. The expression was detected at all developmental stages, but it was significantly higher in the fourth-instar larvae (fig. 3a). And in the fourth-instar larvae, the high expression of *CYP321E1* was observed in the midgut, followed by fat bodies, and then epidermis, but its expression was relatively low in the Malpighian tubule and hemocytes (fig. 3b).

Expression response of CYP321E1 to insecticide exposures

In order to examine and confirm which insecticide could be effective at inducing the expression of *CYP321E1* in *P. xylostella*, alphamethrin, abamectin and chlorantraniliprole were selected in this study. As the results show in fig. 4, all three insecticides increased gene expression of *CYP321E1* at the dose of LC_{15}, the maximum effect of chlorantraniliprole was observed (8.64-fold, $P<0.05$), followed by abamectin (2.98-fold, $P<0.05$). However, exposure of the insects to alphamethrin at the concentration of LC_{15} did not show significant effect on the expression of this gene (1.31-fold, $P<0.05$).

Functional analysis of CYP321E1 by RNAi

We deemed fourth-instar larvae are the best stage for RNAi not only because the relatively higher expression of *CYP321E1* in this stage, but also because the size of the larvae would be suitable for administration of dsRNA by injecting. RT–qPCR analyses at 12 and 24 h after dsRNA injection of *CYP321E1* showed respectively slightly and significantly reduced by about 1.33- and 5.39-fold expression levels as compared with those in the negative control ($P<0.05$) (fig. 5).

As the *CYP321E1* expression was significantly decreased by RNAi at 24 h, we assessed RNAi effect on larvae resistance to different insecticides. The mortalities of the larvae injected with dsGFP (control) and dsCYP after treatment with chlorantraniliprole at the concentration of 50 μg ml^{-1} (about at the dose of LC_{50}) were 41.7 and 70.0%, the results represent an increased mortality by approximately 1.7-fold after the repression of the *CYP321E1* gene by RNAi ($P<0.05$). However, similar treatment with abamectin (40 μg ml^{-1}, also about at the dose of LC_{50}) in the larvae after RNAi for this gene did not show significant effects on the mortality of the larvae ($P<0.05$) (table 1).

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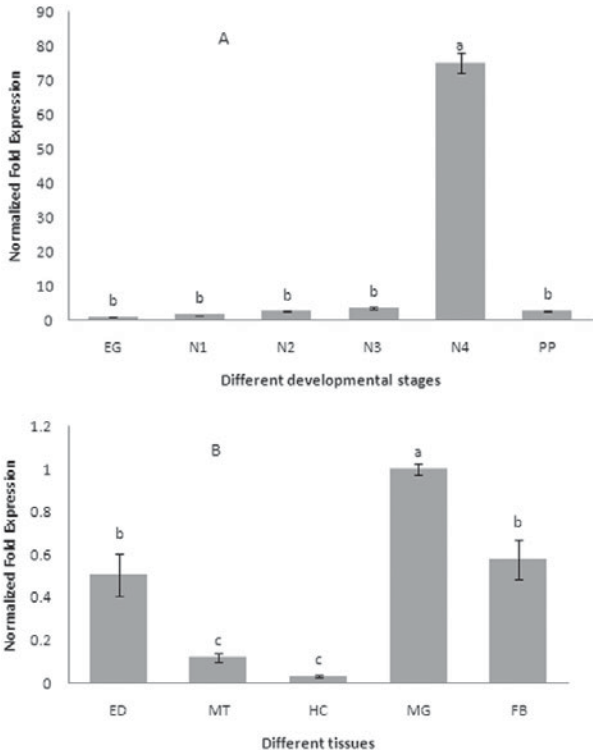

Fig. 3. Expression patterns of *CYP321E1* in different tissues of the fourth-instar larvae and different developmental stages in the whole body as evaluated by RT–qPCR. (A) Stage-dependent expression pattern was examined in six different developmental stages, including eggs (EG), first- (N1), second- (N2), third- (N3), four- (N4) and pupa (PP); (B) Tissue-dependent expression pattern was examined in five different tissues, including hemocytes (HC), fat bodies (FB), epidermis (ED), Malpighian tubule (MT) and midgut (MG). β-*Actin* was used as an internal reference gene. Different letters on the bars indicate that the means of the gene transcript level are significantly difference among the different tissues or developmental stages based on Fisher's LSD multiple comparison test ($P < 0.05$).

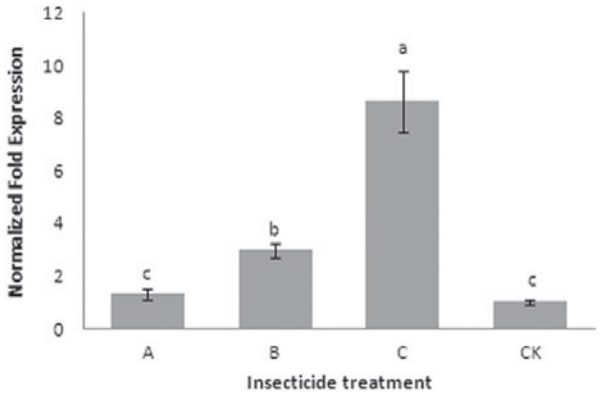

Fig. 4. Effect of alphamethrin (A), abamectin (B) and chlorantraniliprole (C) on the expression of *CYP321E1* after the larvae of *P. xylos*tella treated with each insecticide at LC_{15} dose: 250, 200 and 25 μg ml^{-1}, respectively, and analyzed at 24 h by RT–qPCR. The larvae treated with sterilized water were used as controls (CK). β-*Actin* was used as an internal reference gene. Different letters on the bars indicate that the means of the gene transcript level are significantly difference among the different treatments based on Fisher's LSD multiple comparison test ($P < 0.05$).

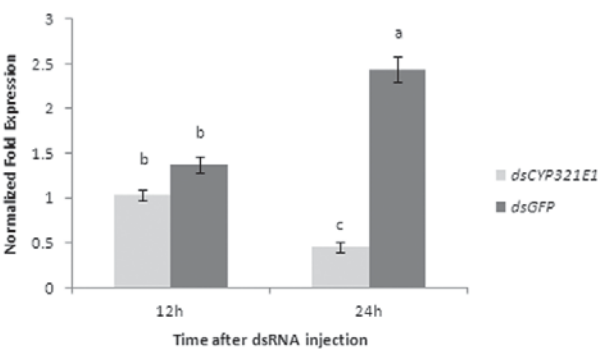

Fig. 5. Changes in the transcript levels of *CYP321E1* after the fourth-instar larvae were injected with its dsRNA. Control larvae were injected with equivalent μg larvae^{-1} of dsGFP. Different letters on the bars indicate that the means of the gene transcript level are significantly difference among the different treatments based on Fisher's LSD multiple comparison test ($P < 0.05$).

Discussion

Previous studies indicated that up-regulation of the cytochrome P450 genes was one of the major factors leading to chlorantraniliprole resistance in *P. xylostella* (Lin *et al.*, 2013). Therefore, identification and characterization of related cytochrome P450 genes have become very attractive topics. To date, however, no cytochrome P450 gene from *P. xylostella* has been reported to be responsible for chlorantraniliprole resistance. Herein, we obtained the full-length cDNA sequence of a novel cytochrome P450 gene *CYP321E1* by sequencing the cDNA after RACE PCR, analyzing the cDNA and deducing the amino acid sequence. Notable in the deduced amino acid sequence of this cDNA sequence is salient characteristics of cytochrome P450s that include the heme-binding decapeptide (FXXGXXXCXG), sequences EXXRXXP and PXRF in helix-K, GXXT in helix-I and WXXXR in helix-C. We also compared transcript expression of *CYP321E1* between different strains, a higher expression level (74.42-fold) in the chlorantraniliprole-resistant strain by comparison to the susceptible strain was observed (data unpublished). These initial findings strengthened the possibility that *CYP321E1* could be involved in chlorantraniliprole resistance in *P. xylostella*.

To investigate the expression profile of *CYP321E1*, we used RT–qPCR to analyze different developmental stages of chlorantraniliprole-resistant strain of *P. xylostella*. The expression of *CYP321E1* was significant higher in the fourth-instar larvae than the larvae of other developmental stages. The different tissues of the fourth-instar larvae were further analyzed, *CYP321E1* was found to be most abundant in the midgut. Chlorantraniliprole is both a stomach and contact toxicant, suggesting that higher expressions of *CYP321E1* in midgut, fat bodies and epidermis may correlate well with how these tissues encounter insecticides. In insects, midgut and fat bodies are generally thought to be the major detoxification organs (Hodgson, 1985; Snyder *et al.*, 1995; Bautista *et al.*, 2009). Our findings of tissue-dependent expression patterns

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Table 1. Toxicity of chlorantraniliprole and abamectin to the fourth-instar larvae injected with dsRNA after 24 h.

Insecticide	Treatment	N[a]	Mortality (%) ± SE	Fold increase in mortality
Chlorantraniliprole	dsCYP	60	70.0* ± 1.71	1.7
	dsGFP	60	41.7 ± 0.96	
Abamectin	dsCYP	60	43.3 ± 1.73	1.3
	dsGFP	60	33.3 ± 1.63	

[a] Total number of insects used.
Asterisk after the number indicates significant difference between the mortality of the dsCYP and dsGFP treatments based on unpaired student's *t*-test at $P < 0.05$.

are consistent with this notion for *CYP321E1*. However, since the epidermis also exhibited relatively high *CYP321E1* expression, it is possible that initial detoxification by *CYP321E1* of chlorantraniliprole absorbed through contact with residues on cruciferous vegetable surfaces, also occurs here. These results may imply an adaptive ability of *P. xylostella* larvae to metabolize chlorantraniliprole upon exposure and the fourth-instar larvae could be important in insecticide resistance.

It is possible that insecticide resistance is due to interactions of a particular insecticide with the regulatory mechanism of induction (Liu & Scott 1996; Le Goff et al., 2006). Recognizing this possibility, we also attempted to examine the inducibility of *CYP321E1* gene by alphamethrin, abamectin and chlorantraniliprole in *P. xylostella*. The low induction concentrations of insecticides and exposure duration in our study were determined according to the report of Guo *et al*. (2012), for that insecticides could play important roles in intoxication rather than induction under high concentration. The induction data show that *CYP321E1* responded differently to the exposures of insect larvae to three different insecticides. Chlorantraniliprole significantly induced the expression of *CYP321E1* at 24 h, abamectin also appeared to induce the expression of *CYP321E1*, but the induction effect was significantly less than that in chlorantraniliprole treated insects. Alphamethrin at present induction concentration and duration did not affect the expression of *CYP321E1*. So we inferred that the up regulation of *CYP321E1* mRNA in *P. xylostella* larvae was correlated with development of chlorantraniliprole resistance because significant increases in the LC_{15} values were detected. These findings are also in agreement with our precious studies on transcriptome analysis of chlorantraniliprole resistance development in *P. xylostella* (Lin *et al*., 2013).

However, all the above-mentioned findings cannot pinpoint whether or not *CYP321E1* is actually involved in insecticide resistance. However, it has been suggested that the major enzymes involved in insecticide detoxification could be identified by a means of induction profiling of insect detoxification enzymes (Poupardin *et al*., 2008). In order to further confirm whether or not the chlorantraniliprole inducible gene *CYP321E1* is involved in the chlorantraniliprole detoxification, we carried out RNAi to silence the target gene by injecting the sequence-specific dsRNA to the fourth-instar larvae of *P. xylostella* followed by insecticides bioassay. A noticeable reduction of the *CYP321E1* expression in the larvae after RNAi 24 h was observed and analysis of knockdown effect of the gene on resistance, based on the bioassay, indicated that *CYP321E1* is responsible for up to 70.0% mortality, which corresponds to 1.7-fold reduction in resistance to for dsRNA-injected larvae. However, from the present data, mortality of abamectin bioassay was not significantly different between dsRNA-injected and control larvae, indicating that *CYP321E1* is not likely involved in abamectin detoxification in *P. xylostella*. Of course, the data on resistance of *CYP321E1* RNAi also suggested that chlorantraniliprole resistance is not due to *CYP321E1* alone, mainly because resistance was not completely abolished. The remaining resistance in *P. xylostella* could be due to other mechanisms such as target-site mutation in the C-terminal membrane-spanning domain of the RyR (Troczka *et al*., 2012).

In summary, we identified and characterized a novel cytochrome P450 gene, which belongs to a cytochrome P450 gene family CYP321, from an important agriculture pest *P. xylostella*. The findings indicate that overexpressed *CYP321E1* is involved in increased chlorantraniliprole detoxification partly leading to resistance. So it is important to further study the difference in the metabolism ability of *CYP321E1* to chlorantraniliprole between the susceptible strain and the resistance strain, and investigate the enzyme properties of this gene in both strains. These studies will influence the practice of chlorantraniliprole resistance management strategies for *P. xylostella*.

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