J Chem Ecol (2015) 41:111–119
DOI 10.1007/s10886-014-0540-z

Expression Analysis of Two P450 Monooxygenase Genes of the Tobacco Cutworm Moth (*Spodoptera litura*) at Different Developmental Stages and in Response to Plant Allelochemicals

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Received: 27 June 2014 / Revised: 25 November 2014 / Accepted: 12 December 2014 / Published online: 30 December 2014

Abstract Cytochrome P450 monooxygenases (P450s) of insects are known to be involved in the metabolism or detoxification of plant allelochemicals and insecticides. *Spodoptera litura* (Lepidoptera, Noctuidae) is a polyphagous moth responsible for severe yield losses in many crops. In this study, two full-length P450 genes, *CYP6B48* and *CYP6B58*, were cloned from *S. litura*. The cDNA sequences encode proteins with 503 and 504 amino acids, respectively. Phylogenetic analysis revealed that *CYP6B48* and *CYP6B58* belong to the CYP6B subfamily of P450s. Quantitative real-time PCR analyses showed that *CYP6B48* and *CYP6B58* were expressed only at larval stage, but not at pupal and adult stages. The highest levels of transcripts were found in the midguts and fat bodies of the larvae. No expression was detected in the ovary or hemolymph. Feeding with diets containing cinnamic acid, quercetin, or coumarin did not affect expression of *CYP6B48*. In contrast, diet supplemented with xanthotoxin dramatically increased the levels of *CYP6B48* transcript in the midgut and fat bodies. Larvae fed with flavone had high levels of transcript of *CYP6B48* in the midgut, whereas only slightly elevated levels were found in the fat bodies. Effects of the tested allelochemicals on *CYP6B58* expression were minor. Hence, our findings show that *S. litura* responds to specific allelochemicals such as xanthotoxin with the accumulation of *CYP6B48* transcripts, suggesting that specific signals in the food control the insect's ability to convert toxic allelochemicals to less harmful forms at the transcriptional level.

Keywords *Spodoptera litura* · Plant allelochemicals · Cytochrome P450 monooxygenases · *CYP6B48* · *CYP6B58* · *Lepidoptera* · *Noctuidae generalist insect pest*

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Introduction

Plants produce a great diversity of secondary compounds, or toxic allelochemicals, which serve as defensive agents against herbivores and pathogens (Li *et al.* 2002a; Riga *et al.* 2014). Herbivorous insects are evolutionarily adapted to the presence of toxins in their diets (Riga *et al.* 2014; Schuler 2011). Detoxification of plant allelochemicals and insecticides in insects frequently relies on cytochrome P450 monooxygenases (P450s or CYPs) that constitute a large superfamily of enzymes (Li *et al.* 2002a; Liu *et al.* 2013; Zeng *et al.* 2007). P450s are NADPH-dependent heme-containing enzymes and important mediators of hydroxylation and epoxidation reactions that result in efficient destruction and degradation of allelochemicals, *e.g.*, in insect guts prior to

absorption (Cano-Ramírez *et al.* 2013; Schuler 2011; Scully *et al.* 2013; Wen *et al.* 2003). In addition to these detoxification reactions, P450s also are involved in insect development, reproduction, ecdysteroid degradation, and various other processes (Cichón *et al.* 2013; Feyereisen 2006; Li *et al.* 2004a, 2007). Insect P450s have been divided into four clades: CYP2, CYP3, CYP4, and mitochondrial P450s (Feyereisen 2006). Clade 3 is subdivided into the CYP6 and CYP9 families (Feyereisen 2006; Li *et al.* 2007). Various CYP6 family members have been demonstrated to detoxify allelochemicals consumed by insects (Grubor and Heckel 2007; Liu *et al.* 2014; Niu *et al.* 2011). For example, CYP6B8 of the moth *Helicoverpa zea* can metabolize many structurally diverse chemicals, including furanocoumarins, flavonoids, and insecticides (Rupasinghe *et al.* 2007). Similarly, the butterflies *Papilio glaucus* and *Papilio multicaudatus* are able to inactivate a broad range of toxins from host plants and their P450s (CYP6B4, CYP6B17, and CYP6B33) can metabolize a wide range of linear and angular furanocoumarins (Hung *et al.* 1997; Mao *et al.* 2007). Furthermore, black pepper (*Piper nigrum*) extracts have insecticidal properties, and consumption by the common fruit fly (*Drosophila melanogaster*) results in stimulated expression of various P450 genes, namely *CYP6A8*, *CYP6D5*, *CYP6W1*, *CYP9B2*, and *CYP12D1* (Jensen *et al.* 2006). Other P450 genes of the common fruit fly are up-regulated in response to caffeine (xanthine alkaloid) and piperonyl butoxide in their diet (Bhaskara *et al.* 2006; Willoughby *et al.* 2006).

The tobacco cutworm moth, *Spodoptera litura* (Lepidoptera: Noctuidae), is a polyphagous insect responsible for considerable yield losses in various crops such as tobacco (*Nicotiana tabacum*), cotton (*Gossypium hirsutum*), peanut (*Arachis hypogaea*), and tomato (*Lycopersicon esculentum*) (Ahmad *et al.* 2007). The management of this pest has become increasingly difficult all over the world. At present, many field populations of *S. litura* have developed multiple resistance against commonly used chemical insecticides, particularly synthetic pyrethroids (Shad *et al.* 2012). The pest status of *S. litura* can mainly be attributed to its resistance to insecticides and its adaptability to a wide range of host plants (Ahmad *et al.* 2007).

Insect P450s play a central role in adaptation to plant allelochemicals and in development of insecticide resistance (Feyereisen 2006; Ismail *et al.* 2013; Wang *et al.* 2014). The efficacy of detoxification may depend on the levels of P450 transcript expressed after exposure, the range of chemicals capable of inducing expression, and the turnover rates of individual P450 proteins involved in detoxification (Chandor-Proust *et al.* 2013; Li *et al.* 2002b; Schuler and Berenbaum 2013). In *S. litura*, expression of two P450 genes, *CYP6B47* and *CYP9A39*, has been analyzed previously. *Spodoptera litura* individuals fed on diet supplemented with lead for several generations showed enhanced tolerance to the insecticide cypermethrin, suggesting that transcript levels of P450 genes are crucial for cypermethrin detoxification (Zhou *et al.* 2012a, b).

As host specificity of *S. litura* may preferentially rely on allelochemicals in the diet, we propose that there is a relationship between P450s of *S. litura* and consumed allelochemicals. In this study, we cloned two additional P450 genes of *S. litura*, *CYP6B48* and *CYP6B58* (originally named *CYP6B29v1* and *CYP6B50*, respectively), which are both members of the CYP6B subfamily of P450s. Using quantitative real-time PCR (qRT-PCR), expression patterns of these genes were determined. Our data indicate that expression levels of *CYP6B48* and *CYP6B58* varied substantially with respect to tissue type, developmental stage, and the presence of allelochemicals in the diet.

Methods and Materials

Insects A laboratory strain of *Spodoptera litura*, originally collected at an experimental farm on the campus of the South China Agricultural University in Guangzhou, China (N 23°16′, E 113°34′), was used. The larvae were reared in an insectary (25±2 °C, 70±5 % relative humidity at a light:dark photoperiod of 14:10 h) and fed with artificial diet. Adults were kept under the same conditions and fed with a 10 % honey solution (Zhou *et al.* 2012b).

RNA Extraction Total RNA was isolated from the midgut of fifth instars of *S. litura* using TRIzol (Invitrogen). RNA was quantified based on its absorbance at 260 nm. The integrity of the RNA was checked by formaldehyde-agarose gel electrophoresis followed by staining with ethidium bromide.

Cloning of Full-Length CYP6B48 and CYP6B58 cDNA Double-stranded cDNA was synthesized using a SMART™ cDNA amplification kit (Clontech). PCR products were purified using a QIAquick PCR purification kit (Qiagen). A cDNA library was PCR-cloned into pGEM-T Easy Vector (Promega, WI., USA) using the synthesized cDNA and the degenerate primers P450F1 5′-TTYTWCRTIGCBGGHTWY-3′ and P450R1 5′-CCDATRC ARTTBCKIKGHCC-3′. Among the sequenced clones, two expressed sequence tags (ESTs) were obtained, named jbi-i (431 bp) and jb5-13 (445 bp). These ESTs showed sequence similarities with the CYP6B subfamily of P450s. To obtain corresponding full-length cDNAs, 5′- and 3′-RACE were performed using the primers CYP6B 58-SP1 (5′-ATCTCG TCTTGGAGGCTGTCAT-3′), CYP6B58-SP5 (5′-AGAGGT TGACCGAGGAGATTGA-3′), CYP6B48-SP1 (5′-CGGG TGTTATTTCCACAGGCTT-3′), and CYP6B48-SP5 (5′-CCATCCGTGTGCTTACTTGCCA-3′). The sequences of the cloned genes *CYP6B48* and *CYP6B58* (originally named

CYP6B29v1 and *CYP6B50*, respectively) were deposited in GenBank (accession numbers GU263827 and GU263828). Amino acid sequences were aligned with PRALINE. Calculation of the theoretical isoelectric point (pI) and molecular weight were performed with the Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html).

Phylogenetic Analysis The cDNA sequences CYP6B48 and CYP6B58 were compared with those of publicly available CYP6B family sequences using the "BLAST-N" or "BLAST-X" tools at the website of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nih.gov/). A phylogenetic tree was constructed with the MEGA 4.0 program based on a multiple alignment of the amino acid sequences performed by Clustal X 1.83, and using the neighbor-joining algorithm with the bootstrap values determined by 1000 replicates (Niu *et al.* 2011; Zhou *et al.* 2012b).

Plant Allochemicals Cinnamic acid [(*E*)-3-phenylprop-2-enoic acid; ≥98 %], quercetin [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one; ≥98 %], coumarin (2*H*-chromen-2-one; ≥99 %), and xanthotoxin (9-methoxy-7*H*-furo[3,2-*g*]chromen-7-one; ≥98 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Flavone (2-phenyl-4*H*-chromen-4-one; ≥98 %) was obtained from Pubo Instrument Co., Ltd (Guangzhou, China). These five chemicals represent natural allochemicals in host plants of *S. litura*. Coumarin (*e.g.*, in *Fragaria chiloensis*, *Ribes nigrum*, *Prunus salicina*, and *Prunus pseudocerasus*), flavone (*e.g.*, in *Arachis hypogaea*, *Apium graveolens*, *Daucus carota*, *Citrus grandis*, and *Glycine max*), and quercetin (*e.g.*, in *Malus domestica*, *Lycopersicon esculentum*, *Citrus sinensis*, *Vitis vinifera*, and *Lycopersicon esculentum*) are typical allochemicals of plants frequently attacked by *S. litura*. (Ahmad *et al.* 2013; Niu *et al.* 2011; Qin *et al.* 2006; Zeng *et al.* 2007). Cinnamic acid (*e.g.*, in *Cinnamomum cassia* and *Lycium barbarum*) and xanthotoxin (*e.g.*, in *Zanthoxylum bungeanum* and *Illicium verum*) are found in less frequent host plants (Ahmad *et al.* 2013; Niu *et al.* 2011; Qin *et al.* 2006; Zeng *et al.* 2007).

Effects of Diet on Expression of CYP6B48 and CYP6B58 in Fourth Instars Twenty synchronously developing 1-d-old fourth instars of *S. litura* were fed with artificial diet containing 0.25 mg g^{-1} xanthotoxin, 1 mg g^{-1} flavone, 1 mg g^{-1} coumarin, 1 mg g^{-1} cinnamic acid or 1 mg g^{-1} quercetin. Twenty control larvae were offered artificial diet without any supplement. After consumption of the diet for 48 h, the insect midguts and fat bodies were dissected and frozen immediately. Tissues of the midguts or fat bodies from four *S. litura* larvae were pooled for extraction of total RNA. The RNA was reverse transcribed using the ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Three independent RNA extractions (resulting in three cDNAs) were performed for all treatments (three biological replicates).

Expression of CYP6B48 and CYP6B58 in Different Tissues Total RNA was extracted from cuticle, brain, midgut, fat body, ovary, and hemolymph of four *S. litura* using the method described above. Three independent RNA extractions (corresponding to four *S. litura* individuals per extraction) were performed to obtain three cDNAs per tissue (three biological replicates).

Expression of CYP6B48 and CYP6B58 Across Different Developmental Stages Larvae (whole body) from first to sixth instar larvae, pupae, and adults of *S. litura* were collected, frozen in liquid nitrogen, and used for extraction of RNA as described above (four individuals per extraction). Three independent replicates (RNA extractions and cDNAs, respectively) were used for each treatment (three biological replicates).

qRT-PCR Analysis The expression levels of *CYP6B48* and *CYP6B58* were determined by quantitative real-time PCR (qRT-PCR). Gene-specific forward/reverse primer sets used in amplification reactions were (5′-GAGCCATCAAAGAA TACTGC-3′/5′-GCCTTCAACTTTCTAGGAGC-3′) for *CYP6B48*, (5′-AGCAAAGAAGGACTGGGACA-3′/5′-CAAAGGCACAGGCGGAAAT-3′) for *CYP6B58*, and (5′-CCCATACAGCGAATCCCGT-3′/5′-AATGTTGTCTCCGT GCCAGC-3′) for *EF-1α* (deduced from the sequence U20129.1), which was used as an internal control to normalize the transcript abundance among different samples. Primers were designed from the ESTs (jbi-i and jb5-13, respectively). The qRT-PCR experiments were performed with an Opticon MJ Research instrument (Bio-rad Inc., USA) in a volume of 25 μl containing 0.2 M of each primer, 12.5 μl of 2×SYBR Green I (Roche), and 1 μl of the cDNA template. The PCR cycling conditions were: 50 °C for 2 min; 95 °C for 2 min; 34 cycles of 95 °C for 15 sec, 65 °C for 30 sec, and 72 °C for 15 sec. To assess the specificity of the PCR amplification, a melting curve analysis was performed at the end of the reaction by increasing the temperature from 65 to 95 °C (5 sec for every increment of 0.5 °C), and a single peak was observed. The ratios of P450 gene/*EF-1α* gene signals were analyzed according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Data obtained for each cDNA were combined (two technical repeats). Expression data shown in the figures are means ± standard error (SE) for three cDNAs derived from three RNA extractions (three biological replicates).

Statistical Analysis Statistical analysis of data from qRT-PCR experiments was performed with the normalized transcript levels of the *CYP6B48* and *CYP6B58* genes. Statistically significant differences ($P<0.05$) were obtained by one-way

ANOVA followed by the Duncan's multiple range test using the SPSS 13.0 Software Package.

Results

Cloning of CYP6B48 and CYP6B58 from S. litura cDNA Two new P450 genes of *S. litura*, *CYP6B48* (originally named *CYP6B29v1*) and *CYP6B58* (originally named *CYP6B50*) were cloned, and the cDNA sequences were deposited in GenBank under the accession numbers GU263827 and GU263828, respectively. The full-length cDNA sequence of *CYP6B48* is 2050 bp in length and contains a 5′ untranslated region (UTR) of 37 bp and a 3′ UTR of 501 bp. A single open reading frame (ORF) at nucleotide positions 38–1546 encodes a predicted protein of 503 amino acids. The protein has a predicted molecular weight of 57.60 kDa and a theoretical pI of 8.40. The *CYP6B58* cDNA has a full-length sequence of 1697 bp with an ORF of 1515 bp, a 44-bp 5′ UTR, and a 138-bp 3′ UTR. The ORF encodes a predicted protein of 504 amino acids. Its predicted molecular weight is 57.60 kDa

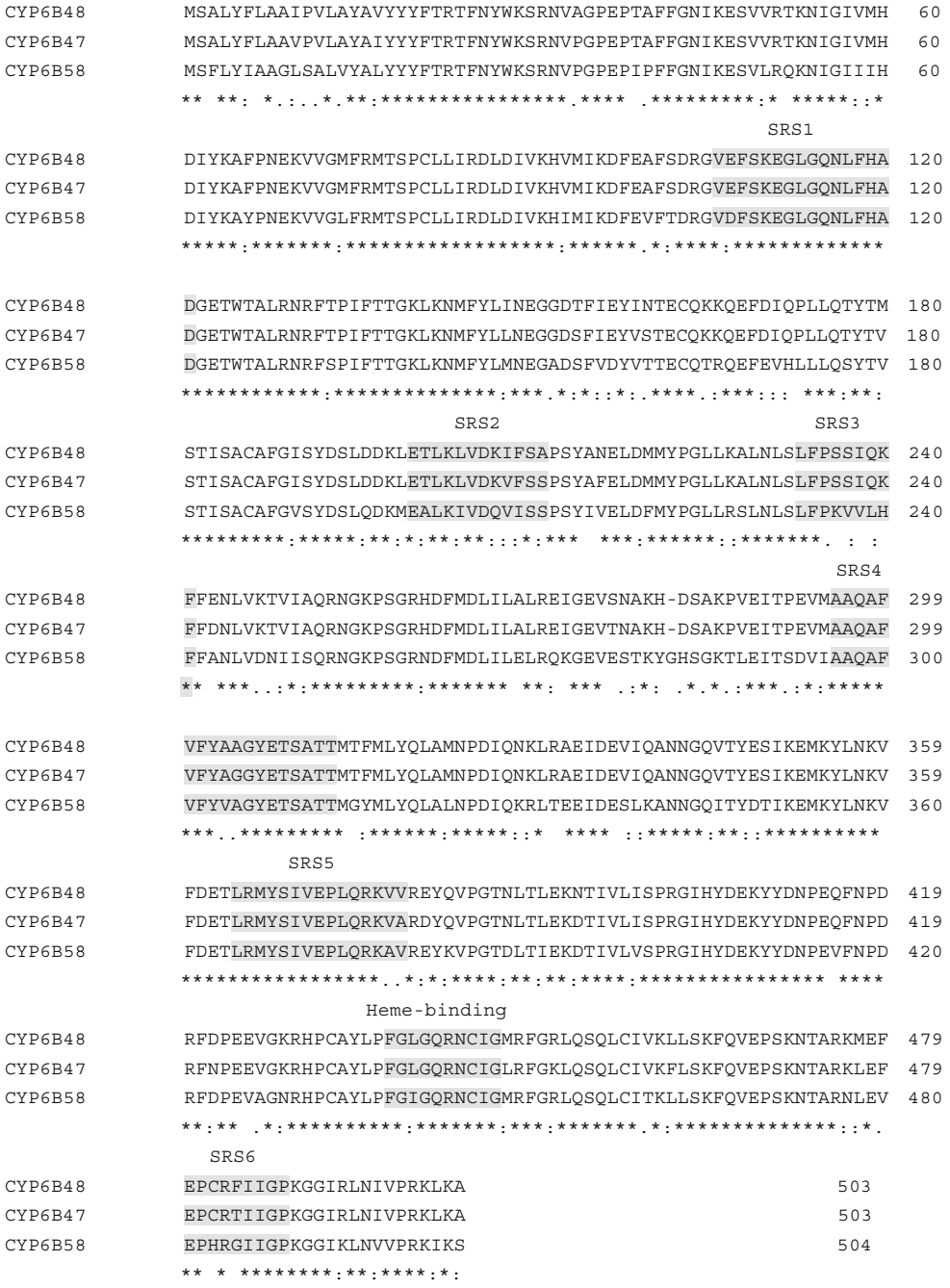

Fig. 1 Alignment of the amino acid sequences deduced from *CYP6B48*, *CYP6B47*, and *CYP6B58* of *Spodoptera litura*. Substrate recognition sites (SRS) are indicated with *gray boxes*. Identical residues are marked by *asterisks*, conserved substitutions by two dots and semi-conserved substitutions by a single dot

and its theoretical pI is 7.84. Figure 1 shows the aligned deduced amino acid sequences of *CYP6B48*, *CYP6B58*, and the previously identified *CYP6B47* gene of *S. litura* (accession number GQ465039.2; Zhou *et al.* 2012b). The CYP6B48 and CYP6B58 sequences are similar to CYP6B47 (95.4 and 77.3 % amino acid identity, respectively). Sequence comparisons with available sequences from GenBank indicate that these three proteins share various characteristics with other P450s. For example, the classic heme-iron ligand P450 signature motif F××G×××C×G (Li *et al.* 2004b; Niu *et al.* 2011) and the substrate recognition sites SRS1 to SRS6 (predicted substrate-binding residues; Gotoh 1992) are conserved in the three P450s (Fig. 1). SRS6 possesses the classic E××R motif, which is almost identical in all P450 sequences (Li *et al.* 2004b; Niu *et al.* 2011; Zhou *et al.* 2012b).

Phylogenetic Relationship of CYP6B48 and CYP6B58 with Other CYP6B Family Members To investigate the relationship of the cloned *CYP6B48* and *CYP6B58* with other P450s, a phylogenetic analysis was performed that included related amino acid sequences obtained from GenBank (Fig. 2). CYP6B48, CYP6B47, and CYP6B58 of *S. litura* represent typical members of the CYP6B subfamily of P450s. Notably, all three proteins show high levels of similarity with CYP6B subfamily members of *H. zea*, namely CYP6B27, CYP6B28, and CYP6B8 (amino acid identities range from 71 and 75 %).

Tissue and Development Specific Expression of CYP6B48 and CYP6B58 Transcript levels of *CYP6B48* and *CYP6B58* in first to sixth instar larvae, pupae, and adults of *S. litura* were determined using qRT-PCR. The expression levels of *CYP6B48* and *CYP6B58* were highest in fifth and sixth instar larvae. Compared to first instars, transcript levels of *CYP6B48* and *CYP6B58* in sixth instars were significantly greater by 42.0 and 295.7 %, respectively. Transcripts of these genes were not detected in pupae and adults (Figs. 3a, 4a). The expression patterns of *CYP6B48* and *CYP6B58* also were investigated for different tissues of *S. litura* larvae. Transcript levels of *CYP6B48* and *CYP6B58* were highest in the midgut and fat body, whereas lower amounts were found for the cuticle and brain (Figs. 3b, 4b). Moreover, *CYP6B58* was expressed at very low levels in the ovary, whereas no expression was detected for *CYP6B48* expression in this tissue. Transcripts of *CYP6B48* and *CYP6B58* were not detected in hemolymph.

Induction of CYP6B48 and CYP6B58 Transcripts by Plant Allelochemicals To determine the effect of plant allelochemicals on the transcription of *CYP6B48* and *CYP6B58* in *S. litura*, qRT-PCR was performed using 1-d-old fourth instars fed for 48 h with artificial diet supplemented with cinnamic acid, quercetin, coumarin, flavone, and xanthotoxin. Cinnamic acid, quercetin, and coumarin did not affect C*YP6B48* expression in the midgut and fat body. However, compared to the control without allelochemicals, C*YP6B48* transcripts in the midgut accumulated in response to flavone (21.6-fold) and xanthotoxin (18.2-fold). A similar increase in C*YP6B48* gene expression, albeit weaker, was seen for RNA samples isolated from the fat body, with a 4.2-fold induction observed for flavone and 16.1-fold induction for xanthotoxin (Fig. 5a). A different gene expression pattern was observed for *CYP6B58*. In the midgut, transcript levels of *CYP6B58* were slightly, but significantly enhanced by coumarin (1.5-fold) and xanthotoxin (1.7-fold) in the diet. Reduced transcript levels were observed for flavone, whereas cinnamic acid and quercetin showed no effects on C*YP6B58* expression. The transcript levels of *CYP6B58* in the fat body were significantly increased by diet supplemented with quercetin (1.4-fold), flavone (1.7-fold), and xanthotoxin (2.0-fold).

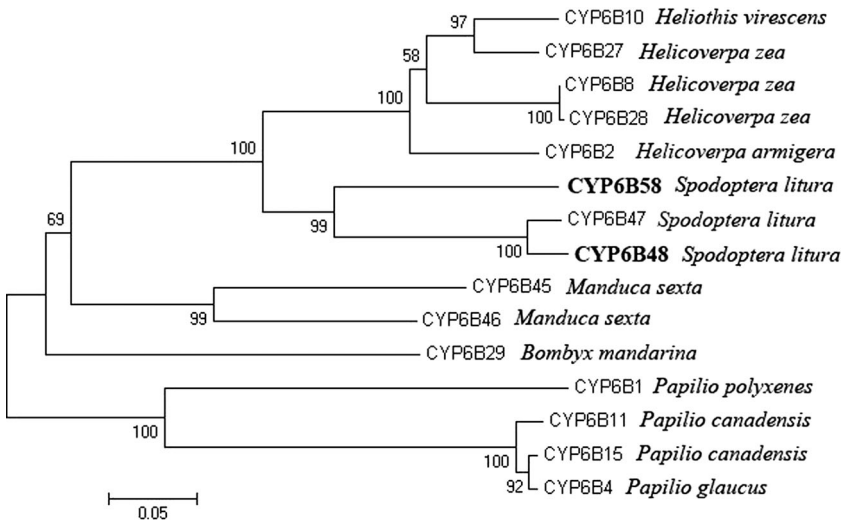

Fig. 2 Phylogenetic analysis of CYP6B48 and CYP6B58 of *Spodoptera litura* and related P450s. The tree was constructed using the neighbor-joining method using the Mega 4.0 software. The scale *bar* indicates 0.05 amino acid substitutions per site. Bootstrap analysis was performed with 1000 replications

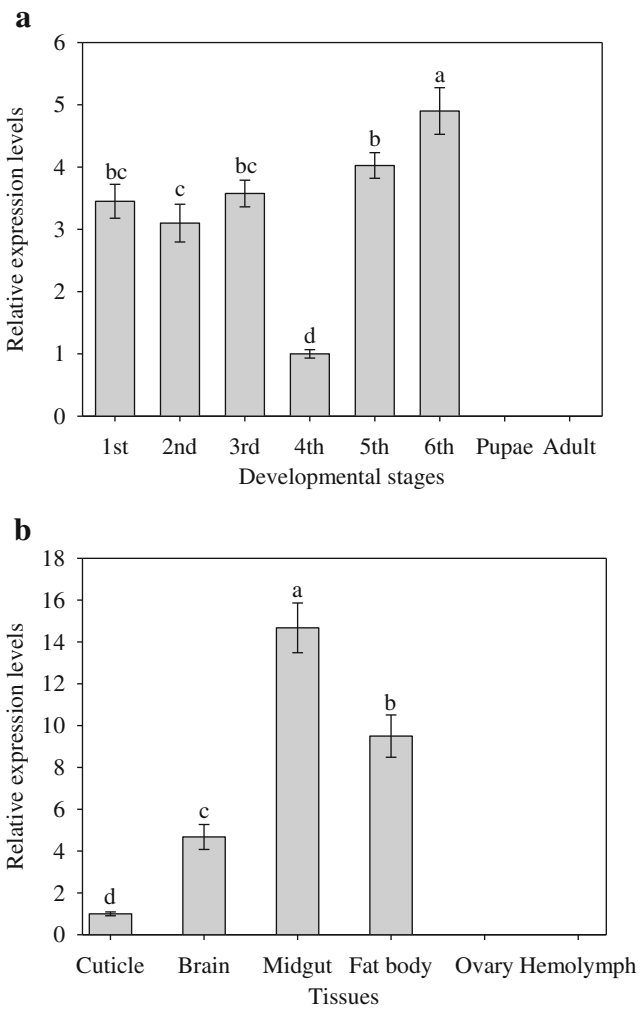

Fig. 3 Transcript levels of *CYP6B48* at different developmental stages (**a**) and in different tissues (**b**) of *Spodoptera litura*. Real-time qRT-PCR analysis was used to determine the relative transcript levels shown by qRT-PCR. Data shown are mean ± SE derived from three biological replicates. Different letters above *bars* indicate significant differences ($P<0.05$) according to the Duncan's multiple range test

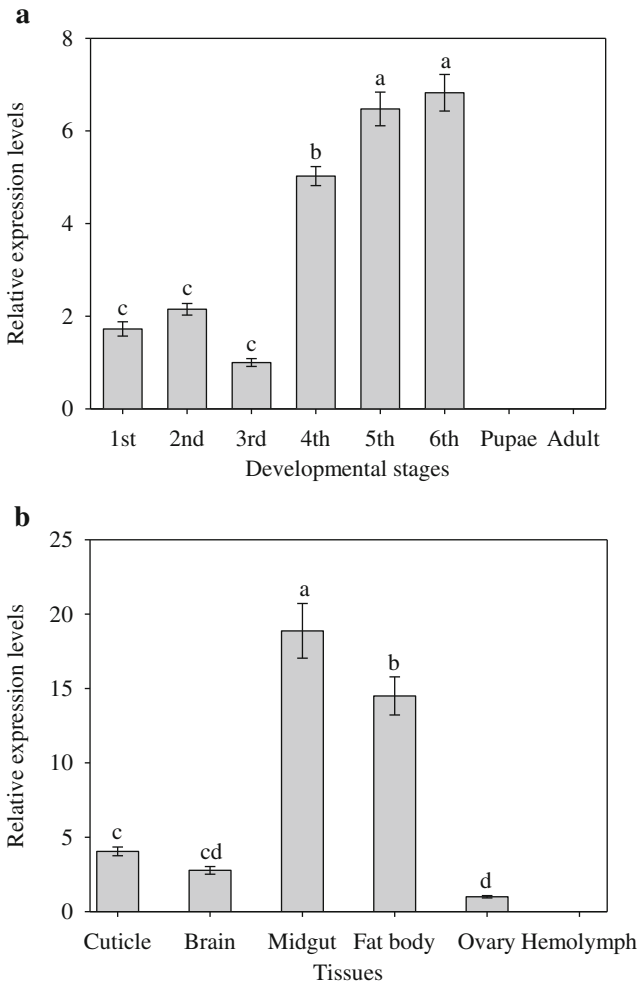

Fig. 4 Transcript levels of *CYP6B58* at different developmental stages (**a**) and in different tissues (**b**) of *Spodoptera litura*. Real-time qRT-PCR analysis was used to determine the relative transcript levels shown by qRT-PCR. Data shown represent means ± SE derived from three biological replicates. Different letters above *bars* indicate significant differences ($P<0.05$) according to the Duncan's multiple range test

In contrast, expression of *CYP6B58* was significantly reduced when the larvae were fed with cinnamic acid and coumarin, respectively (Fig. 5b).

Discussion

The fitness of herbivorous insects largely depends on food quality, which is negatively affected by the plant's ability to accumulate specific allelochemicals. Cytochrome P450 genes of the moth *S. litura*, a pest responsible for severe yield losses in many crops (Ahmad *et al.* 2007), represent an ideal subject for investigating the adaptation of polyphagous insects to plant allelochemicals. The P450 detoxification system might allow *S. litura* to survive on a broad range of host plants, thereby rapidly profiting from available food resources in a changing environment. The findings of this study provide key information on the expression of the P450 genes *CYP6B48* and *CYP6B58* in *S. litura* in response to different allelochemicals in the diet.

Analysis of substrate recognition sites in P450 amino acid sequences can provide clues to possible substrates (Gotoh 1992; Li *et al.* 2002a; Niu *et al.* 2011). For example, SRS1, SRS4, and SRS5 in P450s may play a role in the recognition of substrates containing fatty acids (Hlavica 2011). Interestingly, the SRS1, SRS4, and SRS5 sequences in CYP6B48 and CYP6B58 of *S. litura* (Fig. 1) are very similar to those of CYP6B8 (accession number AF102263.1) of *H. zea* (amino acid identity ranging from 86.7 to 100 % for CYP6B48 and 93.3 to 94.4 % for CYP6B58). The CYP6B8 enzyme of *H. zea* belongs to the category of "generalists", *i.e.*, is a P450 with a broad range of substrates. Known substrates of

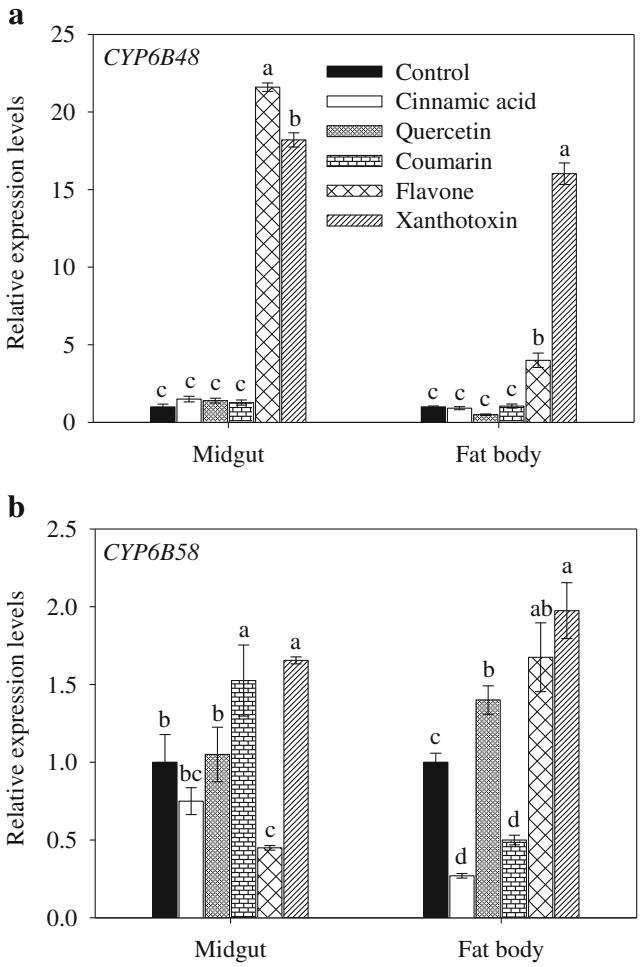

Fig. 5 Transcript levels of *CYP6B48* (**a**) and *CYP6B58* (**b**) in fifth instars of *Spodoptera litura* fed with allelochemicals for 48 h. Total RNA pooled from 20 midguts and fat bodies of fifth instar *S. litura* larvae reared on plain control diet, and diets containing 0.25 mg g^{-1} xanthotoxin, 1 mg g^{-1} flavone, 1 mg g^{-1} coumarin, 1 mg g^{-1} cinnamic acid or 1 mg g^{-1} quercetin. Control larvae were fed with diet alone. Real-time qRT-PCR analysis was used to determine the relative transcript levels shown by qRT-PCR. Data shown are means ± SE derived from three biological replicates. Different letters above *bars* indicate significant differences ($P<0.05$) according to the Duncan's multiple range test

CYP6B8 include natural plant allelochemicals such as flavone, chlorogenic acid, furanocoumarins, and indoles as well as various synthetic insecticides, namely diazinon, α-cypermethrin, or aldrin (Li *et al.* 2004b). Based on these similarities, we suggest that CYP6B48 and CYP6B58 possess similar substrate preferences for allelochemicals and insecticides. This prediction is supported by the fact that transcript levels of *CYP6B48* and *CYP6B58* in *S. litura* larvae are up-regulated in the midgut (Figs. 3b, and 4b) and in response to allelochemicals (putative P450 substrates) supplemented in the diet (Fig. 5). Remarkably, transcripts of *CYP6B48* and *CYP6B58* were not detected in pupae and adults of *S. litura*, suggesting that expression of these genes in larvae is related to the intake of diet containing allelochemicals (Figs. 3a, 4a). A similar expression pattern has been found for P450 genes in other insect species, such as larval expression of *CYP6B1* of the black swallowtail (*Papilio polyxenes*) (Harrison *et al.* 2001).

Tissue-specific expression of P450 genes provides important clues to specific biological functions (Chung *et al.* 2009). Various studies have shown that the midgut of insect larvae is an important detoxification tissue, in which ingested allelochemicals can be efficiently detoxified prior to food adsorption (Zeng *et al.* 2013; Zhang *et al.* 2012). The *CYP6B47* gene of *S. litura* is constitutively expressed in larvae, particularly in the midgut and fat body (Zhou *et al.* 2012b). In the present study, *CYP6B48* and *CYP6B58* in *S. litura* showed similar expression patterns. *CYP6B48* and *CYP6B58* expression levels were highest in the larval midgut, suggesting a role in detoxification of allelochemicals (Figs. 3, 4). These findings also are reminiscent of the strong constitutive expression of four CYP6 family genes in the midgut of *H. zea* (Li *et al.* 2002a).

CYP6 family members of insect P450s appear to be particularly important for the breakdown of plant allelochemicals in the diet of moth larvae. Accordingly, a number of studies have reported that the expression of CYP6 family genes in insect larvae is modulated by allochemicals supplemented in the diet. In *Helicoverpa armigera* larvae, for example, xanthotoxin stimulates expression of *CYP6B7* and *CYP6AE14* in the midgut and also enhances expression of *CYP6B2*, *CYP6AE14*, and *CYP6AE12* in the fat body (Zhou *et al.* 2010). In *H. zea*, four CYP6B genes (*CYP6B8*, *CYP6B9*, *CYP6B27*, and *CYP6B28*) are induced in response to a number of plant allelochemicals, including xanthotoxin, flavone, coumarin, and quercetin. (Li *et al.* 2002a), as well as by the plant defense-related phytohormones, jasmonate, and salicylate (Li *et al.* 2002b). Another study in *H. zea* showed that *CYP6B8* transcripts were induced in larvae consuming diets containing flavone, visnagin, and imperatorin. In contrast, *CYP6B27* expression was not significantly stimulated by these compounds (Wen *et al.* 2009). Furthermore, expression of *CYP6B29* and *CYP6AB5* in the silkworm (*Bombyx mori*) was induced by quercetin, particularly after a short-term dietary treatment (Zhang *et al.* 2012). In the present study, the *CYP6B48* transcripts of *S. litura* in the midgut and fat body were strongly induced by flavone and xanthotoxin, suggesting that CYP6B48 is involved in the breakdown of these allochemicals (Fig. 5a). Such an induction of gene expression could allow the consumption of a diet rich in flavone and xanthotoxin that would otherwise be toxic for the insect. We hypothesize that the larvae possess chemosensory cells that can perceive flavone and xanthotoxin or their degradation products. This would then result in the induction of a specific signal transduction pathway and activation of transcription factors that regulate expression of specific genes including *CYP6B48*. In the beetle *Callosobruchus maculates*, for

example, consumption of the furanocoumarin bergapten alters expression of 543 midgut genes in response to bergapten in the diet (Guo *et al.* 2012). In fact, molecular mechanisms underlying the induction of P450 genes in insects remain largely unknown but point to a complex genetic regulatory network that controls gene expression in response to specific dietary signals (Bhaskara *et al.* 2008; McDonnell *et al.* 2004; Zhang *et al.* 2010).

In contrast to *CYP6B48*, expression of *CYP6B58* in *S. litura* was differentially affected by plant allelochemicals. Transcript levels of *CYP6B58* in the midgut and fat body were not or only slightly induced by allochemicals in the diet (Fig. 5b). Remarkably, expression of *CYP6B58* in the midgut was considerably reduced by flavone and in the fat body by cinnamic acid and coumarin. Down-regulation of P450 genes by allochemicals and pesticides has been reported for several insect species. For example, *P. polyxenes* efficiently metabolizes furanocoumarins of host plants. Furanocoumarin degradation by CYP6B1, however, is inhibited by a range of compounds, including coumarins, furanochromones, flavonoids, and alkaloids (Wen *et al.* 2006).

In summary, we have cloned two P450 genes of *S. litura* and analyzed their expression patterns during various developmental stages and in different tissues. Feeding experiments with larvae suggest that these P450s, especially CYP6B48, are likely involved in the oxidative degradation of plant allelochemicals. Our data also provide basic information for further characterization of these P450s on a protein level in order to determine their substrate preference for allochemicals and insecticides. At present, many *S. litura* larvae show high resistance against various insecticides (Shad *et al.* 2012) and these populations should be compared with respect to P450 activities and gene expression patterns at the genomic level.

Acknowledgments This research was supported by the National Natural Science Foundation of China (31070388, 31100286, 31470576), National 973 Program of China (2011CB100400), Guangdong Province Universities and Colleges Pearl River Scholar Funded Scheme (2010), Guangdong Natural Science Foundation of China (S2011040004336), and Ph.D. Foundation of the Ministry of Education of China (20104404110004). The authors thank Dr. David Nelson for naming the P450 genes.

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