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Identification and expression patterns of Halloween genes encoding cytochrome P450s involved in ecdysteroid biosynthesis in the cotton bollworm *Helicoverpa armigera*

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Abstract

20-Hydroxyecdysone (20E) is a key hormone which regulates growth, development and reproduction in insects. Although cytochrome P450 enzymes (P450s) participating in the ecdysteroid biosynthesis of 20E have been characterized in a few model insects, no work has been published on the molecular entity of their orthologs in the cotton bollworm *Helicoverpa armigera*, a major pest insect in agriculture worldwide. In this study, four cytochrome P450 homologs, namely *HarmCYP302A1*, *HarmCYP306A1*, *HarmCYP314A1* and *HarmCYP315A1* from *H. armigera*, were identified and evolutional conservation of these Halloween genes were revealed among lepidopteran. Expression analyses showed that *HarmCYP302A1* and *HarmCYP315A1* were predominantly expressed in larval prothoracic glands, whereas this predominance was not always observed for *HarmCYP306A1* and *CYP314A1*. The expression patterns of Halloween genes indicate that the fat bodies may play an important role in the conversion of ecdysone into 20E in larval–larval molt and in larval–pupal metamorphosis, and raise the possibility that *HarmCYP315A1* plays a role in tissue-specific regulation in the steroid biosynthesis in *H. armigera*. These findings represent the first identification and expression characterization of four steriodogenic P450 genes and provide the groundwork for future functional and evolutionary study of steroid biosynthesis in this agriculturally important pest.

Keywords: Cytochrome P450, Ecysteroid, Expression pattern, Halloween genes, *Helicoverpa armigera*

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Introduction

Insect growth and developments are regulated by hormones. Steroid hormones play key roles in the coordinated regulation of many developmental and physiological events such as molting, metamorphosis and diapause in insects (Niwa & Niwa, 2014). For example, the precise timing of molting is strictly guided by precisely timed changes of the molting hormone 20-hydroxyecdysone (20E) (Iga & Smagghe, 2010). Insect metamorphosis from larva over pupa to a reproductive adult is under the control of 20E as well (Iga & Smagghe, 2010). It is generally recognized that insects synthesize ecdysone in the prothoracic gland (PG) from dietary cholesterol or phytosterol, and the synthesized ecdysone (E) is secreted in the hemolymph, and then converted into 20E in various peripheral tissues predominantly in midgut (MG) or fat body (FB) (Gilbert *et al.*, 2002).

Ecdysteroids are biosynthesized from the embryonic to the adult stage. Ecdysone biosynthesis involves a series of enzymatic oxidation reactions (fig. S-1) (Niwa & Niwa, 2014). It has

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demonstrated that *Neverland* (*Nvd*) (the [2Fe–2S] Rieske oxygenase) catalyzes the first step from cholesterol to 7-dehydrocholesterol (7-DC) (Yoshiyama-Yanagawa *et al.*, 2011). However, the conversion process from 7-DC to ketodiol remains unclear, commonly referred as the 'Black Box'. The 'Black Box' is hypothesized to be catalyzed by at least three enzymes, including the cytochrome P450 monooxygenases Spook (Spo) and CYP6T3, and the short-chain dehydrogenase/reductase Shroud (Sro) (Niwa & Niwa, 2014). In addition, it is still possible that other uncharacterized enzymes play a role in ecdysteroid biosynthesis. Recent studies have demonstrated that a glutathione S-transferase encoding gene, *noppera-bo* (*nobo*), plays a crucial role in regulating ecdysone biosynthesis in insects (Enya *et al.*, 2014, 2015).

Up to now, four cytochrome P450 enzymes encoded by Halloween genes (*phm, dib, sad* and *shd*) have been characterized in *Drosophila melanogaster*, *Bombyx mori* and *Manduca sexta*. Phm (CYP306A1), Dib (CYP302A1) and Sad (CYP315A1) sequentially convert the precursor of ecdysone, 2, 22, 25-trideoxyecdysone (ketodiol), to 22, 2-dideoxyecdysone (ketotriol), 2-deoxyecdysone and E. The product of *shd* (CYP314A1) hydroxylates E–20E, an active form of ecdysteroid. During the past decade, the orthologs of these Halloween genes have been also identified or inferred in several other insects (Christiaens *et al.*, 2010; Iga & Smagghe, 2010; Jia *et al.*, 2013; Wan *et al.*, 2014*a*; Cabrera *et al.*, 2015). Although many ecdysteroidogenic enzyme-encoding genes are well conserved in the genomes of insects (Niwa & Niwa, 2014), previous studies also raise the possibility that ecdysteroid biosynthesis is differentially regulated among various insect species (Enya *et al.*, 2014). This situation highlights the necessity to know the specific molecular mechanism of ecdysteroid synthesis in certain insects of interest.

From the aspect of pest control, P450 enzymes that catalyze physiologically important reactions offer insect-selective targets for discovering and developing new insecticides (Niwa & Niwa, 2014; Feyereisen, 2015). Several studies have showed that such gene can serve as a potential target gene for RNA-interference-based pest management (Luan *et al.*, 2013; Kong *et al.*, 2014; Wan *et al.*, 2014*b*; Wan *et al.*, 2015). For example, knockdown of *CYP314A1* in *Leptinotarsa decemlineata* by dietary introduction of double-stranded RNA into the secondary instar larvae caused larval lethality, delayed development and reduced pupation ratio (Kong *et al.*, 2014). Similarly, knockdown of CYP306A1 in the white-backed planthopper *Sogatella furcifera* through dietary delivery into the 2nd instars caused lethality and slowed down ecdysis during nymphal stages (Wan *et al.*, 2014*b*), and oral delivery of double-stranded RNA of *Laodelphax striatellus* CYP315A1 at the nymph stage caused nymphal lethality and delayed development in a dose-dependent manner (Wan *et al.*, 2015).

The cotton bollworm *Helicoverpa armigera* is a dreaded pest worldwide (Fitt, 1989). Although molting and metamorphosis are known to be controlled by 20E in this holometabolous insect, the enzymes in ecdysteroid synthetic pathway remain to be characterized. As the first step toward characterizing the enzymes involved in ecdysteroid biosynthesis, in this study, we tried to probe *H. armigera* for Halloween gene orthologs, and investigated their transcriptional patterns. This effort may advance our knowledge about the molecular details of ecdysteroid biosynthesis in insects and may contribute to developing new insecticides targeting these ecdysteroidogenic enzymes for a smart control of this important pest.

Materials and methods

Insects

A colony of cotton bollworm *H. armigera* (Hübner) was established from a field collection from Henan Province, China in 2005 and was maintained in the laboratory. Larvae were individually reared in glass tubes on wheat germ-based artificial diets (Wu & Gong, 1997), at 25 ± 1°C and relative humidity of 70% with a photoperiod of 16-h light/8-h dark. Adults were kept under the same temperature and light conditions, and provided with a 10% honey solution.

Cloning of full-length transcripts of Halloween genes

Total RNA was extracted from entire 2nd-instar larvae of *H. armigera* by using TRIzol (Invitrogen, CA, USA) according to the manufacture's protocol. First-strand cDNA was synthesized from total RNA (1 µg) using PrimeScript First Strand Synthesis Kit according to the manufacturer's instructions (Takara, Dalian, China).

All the oligonucleotide primers (table 1) used in this study were commercially synthesized by Invitrogen (China). The cloning of each gene was performed by three steps. Firstly, a fragment of cDNA was amplified by PCR using degenerate primers. Then the 3′-end and 5′-end of this cDNA fragment were obtained respectively via rapid-amplification of cDNA ends (RACE) using SMART™ RACE cDNA amplification kit according to the manufacturer's protocols (Clontech, CA, USA) with gene-specific primers used for 3′- and 5′-RACE. Lastly, the full-length open reading frames were amplified for sequence confirmation using corresponding primers (table 1), which were designed based on the sequence information obtained from 3′- and 5′-RACE ends. Products which amplified using the high-fidelity DNA polymerase (*Pfu*) were gel purified (Takara, Dalian, China) and then subcloned into pEasy-T1 and transformed the *Escherichia coli* Trans 5α strain (Transgen, Beijing, China). At least three positive clones were sequenced for each gene.

Sequence identification and phylogenetic analysis

Bioinformation analysis was conducted using on line software (http://web.expasy.org/protparam/). Alignment of amino acid sequence was performed by using Sequence Alignment tool Clustal W program, and a neighbor-joining phylogenetic tree was generated by MEGA 6 (Tamura *et al.*, 2013).

Spatial and temporal expression analyses using RT–PCR

The expression profiles were evaluated in various tissues and at various developmental stages of *H. armigera* by RT–PCR. Total RNA from eggs (12 h old), selected tissues (head, MG, Malpighian tubules, FB and epidermis) of the 5th-instar larvae (1 day old), female and male pupae (4–5 days old), and different parts from female and male adults (3–4 days old), were prepared using TRIzol according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Ten individuals were used for each preparation. To remove potential genomic DNA contamination, All RNA samples were treated with RNase-free DNase I (Takara, Dalian, China).

The first-strand cDNA was synthesized from 1 µg RNA with an oligo (dT) primer using the MLV reverse transcriptase.

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Table 1. Oligonucleotide primers used in this study.

Gene name	Sequence (5′–3′)	Reference
	Degenerate primers for isolation of Halloween P450 genes	
CYP302A1	Forward: ACNAAYGGWCCNGANTGGTGG Reverse: TABGMWGTKGTATCKATKGCAGCCA	Iga & Smagghe (2010)
CYP306A1	Forward: GGNGTNGTNAAYTTYTTGCCST Reverse: CAWCCRTGNGGWATKCCSASKGG	Iga & Smagghe (2010)
CYP314A1	Forwards: MGRTACAARATGAMYAARCTRCACGA Reverse: GTTKGCTARCGTYTCWATRCCRGC	
CYP315A1	Forward: ARMGRGGWCTNTTYTTYATGRASGG Reverse: GTCGTRTCDCCWGCNGCRATVAC	Iga & Smagghe (2010)
	Gene-specific primers for 3′ RACE	
CYP302A1	GGCTCCTTTCTTCAACAACCCAA	
CYP306A1	ATAGGGTTGCCACCAATAAAGGAGGC	
CYP314A1	ACTCCAAAAGTTCGAGGAAATC	
CYP315A1	AGGCAGTTCTTCATCTCTTCAGCG	
	Gene-specific primers for 5′ RACE	
CYP302A1	TATCGACCATCATTCCTACGACGTCT	
CYP306A1	GGCCTTCTGGGTGTTCCGTAAAG	
CYP314A1	TAGATTCCAAGCCCATCCTGT	
CYP315A1	CCTCGTAATGTCTATCTCGCTGA	
	Primers for amplification of full-length coding region	
CYP302A1	Forward: ATGTTGAAGTTATCTAAAAAGTA Reverse: TTCCTACAAATTCCTTGGTGT	
CYP306A1	Forward: ATGGATTTTTTCTTTTTATGGC Reverse: TATAGGATCGCAATAGTAGGAAACTG	
CYP314A1	Forward: CTACGATGTCTCTTCCGGGA Reverse: CCATTCCACTAGTCGCAGAGACACA	
CYP315A1	Forward: ATGCAACGCATTAACACAAC Reverse: TTTTTCTGGTTGACGTAGTC	
	Primers for expression profile analysis	
EF-1α	Forward: GACAAACGTACCATCGAGAAG Reverse: GATACCAGCCTCGAACTCAC	Liu *et al.* (2014)
CYP302	Forward: ACGTCGTGGGAATGATGGTC Reverse: GTCAGTCGTCCCACACCAAT	
CYP306	Forward: TTACGGAACACCCTGAAGGC Reverse: GATCGAATGCGCTGGGTTTC	
CYP314	Forward: TTTTCGTGGAGACCACTCCG Reverse: TGGTGCCATTTTTCGCCTTG	
CYP315	Forward: TAGCAAGGATGGCGATGGAC Reverse: GAGGCTATGATCGGAGTGCC	
	Primers for qPCR analysis	
CYP302	Forward: AATACTGGCGGATTACTG Reverse: ACTTTGAGGACTTGTGAAA (Amplicon: 87 bp)	
CYP306	Forward: GAACTTTTAGAAAATGTAGAGAA Reverse: CAGGAGGAAACTTGAATC (Amplicon: 118 bp)	
CYP314	Forward: TTTCTTTGTCTTGGCTTCC Reverse: TCTACAGGACATTCGTGAA (Amplicon: 80 bp)	
CYP315	Forward: CAGTTGACGGATCACTATCA Reverse: TTATCAGTCCATCGCCATC (Amplicon: 81 bp)	
GAPDH	Forward: GTCTACCTCTTCAAGTAC Reverse: GAGATGATGACCTTCTTAG (Amplicon: 121 bp)	Shakeel *et al.* (2015)

At the same time of cDNA synthesis, samples without reverse transcriptase were prepared for evaluating the genomic contamination. The RT–PCR amplifications were carried out in a final volume of 25 µl reaction containing 2 µl of 10× diluted template cDNA, 12.5 µl Taq Master Mix (Tiangen, Beijing, China), 0.5 µl (10 µM) of each primer and sterilized water up to the final volume. The primers used for RT–PCR analysis were listed in table 1. The elongation factor-1α gene (EF-α) was used as a reference gene (Zhou *et al.*, 2009). The thermal cycling profile consisted of an initial step of denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55–60°C for 30 s and 72°C for 30 s, and a final extension step of 72°C for 5 min. Aliquots of PCR products were analyzed on a 2% agarose gel. In order to confirm the fidelity of the RT–PCR, the PCR products were gel purified and sequenced.

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H.armigera       ......MLKLSKKYCGHNKSLSFASN.VASYSDTIEVHNGRGNVKSFEEIPGPKSYPVVGTLYKYMPYMGDYNVEA    69
B.mori           MFVRLTVKNNIPYRARKCVYRRASEN.FVGSEHASKVNEQGDNLMNFEDIPGPRSYPIIGTLHKYLPLIGDYDAEA    75
M.sexta          .....MYRLLNNYKCVPCRKFIQRNYTMEVSHNIIQKEPQKESLLHLNDIPGPKCYPVIGTLHKYLPFIGDYDAEA    71
M.brassicae      ......MLKLSKTFTNNGKCVRFVSN.AACSGENKEVQNEKGHVKSFEEIPGPKCYPIVGTLYKYAPYIGDYNVEK    69
S.littoralis     ......MYKSSKIFSNRLKYVNLVSN.VAKYGSGKTQNC...NVRSFEEIPGPKSYPIIGTLHQYAPFIGDYDVET    66
D.melanogaster   .......................MLTKLLKISCTSRQCT..FAKPYQAIPGPRGPFGMGNLYNYLPGIGSYSWLR    50

H.armigera       LDKNAWLNYRRYGNLVREAPGV.NLLHVYDPEDIETVFRQDHRYPARRSHVAMYHYRTNKPHVYNTGGLLSTNGPE   144
B.mori           LDKNAILNWRRYGSLVREKPIV.NLVHVYDPDDIEAVFRQDHRYPARRSHTAMNYYRTNKPNVYNTGGLLATNGPD   150
M.sexta          LDKIAWLNYRRYGCLVREVPGV.NVIHVYDPDDIEAVFRQDDRFPARRSHLAMYHYRKNKPQVYNTGGILSTNGPE   146
M.brassicae      LDRNSLMNWRRYGSLVREAPGV.RLLHVYDPEDIEVVFRQDHRFPARRSHIAMLHYRLSKPHVYNTGGLLSTNGSE   144
S.littoralis     LDKNAWLNYRRYGSLVRETPGV.NVLHVYDPEDIETVFRQDHRFPARRSHIAMYHYRMNKPDVYKTGGLLSTNGEE   141
D.melanogaster   LHQAGQDKYEKYGAIVRETIVPGQDIVWLYDPKDIALLLNERDCPQRRSHLALAQYRKSRPDVYKTTGLLPTNGPE   126

H.armigera       WWRLRSTFQKNFTSPQSVKNHIESTDSIVTEFVQWLKQINITHNEDFLPYLNRLNLEVIATVAFNERFNSFSSEEQ   220
B.mori           WWRLRSIFQKNFTSPQSVKTHVSDTDNIAKEFVEWIKRDKVSSKNDFLTFLNRLNLEIIGVVAFNERFNSFALSEQ   226
M.sexta          WWRLRSTFQKNFTSPQSVKAHVECTDDIAKEFVQWVHNRKFRCNEDFLIYLNRLNLEIIGAVAFNERFDSFSPQEQ   222
M.brassicae      WWRLRSTFQKNFTSPQSVKNHVERTDGVITEFVQWIKERNISHNEDFLPYLNRLNLEVIGTVAFNERFESFSPQEQ   220
S.littoralis     WWRLRSTFQKNFTSPKSAKSHIESTEIVIREFINWIKERNVTHNEDILPYLNRLNLEVIGVVAFNERFRSFSPEEQ   217
D.melanogaster   WWRIRAQVQKELSAPKSVRNFVRQVDGVTKEFIRFLQESRNGGAIDMLPKLTRLNLELTCLLTFGARLQSFTAQEQ   202
                 helix-c

H.armigera       DLNSRSSKTIAAAFGSNSGIMKLDKGFMWKIFKTPLYKQLADSQEYLEKVSTEILLERVPFFKHADNNDVSLLGS.   295
B.mori           DPESRSSKTIAAAFGSNSGVMKLDKGFLWKMFSTPLYKKLVNSQIYLEKISTDILIRKINLFESDDSKNDKSLLKT   302
M.sexta          HSKSRSSKIIQAAFGSNSGVMKLDKGFLWKFFKTPLYRKLAQSQEYLEKVSTEILMKKMKFFESNESDNDKSLLSS   298
M.brassicae      DSNSRSSKTIQAAFGSNSGIMRLDKGLLWRLFKTPLYKKLADSQEYLEKVSKEILMKRVTFFVHPDDNDNSLLGS.   295
S.littoralis     DPTSRSSKTIDAAFGSNCGIMKLDKGFMWKIFQTPVYKRLADSQTYLEKVSTDILYNRIHYFEQPEDGDISLLGS.   292
D.melanogaster   DPRSRSTRLMDAAETTNSCILPTDQGLQLWRFLETPSFRKLSQAQSYMESVALELVEENVRNGSVGSSLISAYVK.   277

H.armigera       FLQQPNLDLKDVVGMMVDILMAAIDTTAYTTSFALYHISRNPDIQQKMFDE.ILQLLPTKDTRISSEIVSKAVYVR   370
B.mori           FLQQPQLDHKDIMGMMVDILMAAIDTTAYTTSFVLYHIARNKRCQDEMFEE.LHTLLPKKDDEITADVLSKASYVR   377
M.sexta          FLQQPNIDQKDIIGMMVDILMAAIDTTSYTTSFALYHIARNPECQEKIFTE.VETLLPEKDSSITPNVISKAVYTR   373
M.brassicae      FLKQPNLDLKDVLGMMVDIIMAAIDTTAYTTSFALYHIGRNPEVQKKMYNE.ILALLPSKDAKISSDIVSKAIYVR   370
S.littoralis     FLKQPNVDLKDVIGVMVDILMAAIDTTAYSTSFALYHIGRNPEVQQKMFEE.ISTLLPTDDAKITPDILSKATYVR   367
D.melanogaster   ...NPELDRSDVVGTAADLLLAGIDTTSYASAFLLYHIARNPEVQQKLHEEARRVLPSAKDELSMDALRTDITYTR   350
                                     helix-I

H.armigera       SCVKESLRLNPVSIGVGRLTQKEFILRDYLIPKGTVIVTQNMVSSRLQQYVNDPLEFKPDRWLRGSASYENIHPFL   446
B.mori           SSIKESLRLNPVSIGIGRWLQKDIVLKGYSIPKGTVIVTQNMTSSRLPQFIRDPLTFKPERWMRGSPQYETIHPFL   453
M.sexta          SSIKESLRLNPVSVGIGRLLQKDITLKGYFIPKDTVIVTQNMVSCRLPQYVREPLLFKPERWLRGSSEYENIHPFL   449
M.brassicae      SCVKESLRLNPVSIGVGRLTQKDFVLRGYLIPEGTVIVTQNMLASRLPQYIKDPLKFKPERWLRGSEGHENIHPFL   446
S.littoralis     ACLKESLRLNPVSVGIGRLTQKDFVLRGYLIPEGTVIVTQNFVASRMPQYVKDPLKFKPERWIRDSESYENIHPFL   443
D.melanogaster   AVLKESLRLNPIAVGVGRILNQDAIFSGYFVPKGTTVVTQNMVACRLEQHFQDPLRFQPDRWLQ...HRSALNPYL   423
                    helix-k                                   PERF-motif

H.armigera       SLPFGFGPRSCIARRLAEQNICIILIRLIREYNLKWMGGEL..GVQTLLINKPDKPVSLSFTPRNL...         510
B.mori           SLPFGHGPRSCIARRLAEQNICIIIMRLIREFEIQWAGEEL..GVKTLLINKPNKPVSLNFIPRSS...         517
M.sexta          SLPFGFGPRSCIARRLAEQNMCIIIIRLIRDFQIQWKGREL..GIRTLLINKPDQPVTLSFVPRLPRRL         516
M.brassicae      SLPFGFGPRSCIARRLAEQNICIIILIRLIREFNIKWMGDEL..GIRTLLINKPDKPVSLSFTPRNE...        510
S.littoralis     SLPFGFGPRSCIARRLAEQNICIFLMRLIRDFNVTWMGEDL..GIKTLLINKPDKPVSLSFTSRIV...         507
D.melanogaster   VLPFGHGMRACIARRLAEQNMHILLLRLLREYELIWSGSDDEMGVKTLLINKPDAPVLIDLRLRRE...         489
                  heme-binding site
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Fig. 1. Alignment of selective CYP302A1 sequences using the Clustal W program. Identical residues are highlighted. Dots indicate gaps inserted for optimizing the alignment. The conserved motifs are underlined.

Quantitative real-time PCR (qPCR) analysis of gene expression

A sensitive quantitative analysis was performed to compare the expression profile of each Halloween gene in the PG and two main peripheral tissues (the MG and FT) during larval development. Three independent cDNA samples originating from different tissues of 5–10 animals for each time point were used. qPCR assays were designed and performed according to the MIQE guidelines (Bustin *et al.*, 2009), using SYBR premix Ex Taq II (Perfect Real Time) kit (Takara) on MX3005P machine (Strategene). The primers used for qPCR, amplifying a product with a size between 80 and 121 bp, were listed in table 1. The reaction of 20 µl in total volume contained 10 µl 2xSYBR premix Ex Taq™ II, 0.4 µl 50× ROX II and 0.4 µl each primer and 2 µl cDNA template using the cycling parameters: 95°C for 15 s, followed by 40 cycles of 95°C for 5 s, 56°C for 34 s and 68°C for 30 s, and a cycle of 95°C for 15 s and 60°C for 1 min. The melting curves of amplicons were measured by taking continuous fluorescence reading while increasing temperature from 56 to 95°C with 0.5°C increments for 10 s. Potential contamination was checked by including non-reverse transcriptase preparation and non-template controls. PCR fidelity was confirmed by checking the amplicon size and sequencing. PCR product homogeneity was evaluated by melting curve analysis. Amplification efficiency was calculated by serial dilutions from 1- to 1000-fold of each cDNA templates. mRNA levels of the target genes were normalized to *GAPDH* (encoding glyceraldehyde-3-phosphate dehydrogenase) after correcting for differences in amplification efficiency. In a pilot study, we considered several housekeeping genes encoding GAPDH, EF-1α, ribosomal protein L28, ribosomal protein S3 as the candidate reference genes based on a literature review, and found that *GAPDH* gene was stably expressed. Under the conditions described in this study, the amplification efficiency for each gene was between 102 and 108%, and the gene-specific PCR products were achieved. All qPCR analyses were performed with three independent biological replicates and three technical

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H.armigera       ...................MDFFFLWLVTFVAGFWIFKKIK..QWQSLPPGPWGLPVVGYLPFLDRHQPHRTLAK    54
B.mori           ...................MDLYFIWLVTFVAGFWIFKKIK..EWQNLPPGPWGLPIVGYLPFIDRYHPHITLTN    54
M.sexta          ...................MDLFFIWLVTFVAGFWICKKIK..EWQNLPPGPWGLPILGYLPFIDPRQPHITLTK    54
M.brassicae      ...................MDFFFLWLVTFVAGFWIFKKLK..QWQSLPPGPWGLPVVGYLPFLDRHQPHLTLTK    54
S.littoralis     ...................MDFFFLWLVTFVAGFWIFKKIK..EWQNLPPGPWGLPIVGYLPFLDRHQPHLTLTK    54
D.melanogaster   MSADIVDIGHTGWMPSVQSLSILLVPGALVLVILYLCERQCNDLMGAPPPGPWGLPFLGYLPFLDARAPHKSLQK    75
                                                                P/G rich

H.armigera       LSKEYGSIYGIGMGSVYAVVLSDHKLVRDAFAKENFSGRAPLYLTHGIMKGNGIICAEGGLWKDQRKLITTWLKS    129
B.mori           LSKTYGAIYGLKMGSIYAVVLSDHKLVRDAFSKDSFSGRAPLYLTHGLMNGNGIICAEGGLWRDQRKLITSWLKS    129
M.sexta          LSKLYGPIYGLKMGNIYAVVLSDHKLIRDAFAKENFSGRAPLYLTHGIMHGNGIICAEGGLWKDQRKLITTWLKS    129
M.brassicae      LAKQFGSIYGIGMGSVYAVVLSDCKLVREAFAKESFSGRAPLFLTHGIMKGNGIICAEGGLWKDQRKLITTWLKS    129
S.littoralis     LSKQYGPIYGIGMGSVYAVVLSDHKLIRDAFAKDNFSGRAPLYLTHGIMHGNGIICAEGSLWKDQRKLITMWLKS    129
D.melanogaster   LAKRYGGIFELKMGRVPTVVLSDAALVRDFFRRDVMTGRAPLYLTHGIMGGFGIICAQEDIWRHARRETIDWLKA    150
                                                                    helix-c

H.armigera       FGMSKHS.VSREKLEKRIASGVYELLENVE....KAAGSPMDLTHMLSNSLGNVVNEIIFGFKFPPEDKTWHWFR    199
B.mori           FGMSKHS.VSREKLEKRIASGVYEILENIE....KTSDAALDLPHMLTNSLGNVVNEIIFGFKFPPEDKTWQWFR    199
M.sexta          FGMSKHS.ALRDKLEKRIASGVYELLDSIE....KSPKEPIDLPHMVSNSLGNVVNEIIFGFKFPSDDKTWQWLR    199
M.brassicae      FGMSKHS.VSREKLEKRIASGVYELLENVE....KAAGSPMDLSQMLSNSLGNVVNEIIFGFKFPPEDKTWHWFR    199
S.littoralis     FGMSKHS.VSREKLEKRIASGVYELLENVE....KSEGSPMDLSHMLTNSLGNVVNEIIFGSKFPPDDKTWNWFR    199
D.melanogaster   LGMTRRPGELRARLERRIARGVDECVRLFDTEAKKSCASEVNPLPALHHSLGNIINDLVFGITYKRDDPDWLYLQ    225

H.armigera       QIQEEGCHEMGVAGVVNFLPFIRFISSSTQKTIEVLTRGQAQTHRLYASIIDRRRKIIGLPPIKEAAYAEHKNLF    274
B.mori           QIQEEGCHEMGVAGVVNFLPFIRHVSPSTRKTIEVLVRGQAQTHTLYASMIDRRRKMLGLEKPKGAEYAPHENLL    274
M.sexta          QIQEEGCHEMGVAGVVNFLPFVRFFSPTIRKTIQMLIRGQAQTHTLYASVIARRRNMLGLEKPKGAEHLPHSNLF    274
M.brassicae      QIQEEGCHEMGVAGVVNFLPFVRFISSSTQKTMEVLTRGQAQTHRLYASIINRRRKIIGLPPIKEAAYPPHDNLF    274
S.littoralis     QIQEEGCHEMGVAGVVNFLPFIRFISASTRKTIEVLTRGQAQTHRLYASIIDRRRKVLGLAPVKEAAYPLHENLF    274
D.melanogaster   RLQEEGVKLIGVSGVVNFLPWLRHLPANVRN.IRFLLEGKAKTHAIYDRIVEACGQRLKEKQKVFKELQEQKRLQ    299

H.armigera       TEHPEGHIKCIKYSKHASNT...EEHLFDPNILIPTDGECVLDNFLLEQKRRFESGDESARYVTDEQMLYLLADM    346
B.mori           KLYPNGHIKCIKYSKVSPN....TEHFFDPNTLIPTEGDCILDNFLLEQKKRFESGDPTALYMRDEQLHFLLADM    345
M.sexta          NEYPDGKLKCIKYSKHAPN....TEHIFDPKTLIPTEGECILDSFLLEQKKRFENGDETARYMTDEQLHFLLADM    345
M.brassicae      SEHPEGHMKCIKYSKHASNT...EEHFFDPNILIPTDGECILDNFLLEQKRRFETGDEGTKYVTDEQMLYLLADM    346
S.littoralis     NEHPDGHIKCIKYSKHASEH...EEHYFDPNILIPTDGECILDNFLLEQKRRYESGDEGAKYMTDEQMLYLLADM    346
D.melanogaster   RQLEKEQLRQSKEADPSQEQSEADEDDEESDEEDTYEPECILEHFLAVR.......DTDSQLYCDDQLRHLLADL    367

H.armigera       FGAGLDTTSVTLAWFLLYMALYPEEQELVRKEILSVYPEDGEVDSSRLPHLMAAICETQRIRSIVPVGIPHGCIE    421
B.mori           FGAGLDTTSVTLAWFLLYMALFPEEQEEIRKEILSVYPYDDDVDSSRLPLLMAAICETQRIRSIVPVGIPHGCIE    420
M.sexta          FGAGLDTTSVTLAWFLLYMALYPDEQEIVRKEILSEYPEECEVESSRLPRLMAAIYETQRIRSIVPVGIPHGCLE    420
M.brassicae      FGAGLDTTSVTLAWFLLYMALYPEEQELVREEIVSVYPEDAEVDGSRLPHLMAAICETQRIRSIVPVGIPHGCVE    421
S.littoralis     FGAGLDTTSVTLSWFLLYMALYPEEQEIVRKEILSVYPEDGEVDGSRLPHLMAAICETQRIRSIVPVGIPHGCLQ    421
D.melanogaster   FGAGVDTSLATLRWFLLYLAREQRCQRRLHELLLPLGPSPTLEELEPLAYLRACISETMRIRSVVPLGIPHGCKE    442
                   helix-I                                               helix-k

H.armigera       DTYLGNYRIPKGTMVIPLQWAIHMDPDVWENPDEFRPSRFLGSDGSLLKPPEFIPFQTGKRMCPGDELSRMLACG    496
B.mori           DAYLGNYRIPKNAMVIPLQWAIHMDPNVWEEPEKFKPRRFLAQDGSLLKPQEFIPFQTGKRMCPGDELSRMLSCG    495
M.sexta          DTYLGNYKIPKGAMVIPLQWALHMDPDVWEDSEEFKPSRFLAPDGSLLKPQEFIPFQTGKRMCPGDELSRMLASG    495
M.brassicae      DTYLGNYRVPKGAMVIPLQWAMHMDPDVWENPEEFRPSRFLGPDGSLLKPQEFIPFQTGKRMCPGDELSRMLACG    496
S.littoralis     DTFIGNYRIPKGAMVIPLQWALHMDPDVWEDPEVFRPQRFLAEDGSLLKPQEFIPFQTGKRMCPGDELSRMLACG    496
D.melanogaster   NFVVGDYFIKGGSMIVCSEWAIHMDPVAFPEPEEFRPERFLTADGAYQAPPQFIPFSSGYRMCPGEEMARMILTL    517
                                                  PERF-motif        heme-binding site

H.armigera       LVARLFRRRRVRLASDPPSEEEMKGTVGVTLTPPTVSYYCDPI................    539
B.mori           LVSRLFRKQRIRLASKIPTAEEMRGTVGVTLAPPPVKYYCEPI................    538
M.sexta          FIARLFRRKRVRLAS.IPSQEDMQGTVGVTLAPPRVQYFCDPI................    537
M.brassicae      LVARLFRRRRVRLASDRPSEEEMRGTIGLTLCPPRVSFYCDSV................    539
S.littoralis     LVARLFRRRRVRLATDPPSTKDMQGTVGVTLSPPTVSYYCDPL................    539
D.melanogaster   FTGRILRRFHLELPS..GTEVDMAGESGITLTPTPHMLRFTKLPAVEMRHAPDGAVVQD    574
```

Fig. 2. Alignment of selective CYP306A1 sequences using the Clustal W program. Identical residues are highlighted. Dots indicate gaps inserted for optimizing the alignment. The conserved motifs are underlined.

replicates. The relative expression levels of target genes were calculated by the comparative C_t method as described by Livak & Schmittgen (2001). Gene expression data (relative expression) were statistically analyzed by the analysis of variance (ANOVA) followed by Tukey's HSD multiple comparisons for mean separation among different tissues using SPSS for Windows (PASW 18, Chicago, IL, USA). A *P*-value of <0.05 was considered significant.

Results

Cloning and identification of Halloween P450 homologs from H. armigera

Full-length transcripts of four Halloween P450 orthologs from *H. armigera*, namely *HarmCYP302A1* (GenBank number: KP764853), *HarmCYP306A1* (KP764854), *HarmCYP314* (KP764856) and *HarmCYP315A1* (KP764855) respectively,

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H.armigera      MSLPGVFLFSHYVESFWSTPPPLVDWSYVPTIVLAVILVVVAATALAAR.AADGKQ..ATRLPGPQALPFLGTRW...   72
B.mori          MSLPGVFLFSHYVESFWSTSPPLLDWSCVPTIVLAVIAVVVAVTALLTR.TSDAKH..SCRLPGPQPLPFLGTRW...   72
M.sexta         MSLPGVFLFSYYMKNVWSTPTPLLDWSCVPTIVLIVALVVVTVTAVLTR.AVDTKN..PSRLPGPQPLPFLGTRW...   72
M.brassicae     MSLPGAFLFSHYVESFWGTPPPLVDWSYVPTIVLAVILVLVAATALAAR.AADGKQ..STRLPGPQALPFLGTRW...   72
S.littoralis    MSLPGVFLFSHYVESFWVAPPPLVDWTCIPTIVLVVLLVLVVASALAARAMADVRP..VERLPGPLPLPFVGTRW...   73
D.melanogaster  MAVILLLALALVLVCYCALHRHKLADIYLRPILKNTLLEDFYHAELIQFEAPKRRRRGIWDIPGPKRIPFLGTKWIFL   78
                                                                              P/G rich

H.armigera      LFWGRYKMNKLHEAYEDMFRRYGPVFVETTPGGASVVSIAERAALEAVIRAPAKRPYRPPTEIVQVYRRSRPDRYAST  150
B.mori          LFWSRYKMNKLHEAYADMFKRYGPVFMETTPGGVAVVSIAERTALEAVIRSPAKKPYRPPTEIVQMYRRSRPDRYAST  150
M.sexta         LFWSRYKMNKLHEAYEDMFRRYGPVFAETTPGGATIVSIADREALDAVIKAPAKRPYRPPTEIVQVYRRSRPDRYAST  150
M.brassicae     LFWSRYKMNKLHEAYEDMFRRYGPVFVETTPGGASVVSIAERAALEAVIRAPAKRPYRPPTEIVQVYRRSRPDRYAST  150
S.littoralis    LFWSRYKMNKLHEAYEDMFRRYGLVFAEIHPGGATMVSIAEREALEAVIRAPSRRPYRPPTEIVQVYRRSRPDRYAST  151
D.melanogaster  LFFRRYKMTKLHEVYADLNRQYGDIVLEVMPSNVPIVHLYNRDDLEKVLKYPSKYPFRPPTEIIVMYRQSRPDRYASV  156

H.armigera      GLVNEQGEKWHHLRRHLTAELTSPNTMQGFLPELNNICDDFLELLESCRRSDGTVAG.FDQLTNRMGLESVCGLMLGS  227
B.mori          GLVNEQGEKWYHLRRNLTTDLTSPHTMQNFLPQLNTISDDFLELLNTSRQSDGTVYA.FEQLTNRMGLESVCGLMLGS  227
M.sexta         GLVNEQGDKWLHLRRHLTVELTSPNTMQGFMPELNSICEDFLDLLQSSRQANGTVHG.FDQLTNRMGLESVCGLMLGS  227
M.brassicae     GLVNEQGEKWHHLRRHLTAELTSPNTMQGFLPELNNICDDFLVLLDSCRRSDGTVAG.FDQLTNRMGLESVCGLMLGS  227
S.littoralis    GLVNEQGEKWYHLRRHLTAELTSPSTMQGFLPELNTICDDFLELVNTTRRADGIVPG.FDQLTNRMGLESVCALMLGS  228
D.melanogaster  GIVNEQGPMWQRLRSSLTSSITSPRVLQNFLPALNAVCDDFTELLRARRDPDTLVVPNFEELANLMGLEAVCTLMLGR  234
                          helix-c

H.armigera      RLGFLERWMSG..RAATLAAAVKAHFRAQRDSYYGAPLWKFAPTTLYRTFVKSEETIHTIVSELMEEAKTKKQGTAND  303
B.mori          RLGFLERWMSG..RAMALAAAVKNHFRAQRDSYYGAPLWKFAPTALYKTFVKSEETIHAIVTELMEEAKSKTTGMAQD  303
M.sexta         RLGFLERRMSG..RAATLAAAVKTHFRAQRDSYYGAPLWKFAPTTLYKTFVRSEETIHTVVSELMEEAKARTNGSAKD  303
M.brassicae     RLGFLERWMSG..RAATLAAAVKAHFRAQRDSYYGAPLWKFAPTTLYRTFVKSEETIHTIVSELMEEAKNRSQGTAND  303
S.littoralis    RLGFLERWMSG..RAATLASAVKAHFRAQRDSFYGAPLWKFAPTTLYRTFAKSEDTIHTIVSDLMEEAKLKTQKNASD  304
D.melanogaster  RMGFLAIDTKQPQKISQLAAAVKQLFISQRDSYYGLGLWKYFPTKTYRDFARAEDLIYDVISEIIDHELEELKKSAAC  312

H.armigera      D.....AMREIFLRIILENPAVDMRDKKAAVIDFITAGIETLANSLVFLLYLLSGRPDWQRVIRSELPSC..STLTAED  374
B.mori          E.....AIQEIFLKIILENPALDMRDKKAAIIDFITAGIETLANSLVFLLYLLSGRPDWQRKINSELPPY..AMLCSED  374
M.sexta         D.....GMQEIFLRIILENPALDMRDKKAAVIDFITAGIETLANSLVFLLYLLSGRPDWQHTIRSELPSC..SRLSADD  374
M.brassicae     D.....AMQEIFLRIILENPALDMRDKKAAVIDFITAGIETLANSLVFLLYLLRGRPDWQRTIRSELPSC..STLSAED  374
S.littoralis    E.....AMREIFMRIILENPALDMRDKKAAVIDFITAGIETLANSLVFLLYLLSVRPDWQRTIRSELPSC..STLTVED  375
D.melanogaster  EDDEAAGLRSIFINIILELKDLDIRDKKSAIIDFIAAGIETLANTLLFVLSSVTGDPGAMPRIISEFCEYRDTNILQDA  390
                                                         helix-I

H.armigera      LAAAPSVRAAIYEAFRLLPTAPFLARVLDTPMTVGGHKLPAGTFVLAHTAAACRREENFWRATEYLPERWISMRQP..  450
B.mori          LAGAPSVRAAINEAFRLLPTAPFLARLLDSPMTTGGHKIPPGTFVLAHTAAACRREENFWRAEEYLPERWIKVQEP..  450
M.sexta         LSAAPSVRAAINEAFRLLPTAPFLARLLDTPMTLGGHKLPAGTFVLAHTGAACRREENFWRAHEFLPERWSSSRQP..  450
M.brassicae     LAAAPSVRAAIYEAFRLLPTAPFLARLLDSPMTIAGHKLPAGTFILAHTGAACRREENFWRAREFIPERWVKPTAP..  450
S.littoralis    LAAAPSVRAAISEAFRLLPTAPFLARLLETPMVIAGHKLPAGTFVLAHTGAACRRDENFWRAREYLPERWLEPRAP..  451
D.melanogaster  LTNATYTKACIQESYRLRPTAFCLARILEEDMELSGYSLNAGTVVLCQNMIACHKDSNFQGAKQFTPERWIDPATENF  468
                            helix-k                                             PERF-motif

H.armigera      ....HAASLVAPFGRGRRMCPGKRFVELELHLLLAKIMQRWRVEFDGELDVQFDFLLSAKSPVCLRLVEW..        516
B.mori          ....HAYSLVAPFGRGRRMCPGKRFVELELHLLLAKIMQKWRVEFDGELDIQFDFLLSAKSPVTLRLVEW..        516
M.sexta         ....HAASLVAPFGRGRRMCPGKRFVELELHLLLAKIMQRWRVEFDGELDVQFDFLLSPKSPVTLRLVEW..        516
M.brassicae     ....HAAALVAPFGRGRRMCPGKRFVDLELHLLLAKIMQKWRVEFDGELDIQFDFLLAPKSPVSLRLVEW..        516
S.littoralis    ....HAAALVAPFGRGRRMCPGKRFVDLELHLLLAKIMQRWRVEFDGELDIQFDFLLSPKSPVSLRLVEW..        517
D.melanogaster  TVNVDNASIVVPFGVGRRSCPGKRFVEMEVVLLLAKMVLAFDVSFVKPLETEFEFLLAPKTPLSLRLSDRVF        540
                           heme-binding site
```

Fig. 3. Alignment of selective CYP314A1 sequences using the Clustal W program. Identical residues are highlighted. Dots indicate gaps inserted for optimizing the alignment. The conserved motifs are underlined. Two positively charged residues, a signature of mitochondrial enzymes, are boxed.

were obtained. Overall, these transcripts encode proteins with a size consistent with the character of cytochrome P450s. They possessed the conserved structural attributes of many P450s (Feyereisen, 2012), for example the WxxxR motif in Helix-C, the GxE/DTT/S in Helix-I, the ExLR in Helix-K and the heme-binding domain (PFxxGxRxCxG/A) (figs 1–4). Although the PERF motif (PxxFxPE/DRF/W) was easily discernible in HarmCYP302A1, this motif in other three proteins was not very typical. The PERF motif in HarmCYP306A1 was modified by a substitution of *E/D* with *S*, while only PERW and PYRW were visible in HarmCYP314A1 and HarmCYP315A1, respectively. In addition, the N-terminals of HarmCYP302A1 and HarmCYP315A1 carried the distinctive mitochondrial import sequence consisting of several charged residues. HarmCYP306A1 contained the microsomal P450 characteristic N-terminal string consisting of hydrophobic residues followed by a proline–glycine (P/G)-rich region (figs 1–4). The two to three positively charged residues interacting with adrenodoxin (Feyereisen, 2012), a signature of mitochondrial enzymes, were observed in HarmCYP302A1, HarmCYP314A1 and CYP315A1. Additional two positively charged residues near the heme-binding site were found in HarmCYP302A1, HarmCYP314A1 and CYP315A1. Notably, HarmCYP314A1 has a P/G-rich region and lack some of the positively charged residues, indicating that HarmCYP314A1 can be both microsomal and mitochondrial.

The identified Halloween P450 genes are highly conserved in Lepidoptera. *Helicoverpa armigera* orthologs show 66–80% (CYP302A1), 80–92% (CYP306A1), 85–93% (CYP314A1) and 58–80% (CYP315A1) identity to lepidopteran orthologs (tables S1 and S2). On the other hand, the identity between *H. armigera*

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H.armigera        .....................MQRINTTPFLK..LLSYSKM..CLSQNACKTTLSIEDMPRPKSLPIIGTKLEFIAA   52
B.mori            .....................MHRFFSMSSIRSAVRSRNSNRCSMSTKFHKSLRTIDEMPHKKSLPIIGTKFDLFSA   56
M.sexta           .....................MHRMTRLLSKQQKLLIFQRN..AASAECIRTDLTINEMPHPKSMPILGTKLEFFAA   54
M.brassicae       .....................MQRNQGVPFLK..LLKFNK...CLSHNASKTPLSIEHMPRPKSLPLLGTKLEFIVA   51
S.littoralis      .....................MICNRNKALFK..LIKFNK...NLSYDASPSPKSIEFMPRPKSLPIVGTKIDFLAA   51
D.melanogaster    MTEKRERPGPLRWLRHLLDQLLVRILSLSLFRSRCDPPPLQRFPATELPPAVAAKYVPIPRVKGLPVVGTLVDLIAA   77

H.armigera        GSGTKLHEYADRRHKQLGPLYCEKLGGNTDLVFVSDPMLIRSLFINLEGKYPVHILFEPWVLYEKLYGSKRGLFFMN  129
B.mori            GGGKNLHKYIDMRHKQLGPIFYERLTGKTKLVFISDPTHMKSLFLNLEGKYPAHILFEPWVLYEKLYGSKRGLFFMD  133
M.sexta           GGGKKLHEYIDNRHKQLGSIFCENLGGSADLVFISDPTLMKTLFLNLEGKYPAHILFDPWILYEKLYGSKRGLFFMN  131
M.brassicae       GSGTKLHEYADSRHKQLGSIYCEKLGGNTELVFVSDPTLIKTLLLNLEGKYPVHILFEPWEVYEKLYGSKRGLFFMN  128
S.littoralis      GGGSKLHEYVDFRHKQLGPIFCDKLGGRIDLVFVSDPALIKTLFLNLEGKYPIHILFEPWELYEKLYGAKRGLFFMN  128
D.melanogaster    GGATHLHKYIDARHKQYGPIFRERLGGTQDAVFVSSANLMRGVFQ.HEGQYPQHPLPDAWTLYNQQHACQRGLFFME  153

H.armigera        GDEWLNNRRVMNKYLLKENCENWFEIPVKKTVNNLIQRWKIKTEKGQFTPDLET.............EFYKFSIDVI  193
B.mori            GEDWLINRRIMNKHLLREDSDVWLRAPIRTAVFHFICNWKLRAQSGNFSPNLES.............EFYRFSTDVI  197
M.sexta           GEEWLNNRRIMNKHLLKEDSEKWLNDPVKATIKSFINNWKTRAEQGNFIPDLET.............EFYRLSTDVI  195
M.brassicae       GNEWLENRRIMNKFLLKENSVNCFEIPVRNTVNNLIQKWKIKMENGTFVPNLES.............EFYRLSIDVI  192
S.littoralis      GEEWLENRRVVNKHLLKENSEKLFDNPVTNTINQLVQKWIIEAKKGFYVPNLET.............EFYRLSIDVI  192
D.melanogaster    GAEWLHNRRIINRLLLNGN.LNWMDVHIESCTRRMVDQWKRRTAEAAAIPLAESGEIRSYELPILEQQLYRWSIEVL  229
                     helix-c

H.armigera        IAVMLGSSSSIQRDRHYEALLDMFSETVKKIFQTTTKLYGLPVDICQKINLKVWREFKESVDGSLSLAHKIVGEILM  270
B.mori            LAVLQGNSALLKPTPEYEMLLLLFSEAVKKIFSTTTKLYALPVEFCQRWNLKVWRNFKQSVDDSISIAQKIVYEMLH  274
M.sexta           IAILLGSNSSIKTSKQYEMLLCMFSESVKNIFQTTTKLYALPVTWCQRINLKVWRDFKECVDMSLFLAHKIVTEILN  272
M.brassicae       LAVMLGSSTSLKPDKHYDALLTMFSETVKKIFQTTTKLYGLPVNVCQKINLKIWRDFKESVDISLSLVHKIVGEMLL  269
S.littoralis      ISVMLGSSIFHKFSVHSDALLTAFAEEVKKIFQTTTKLYGWPVNMCQKMNLKVWRDFKKSVDISLFLANKIVEEMIN  269
D.melanogaster    CCIMFGTS..VLTCPKIQSSLDYFTQIVHKVFEHSSRLMTFPPRLAQIIRLPIWRDFEANVDEVLREGAAIIDHCIR  304

H.armigera        NSKD.....GDGLINLLVKENVKPDDIKRIIGDFVIAAGDTTSYTSIWTLFLLSRNEKARE....ELYQRDS.YINH  337
B.mori            TKDA.....GDGLVKRLKDENMSDELITRIVADFVIAAGDTTAYTSLWILFLLSKNTEILT....EMNDNDQ.YVKN  341
M.sexta           RRHE.....NDGLIKRLCEDKMSDEDIIRIVADFVIAAGDTTAYTSLWTLLLMAKNKDYVNN...ELPMKDINNIKH  341
M.brassicae       QTKD.....SNGLINLLSEEKMKQEDIKRIIGDFVIAAGDTTSYTSIWTLLLLSRNESVKQ....ELYSRNS.YINY  336
S.littoralis      NKQP.....KDGLINLLIEENLKPEIIKRIIVDFVIAAGDTTSYTTIWTLYLLSKNKDVRQ....ELFKRNS.IANY  336
D.melanogaster    VQEDQRRPHDEALYHRLQAADVPGDMIKRIFVDIVIAAGDTTAFSSQWALFALSKEPRLQQRLAKERATNDSRLMHG  381
                                                          helix-I

H.armigera        VIKESMRLYPVAPFLTRILPKESIFGNYKLSKGTPIIASIYTSGRDEQNFSRANEYILPYRWDRNDARKNELVNHVTS  414
B.mori            VVKEAMRLYPVAPFLTRILPKQCVLGPYLLEEGTPVIASIYTSGRDEQNFSKADQFILPYRWDRNDQRKKDLVNHVPS  418
M.sexta           VVKEAMRLYPVAPFLTRILPKESILGPYKLNEGTPVIASIYTSGRDINNFSRPEEFILPYRWDRNDPRKKELMNHNPS  418
M.brassicae       VIKESMRLYPVAPFLTRILPKECIFGPYKLNKGTPIIASIYTSGRDEQYFSKADEYILPYRWDRNDARRKELVNHVSS  413
S.littoralis      AIKESMRLYPVAPFLTRILPKECIFGPYKLKGGTPIIVSIYTSGRDEQYFSRATEYILPYRWDRMDIRRNDIVNHVSS  413
D.melanogaster    LIKESLRLYPVAPFIGRYLPQDAQLGGHFIEKDTMVLLSLYTAGRDPSHFEQPERVLPERWCIGETEQ....VHKSH  454
                     helix-k                                          PERF-motif

H.armigera        ASIPFALGARSCIGKKLAMLQMTELISEIVQNFEFECLNKDEVTANTSQVLVPSRDIQLSFRLRQPEK........  482
B.mori            ATIPFAFGARSCIGKKMAMLQMTELISQIVKNFDLKSMNNSDVDAVTSQVLVPNKDIKVLILPRSISK........  486
M.sexta           ASIPFALGSRSCIGKKIAMLQITELMSQIVKNFHLECLNKTPVNILTSQVLVPDKNIDIQVSLYDSSKLNKNECWL  494
M.brassicae       ASIPFALGARSCIGKKIAMLQMKELINQIVQNFEFECLNKDEINANTSQVLVPDKDIKLAFTLRKPEMRQ......  483
S.littoralis      ASIPFALGARSCIGKKLAMLQMKELITQMVQNFEFECINKDEVTSKTSQVLVPSQK....................  469
D.melanogaster    GSIPFAIGQRSCIGRRVALKQLHSLIGRCTAQFEMSCLNEMPVDSVLRMVTVPDQTIRLALRPRTE..........  520
                     heme-binding site
```

Fig. 4. Alignment of selective CYP315A1 sequences using the Clustal W program. Identical residues are highlighted. Dots indicate gaps inserted for optimizing the alignment. The conserved motifs are underlined.

and insects of other orders were much lower (<46%) (tables S1 and S2).

A phylogenetic analysis was performed using the putative amino acid sequences of Halloween P450 genes available in GenBank. The phylogenetic tree clearly clustered into two groups, the CYP2 clan (*CYP306A1*) and the mitochondrial clan (*CYP302A1*, *CYP314A1* and *CYP315A1*) (fig. 5).

General spatial and temporal patterns of H. armigera *Halloween P450 genes by RT–PCR*

The general spatial and temporal expressions of the four Halloween genes were determined using the RT–PCR approach with the *EF-α* as a reference. Using templates without the addition of reverse transcriptase, no amplification was observed (data not shown). Transcripts of all the four genes were detected during all stages of development (fig. 6).

HarmCYP302A1 and HarmCYP314A1 were detected in all the selected tissues of both larvae and adults. HarmCYP306A1 was detected in all the selected tissues except that its expression in adult testis was below the reliable detection level. Notably, HarmCYP315A1 was expressed specifically in egg, larval PGs, pupae and adult ovaries.

Spatial and temporal patterns of H. armigera *Halloween P450 genes in the PG and peripheral tissues during larval development by qPCR*

A more sensitive quantitative analysis was performed to compare the expression profile of each Halloween gene in the endocrine tissue (PGs) and two main peripheral tissues (the MG and FT) during the fifth larval instar (fig. 7) and the final instar (fig. 8). HarmCYP302A1 was predominantly expressed in PG at both 5th instar (L5) and the final (6th) instar larvae (L6), and the expression level in PG in L5 is higher than in L6 in PG. Furthermore, developmental variation in the expression of CYP302A1 in PG was obvious. There was an increase from day 0 to day 1 in L5 and then a sharp reduction

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Fig. 5. Phylogenetic tree of the four Halloween P450 genes generated using the neighbor-joining method performed with the amino acid sequences by Mega 6. The scale bar represents the evolutionary distances computed using the Poisson correction method. The information (the full name of animal species and the GenBank access number of the used sequences) was listed in table S1.

Fig. 6. Expression profile of Halloween genes in various tissues at different developmental stages. *EF-α* = the elongation factor-1α; HD, head; EP, epidermis; PG, prothoracid gland; MG, midgut; MT, Malpighian tubules; FT, fat body; MP, male pupae; FP, female pupae; AT, antenna; LG, leg; TS, testes; OV, ovary.

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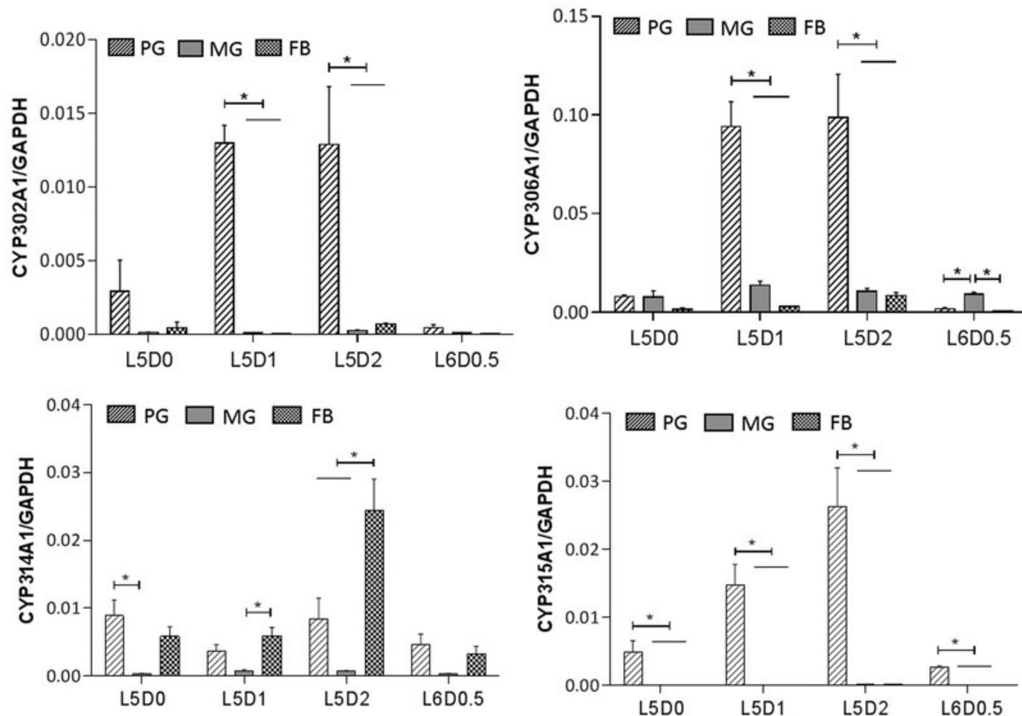

Fig. 7. Developmental expression profile of four Halloween P450 genes of *Helicoverpa armigera* in three tissues during 5–6th instar larval transitions. The expression levels were measured by qPCR. Each datum point represents the mean ± SEM of the expression level relative to *GAPHD*. The relative expression levels were compared among three tissues at each time point. Significant difference is marked with * ($P < 0.05$). PG, prothoracic glands; MG, midguts; FB, fat bodies; L, larval stage; D, day.

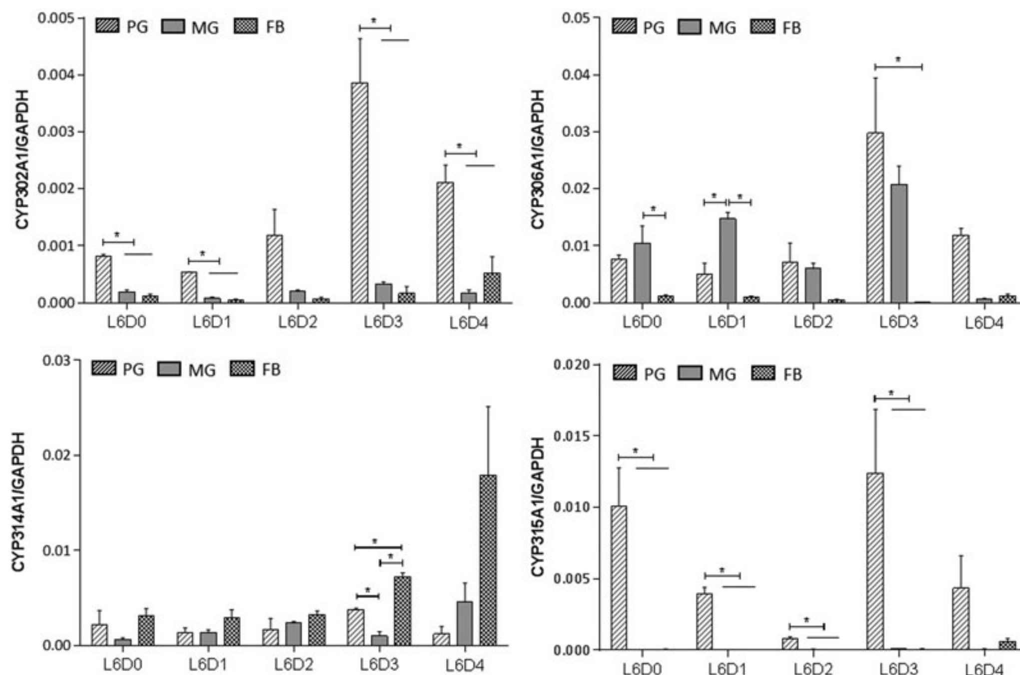

Fig. 8. Developmental expression profile of four Halloween genes in three tissues of 6th larvae of *Helicoverpa armigera*. The expression levels were measured by qPCR. Each datum point represents the mean ± SEM of the expression level relative to *GAPHD*. The relative expression levels were compared among three tissues at each time point. Significant difference is marked with *($P < 0.05$). PG, prothoracic glands; MG, midguts; FB, fat bodies. L, larval stage; D, day.

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from day 2 in L5 to L6 (fig. 7), and an increase later on at day 3 in L6 again (fig. 8). For HarmCYP306A1, higher expression was observed in PG at day 1 and day 2 in L5, while the comparable level of the expression was detected between PG and MG except at day 1 in L6. In addition, the maximal expression level of HarmCYP306A1 was lower in L6 than L5 (L5D2/L6D3 = 3.3-fold). The lowest abundance of HarmCYP314A1 was observed in MG, while the HarmCYP314A1 levels were comparable between PG and FB in L5 except at day 2 (the highest expression was detected in FB). Overall there was no significant difference in the HarmCYP314A1 level among the three selected tissues except at day 3 in L6. HarmCYP315A1 was predominantly expressed in PG of both the 5th instar and the final instar larvae.

Although HarmCYP315A1 and CYP302A1 were dominantly expressed in PG, their expression were detectable in MG and FB by the very sensitive qPCR measurement, with an abundance being much lower compared with that in PG (figs 7 and 8). For example, the relative expression levels of CYP302A1 in MG and FB to that in PG were 5.6×10^{-3} and 2.7×10^{-3}, respectively, while the abundance of HarmCYP315A1 in PG was about 1000-fold higher than that in MG or FB at day 1 in L5 (fig. 7).

Discussion

Here, by using RT–PCR with degenerate primers and RACE approaches, four Halloween P450 genes (*HarmCYP302A1*, *HarmCYP306A1*, *HarmCYP314A1* and *HarmCYP315A1*) putatively encoding enzymes involved in the final hydroxylation steps in the synthesis of ecdysone from the cotton bollworm *H. armigera* were identified. The proteins encoded by these four genes possess typical P450 motifs, and exhibit high similarity to those previously identified from other lepidopteran insects (table S2).

Previous studies showed that the expression of ecdysteroidonenic genes, including *CYP302A1*, *CYP306A1* and *CYP315A1* is restricted to the PG cells at the larval stage in *Drosophila*, *Manduca* and *Bombyx* (Chavez *et al.*, 2000; Warren *et al.*, 2002, 2004; Namiki *et al.*, 2005; Niwa *et al.*, 2005; 2010; Ono *et al.*, 2006; Yoshiyama *et al.*, 2006; Rewitz *et al.*, 2006*a*, *b*). More recently, accumulated evidence suggests that PG is not the only organ for ecdysteroid biosynthesis. Peripheral tissues such as MG and FB have been shown to convert the ketodiol into 20E (Brown *et al.*, 2009). For example, *CYP306A1* was also expressed in MG besides PG in the larvae of *Spodoptera littoralis* (Iga & Smagghe, 2010). Our data showed that *HarmCYP302A1*, *HarmCYP306A1* and *HarmCYP314A1* transcripts were detected in many tissues of larvae (fig. 6). Real-time PCR results show that *HarmCYP302A1* and *HarmCYP315A1* were predominantly expressed in PG. However, this predominance was not observed for *HarmCYP306A1* and *CYP314A1*. For example, the highest expression of *HarmCYP314A1* was detected in FB of larvae (at day 2 in L5, day 3 in L6 (figs 7 and 8), similar to the pattern observed in *S. littoralis* (Iga & Smagghe, 2010)). This work provides another case demonstrating the expression of Halloween P450 genes in non-endocrine tissues. Whether these tissues can serve as secondary sources of primary or secondary ecdysteroids deserves further investigation. High expression of *HarmCYP314A1* in FB (figs 7 and 8) suggests that FB plays a crucial role in the last step of steroid synthesis, i.e. transforms E into the more active 20E.

Steroid hormones are involved in regulating biological processes such as germline development and innate immunity in the adult stage of the ecdysozoan animals, therefore steroid hormones must be synthesized in this stage (Niwa & Niwa, 2014). In the case of *H. armigera*, the four P450s were all expressed in the ovaries, suggesting that the ovaries may be the site of ecdysteroid synthesis in female adults. This observation is in keeping with the generally accepted view that ecdysteroid production is taken over by the gonads in adults (Dubrovsky, 2005). Interestingly, except *HarmCYP315A1*, the expressions of other three P450s were detectable in non-endocrine tissues (e.g. MGs and legs) besides the ovaries in adult females.

In summary, the sequences and expression profiles of four Halloween genes in the cotton bollworm *H. armigera* were described in this paper. All the four P450 genes are evolutionally conserved in Lepidoptera. Overall, these genes are predominantly expressed in PGs, and also detectable in non-endocrine tissues. High expression of HarmCYP314A1 in FB indicates that FB plays important roles in the conversion of E into 20E in larval–larval molt and in larva–pupal metamorhphosis. Further functional studies of these Halloween P450 orthologs are underway in our laboratory.

Supplementary Material

The supplementary material for this article can be found at http://dx.doi.org/10.1017/S0007485316000663

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