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CYP18A1* regulates tissue-specific steroid hormone inactivation in *Bombyx mori

Zhiqian Li^{a,b}, Xie Ge^{a,b}, Lin Ling^a, Baosheng Zeng^{a,b}, Jun Xu^{a,b}, Abu F.M. Aslam^a, Lang You^a, Subba Reddy Palli^c, Yongping Huang^{a,*}, and Anjiang Tan^{a,**}

^aKey Laboratory of Insect Developmental and Evolutionary Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China

^bUniversity of Chinese Academy of Sciences, Beijing 100049, China

^cDepartment of Entomology, College of Agriculture, S-225 Agriculture Science Center North, University of Kentucky, Lexington, KY 40546, USA

Abstract

Insect development and metamorphosis are regulated by two major hormones, juvenile hormone and ecdysteroids. Despite being the key regulator of insect developmental transitions, the metabolic pathway of the primary steroid hormone, 20-hydroxyecdysone (20E), especially its inactivation pathway, is still not completely elucidated. A cytochrome P450 enzyme, *CYP18A1*, has been shown to play key roles in insect steroid hormone inactivation through 26-hydroxylation. Here, we identified two *CYP18* (*BmCYP18A1* and *BmCYP18B1*) orthologs in the lepidopteran model insect, *Bombyx mori*. Interestingly, *BmCYP18A1* gene is predominantly expressed in the middle silk gland (MSG) while *BmCYP18B1* expresses ubiquitously in *B. mori*. *BmCYP18A1* is induced by 20E *in vitro*, suggesting its role in 20E metabolism. Using the binary Gal4/UAS transgenic system, we ectopically overexpressed *BmCYP18A1* in a MSG-specific manner with a *Sericin1-Gal4* (*Ser-Gal4*) driver or in a ubiquitous manner with an *Actin3-Gal4* (*A3-Gal4*) driver. Ectopic overexpression of *BmCYP18A1* in MSG or in all tissues resulted in developmental arrestment of transgenic animals during the final instar larval stage. The 20E titers in the transgenic animals expressing *BmCYP18A1* were lower compared to the levels in the control animals. Although the biological significance of MSG-specific expression of *BmCYP18A1* is unclear, our results provide the first evidence that *BmCYP18A1*, which is conserved in most arthropods, is involved in a tissue-specific steroid hormone inactivation in *B. mori*.

Keywords

20-Hydroxyecdysone; *CYP18A1*; Middle silk gland; *Bombyx mori*; Gal4/UAS

*Corresponding author. Tel.: +86 2154924047; fax: +86 2154924015. **Corresponding author. Tel.: +86 2154924046; fax: +86 2154924015.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ibmb.2014.08.007>.

1. Introduction

Insect growth and development are controlled by two major hormones, juvenile hormones and ecdysteroids (Delanoue et al., 2010; Kozlova and Thummel, 2003; Riddiford et al., 2010). The titers of both hormones are precisely coordinated by biosynthesis and metabolism pathways and the levels of these hormones are tightly correlated with their regulation of physiological and developmental processes. 20-Hydroxyecdysone (20E) is the most active form of ecdysteroids, which plays key roles in regulation of developmental processes including eversion of imaginal discs (Mirth et al., 2009), remodeling of fat body (Bond et al., 2011), patterning of larval and pupal cuticle (Wang et al., 2010, 2009a; Yamaguchi et al., 2013), apoptosis and autophagy during developmental transitions (Denton et al., 2013; Thummel, 2001) in different insect species. 20E exerts its function through the heterodimer composed of the ecdysone receptor (EcR) and ultraspiracle (USP) (Schubiger and Truman, 2000; Yao et al., 1992) and triggers downstream signal transduction in a strictly hierarchical manner (Beckstead et al., 2005).

The biosynthesis of steroid hormone starts from conversion of cholesterol into ecdysone in the prothoracic glands (PGs) (Marchal et al., 2010). Subsequently, ecdysone is released into the haemolymph, transported to target tissues and converted to 20E (Rees, 1995). Although, the process from 7-dehydrocholesterol (7 dC) to 2, 22, 25-trideoxyecdysone (ketodiol) is still a “Black Box” (Iga and Kataoka, 2012), numerous genes involved in ecdysteroid biosynthesis have been identified in insects (Ono et al., 2006; Yoshiyama et al., 2006). In contrast, only a few genes involved in ecdysteroid inactivation have been identified. One of the 20E inactivation routes is the oxidation at C-3 (Takeuchi et al., 2001) by ecdysone oxidase. It catalyzes 20E into 3-dehydroecdysone reversibly, which is followed by irreversible reduction by 3-dehydroecdysone 3 α -reductase to 3-epiecdysone (Chen et al., 1999; Takeuchi et al., 2000). In addition, 26-hydroxylation of 20E mediated by a conserved cytochrome P450 enzyme (CYP18A1) has been reported in *Drosophila melanogaster*, *Spodoptera littoralis* and *Manduca sexta* (Davies et al., 2006; Guittard et al., 2011; Rewitz and Gilbert, 2008). In *D. melanogaster*, *CYP18A1* is responsible for reducing 20E levels thus affecting many biological processes especially during larval-pupal metamorphosis. Both loss-of-function and ectopic overexpression of *CYP18A1* were found to be lethal (Rewitz et al., 2010; Guittard et al., 2011). In the lepidopteran insects *S. littoralis* and *M. sexta*, *CYP18A1* expression is coincident with 20E titer dynamics during the final instar larval stage (Davies et al., 2006). Nonetheless, the physiological functions of *CYP18A1* remain largely unknown, especially in non-drosophilid insects.

In the present study, we identified *CYP18A1* in the domesticated silkworm, *Bombyx mori*, a lepidopteran model insect. *BmCYP18A1* transcript is predominantly expressed in the middle silk gland (MSG) during wandering stage of the larvae. This remarkably tissue-specific expression pattern is distinct from any other insect species reported previously, revealing a unique 20E inactivation pathway regulated by *CYP18A1* in *B. mori*. In addition, another duplicate gene, *BmCYP18B1*, was also identified which shows no tissue-specificity. Ectopic overexpression of *CYP18A1* achieved by Gal4/UAS transgenic system in either constitutive (A3-Gal4 driver) or MSG-specific (*Ser-Gal4* driver) manner caused 20E titer reduction and subsequent lethality during the final (fifth) larval stage, suggesting that *CYP18A1* is

involved in 20E inactivation in this insect. The current study provides the first evidence that *CYP18A1* plays key roles in steroid hormone metabolism in a tissue-specific manner.

2. Materials and methods

2.1. Silkworm strain

A multivoltine, nondiapausing silkworm strain, Nistari, was used for germline transformation and subsequent experiments. Larvae were reared on fresh mulberry leaves at 25 °C under standard conditions (Tan et al. 2005).

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated from the larval middle silk gland (MSG) and other tissues using TRIzol reagent (Invitrogen, CA, USA), following the manufacturer's instructions. The RNAs were treated with DNase I (Invitrogen) to remove genomic DNA. 1 µg of total RNA was used for cDNA synthesis by employing the ReverAid First Strand cDNA Synthesis Kit (Fermentas, Lithuania, EU).

2.3. BmCYP18A1 cloning and quantitative real time PCR (qRT-PCR)

The DNA sequence coding for *BmCYP18A1* was PCR-amplified using a pair of primers-*BmCYP18A1F*: 5'-ATGATAACTATGCTTACGAATTC-3' and *BmCYP18A1R*: 5'-TTAATGTGAGCCGACATGACG-3' designed based on the sequence obtained from the GenBank (NM_001083609). cDNA synthesized from total RNA isolated from the larval wandering stage MSG was used as the template. PCR conditions were as follows: initial incubation of 94 °C for 2 min was followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2min. PCR amplified fragments were gel-purified and cloned into pMD18-T vector (TaKaRa, Dalian, China) and sequenced.

qRT-PCR was carried out to investigate the relative levels of *BmCYP18A1* mRNA in wild type or transgenic animals. PCR conditions were as follows: initial incubation of 95 °C for 1 min, followed by 40 cycles of 95 °C for 10 s and 55 °C for 20 s. The qRT-PCR primers that amplified a 124-bp fragment of *BmCYP18A1* were *BmCYP18A1RTF*: 5'-AGCATTTGTTCTTTGGCAAC-3' and *BmCYP18A1RTR*: 5'-AGAGATTCGGATCCATGTGC-3'. The primer pair (*Bmrp49RTF*: 5'-TCAATCGGATCGCTATGACA-3' and *Bmrp49RTR*: 5'-ATGACGGGTCTTCTTGTTGG-3') amplified a 136-bp fragment of *Bombyx mori* ribosomal protein 49 (*Bmrp49*) was used as an internal control. Other qRT-PCR primes used in this study were as follows: *BmCYP18B1RTF*: 5'-GGATAGGAAGCATCGGACCA-3', *BmCYP18B1RTR*: 5'-GGCGAATTGAGACAGACGAC-3', *BmEcRBRTF*: 5'-AGACGCCGCTGGTCTAATAA-3', *BmEcRBTR*: 5'-ATTCCGTCATCGTAGCTCCA-3', *BmUSPRTF*: 5'-ACACTTCGGGCAGCTAGAA-3', *BmUSPRTR*: 5'-TCCGCGAGTCTACGTTCTCT-3', *BmHR3RTF*: 5'-AACGACATACGAGCCCAAAC-3', *BmHR3RTR*: 5'-TAGAAACATCCTGGGGCTTG-3', *BmFtz-FIRTF*: 5'-TATAGATCGCCGGAAGAAGC-3', and *BmFtz-FIRTR*: 5'-

GTGGTCGGCTAATGAAGGAA-3'. All qRT-PCR analyses were performed with three independent biological replicates.

2.4. Plasmid construction

Three *piggyBac*-based transgenic plasmids were used in the current silkworm Gal4/UAS transgenic system: *pBac[Actin3Gal4-3xp3-DsRed2]* (*A3-Gal4*), *pBac[Sericin1Gal4-3xp3-DsRed2]* (*Ser-Gal4*) and *pBac[UAS-BmCYP18A1-A3-EGFP]* (*UAS-BmCYP18A1*).

The plasmid *A3-Gal4* expresses the Gal4 activator ubiquitously and includes a selection marker, red fluorescent protein DsRed2 under the control of the eye-specific 3xp3 promoter (Tan et al., 2005). For construction of the plasmid *Ser-Gal4*, a 705-bp sericin1 promoter sequence, which drives the target gene expression in an MSG-specific manner (Tatematsu et al., 2010), was PCR-amplified with the following primers: *SerPF*: 5'-TTAGGCGCGCCCTTTGTATCCCTTTTACGAA-3' and *SerPR*: 5'-ATGCTAGCGATCGCTTGATCCTTAGAGA-3' using the genomic DNA as the template. Primers contained *AscI* and *NheI* sites at their 5' ends respectively and the PCR product was cloned to replace A3 promoter sequence in *A3-Gal4* vector. The plasmid *pBac[UAS-3xp3-EGFP]* was used as initial plasmid to construct UAS effector line. The initial 3xp3 promoter was replaced by A3 promoter to generate *pBac[UAS-A3-EGFP]*, which enhances fluorescence screening in the black eye strain, Nistari. The full ORF sequence of *BmCYP18A1* was sub-cloned into *pBac[UAS-A3-EGFP]* using GBclonart seamless cloning kit (GBI, Shanghai, China). The primers used here were *UASBmCYP18A1F*: 5'-AATAGGGAATTGGACTAGTCATGATAACTATGCTTACGAATTC-3' and *UASBmCYP18A1R*: 5'-GGTATGGCTGATTATGATCCTTAATGTGAGCCGACATGACG-3'.

2.5. 20E induction assay

Five tissues including anterior silk gland (ASG), middle silk gland (MSG), posterior silk gland (PSG), fat body (FB) and midgut (MG) were dissected from the day 1 fifth instar (L5D1) larvae and cultured in 24-well plates (Nunc, Denmark) at 27 °C in 0.5 ml Tc100 insect medium supplemented with 10% fetal bovine serum (Gibco, NY, USA). 0.5 µl (2 µM) 20E dissolved in DMSO or equal amount of DMSO used as control were added to the medium. Cultured tissues were collected at 0, 3, 6, 9, 12 and 24 h after 20E treatment and subjected to RNA and cDNA preparation for subsequent qRT-PCR analysis.

2.6. Germline transformation

Transformation plasmids were microinjected with helper plasmid into the preblastoderm G₀ embryos and then incubated at 25 °C in a humidified chamber for 10–12 d until hatching (Tan et al., 2013). Hatched larvae were reared to moth and sib-mated or backcrossed with wild type moths. G₁ progeny were scored for the presence of the marker gene during embryonic stage under a fluorescence microscope (Nikon AZ100, Japan). For the Gal4/UAS system, Gal4 activator lines were crossed with the UAS effector line and the heterozygote progeny were used in subsequent experiments.

2.7. Ecdysteroid titer determination

Ecdysteroids were extracted from the MSG and haemolymph of transgenic or control silkworms at wandering, when 20E titers reached the peak. Samples were homogenized and extracted with methanol as described previously (Warren et al., 2006). The extracts were evaporated, re-dissolved, and subjected to enzyme immunoassays by using EIA kit (Cayman Chemical, MI, USA) as described before (Parthasarathy et al., 2008). A competitive assay was used to quantify 20E using anti-20-hydroxyecdysone EIA antiserum, acetylcholinesterase-conjugated 20E, and standard 20E (Sigma). Acetylcholinesterase activity was quantified by Ellman's buffer and measured at 405 nm by using Thermo Varioskan Flash (Thermo, Illinois, USA).

2.8. Immunoblot analysis

Proteins were extracted from the MSG and other tissues at indicated stages. Samples were homogenized, dissolved in PBS (phosphate-buffered saline) and quantified using BCA kit (Thermo). Extracted proteins were separated by 10% SDS/PAGE and transferred to the nitrocellulose membrane (GE Healthcare, Wisconsin, USA). The polyclonal mouse anti-BmCYP18A1 primary antibody recognizes the amino acids 524–541 of BmCYP18A1 and was used to detect BmCYP18A1 protein (1:200 dilution; ABmart Inc., Shanghai, China). And α -Tubulin detected by the mouse anti- α -Tubulin primary antibody (1:5000 dilution; Vazyme biotech co., ltd., Piscataway, USA) was used as the control. For the secondary antibody, horseradish peroxidase conjugated goat anti-mouse IgG (1:5000 dilution; Beyotime, Shanghai, China) was used. Signal detection was performed using the ECL Plus Western Blotting Detection Kit (GE Healthcare, USA).

2.9. Paraffin embedding and DAPI staining

MSG from L5D4 larvae (*UAS-BmCYP18A1* line and *Ser > BmCYP18A1* line) were dissected on ice and fixed in Qurnah's fixative (ethanol:chloroform:acetic acid = 6:3:1) overnight. The fixed MSGs were dehydrated in 100% ethanol for three times, treated with xylene three times, embedded in paraffin and 5 μ m thick sections were cut using the Leica RM2235 microtome. For 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) staining, sections were dewaxed using xylene and serially rehydrated by 95%, 80%, and 70% ethanol, distilled water and phosphate-buffered saline (PBS). After rehydration, samples were stained with 1 μ g/ml DAPI working solution for 15 min and washed with PBS for 3 times. Sections were photographed under a fluorescence microscope (Olympus BX51, Japan).

2.10. Statistical analysis of data

Statistical analysis was performed using a one-tailed unpaired student *t*-test to compare specific differences. In all cases, $p < 0.05$ was used to determine significance.

3. Results

3.1. Sequences cloning and the spatial and temporal expression

Searching for the NCBI database revealed there are two *BmCYP18* gene duplicates: *BmCYP18A1* (NM_001083609.2) and *BmCYP18B1* (XM_004923159.1). The amino acid identity between *BmCYP18A1* and *BmCYP18B1* was 33.67% (Fig. S1). Both genes have three highly conserved protein domains: proline-glycine-rich domain, I-helix domain and haem-binding domain (Fig. S1). In addition, no signal peptide was identified in two *BmCYP18* genes by SignalP program (<http://www.cbs.dtu.dk/services/SignalP/>) suggesting they are not secretory proteins. Their mRNA levels were quantified at three representative developmental stages: day 3 of the fifth instar (L5D3) during the feeding stage, wandering stage (W) and day 1 pupal stage (P1). For each stage, ten major tissues that include prothoracic gland (PG), fat body (FB), midgut (MG), anterior silk gland (ASG), middle silk gland (MSG), posterior silk gland (PSG), Malpighian tubules (MT), testis (Te), ovary (Ov) and epidermis (Epi) were dissected, total RNA isolated and used in qRT-PCR. The data from qRT-PCR analysis showed the maximum levels of *BmCYP18A1* mRNA in MSG (Fig. 1A), revealing the MSG-specific expression of *BmCYP18A1*. We further investigated developmental profile of *BmCYP18A1* expression in MSG from L4D1 to P1. A slight increase in *BmCYP18A1* mRNA level was detected during L4 to L5 molting stage (Fig. 1B). It was undetectable during L5 stage but increased again and reached the maximum level during the wandering and prepupal stages. However, the mRNA level decreased again and became undetectable by day 1 of pupal stage. Consistent to the transcriptional level, Western blotting analysis showed that *BmCYP18A1* protein expressed predominantly in the MSG at wandering stages (Fig. 1C). No obvious protein expression was detected in MSG during the feeding stages of the final instar larvae, but a significant protein band was detected in the MSG at wandering and PP1 stages (Fig. 1D). Both the transcriptional and translational profiles suggested that *BmCYP18A1* was predominantly expressed in the MSG during larval-pupal metamorphosis. In contrast, *BmCYP18B1* mRNAs were detected in all tissues and stages tested and showed no significant difference among different tissues and developmental stages (Fig. 1E).

3.2. Induction of *BmCYP18* genes by 20E

To investigate whether the transcription of two *BmCYP18* genes is induced by 20E, tissues including ASG, MSG, PSG, MG and FB were dissected from L5D1 larvae and exposed to 20E. As the results, *BmCYP18A1* transcription was induced by 20E in all tissues tested. In MSG, a nine-fold increase in *BmCYP18A1* mRNA level was detected at 3 h after 20E treatment (Fig. 2A). In other tissues, the increase of *BmCYP18A1* mRNA levels was detected at 6 h in ASG, PSG and FB; and at 9 h in MG (Fig. 2A). The maximum induction of *BmCYP18A1* mRNA level in MSG reached to 230-fold at 9 h of 20E exposure. However, it was only 33-fold in ASG, 30-fold in FB, 13-fold in PSG and 5-fold in MG respectively (Fig. 2A). These data showed that *BmCYP18A1* is a 20E-inducible gene and its gene in MSG is most sensitive to 20E treatment in *Bombyx mori*. Unlike *BmCYP18A1*, very little increase in *BmCYP18B1* mRNA levels was detected when tissues were treated with 20E (Fig. 2B). Ubiquitous expression profile and lack of 20E response suggest that *BmCYP18B1*

may not be a major player in ecdysteroid metabolism. Therefore, we focused on *BmCYP18A1* for further characterization as described below.

3.3. Ectopic overexpression of *BmCYP18A1* using Gal4/UAS system

The Gal4/UAS transgenic system was used to elucidate biological functions of *BmCYP18A1* in *Bombyx mori*. Two activator lines were established: a constitutive *A3-Gal4* driver which overexpresses target gene in a ubiquitous manner, and an MSG-specific *Ser-Gal4* driver which overexpresses the target gene specifically in MSG. A *UAS-BmCYP18A1* effector line was also established (Fig. 3A). Transgenic G₁ late embryos expressing *EGFP* or *DsRed* were shown in Figs. 3B–G.

Offspring from a cross between *UAS-BmCYP18A1* effector and *A3-Gal4* or *Ser-Gal4* activator line were used to investigate *BmCYP18A1* expression in their tissues. The L5D4 larvae from *A3>BmCYP18A1* and control (*UAS-BmCYP18A1*) were dissected and four major tissues that include MG, MSG, FB and Epi were isolated. Total RNAs were purified from the dissected tissues and used in qRT-PCR. *BmCYP18A1* mRNA levels in transgenic animals showed an increase of 5-fold in MG and 2-fold in MSG, FB and Epi when compared to that in the wild type animals (Fig. 4A).

In contrast, *Ser > BmCYP18A1* transgenic animals showed increase in levels of *BmCYP18A1* mRNA only in MSG as expected and a significant up-regulation (up to 1000-fold) of *BmCYP18A1* was observed at L5D4 (Fig. 4B). We then quantified *BmCYP18A1* mRNA levels in MSG isolated from L5D1 to P1. The mRNA levels of *BmCYP18A1* significantly increased from L5D3 to P1 in *Ser > BmCYP18A1* (Fig. 4C). More than 30-fold up-regulation was detected in MSG dissected from transgenic animals during the wandering stage when the endogenous *BmCYP18A1* expression reached its peak. *BmCYP18A1* mRNA levels stayed relatively high at PP1 followed by a rapid decrease to the background level after pupal ecdysis in *Ser > BmCYP18A1* transgenic silkworms (Fig. 4C). Up-regulation of *BmCYP18A1* protein in the transgenic MSG was also detected by Western blotting (Fig. 4C).

To investigate how *Ser-Gal4* driver directed targeted gene expression, we examined the transcriptional profile of *Bmsericin1* in the MSG dissected from transgenic insects. The developmental profiles of *sericin1* mRNA were identical in MSG isolated from *Ser > BmCYP18A1* and the control *UAS-BmCYP18A1* insects (Fig. 4D). This expression pattern is similar to the expression pattern of *BmCYP18A1* in *Ser > BmCYP18A1* transgenic animals, suggesting that *sericin1* promoter reproduced temporal and spatial *BmCYP18A1* activity faithfully (Figs. 4C, D). These data showed that the MSG-specific overexpression of *BmCYP18A1* was efficient and tissue-specific.

3.4. *BmCYP18A1* ectopic overexpression arrested larval growth and resulted in lethality of the final instar

Transgenic silkworms, *A3>BmCYP18A1*, *Ser > BmCYP18A1* and the control *A3-Gal4*, *Ser-Gal4*, *UAS-BmCYP18A1* lines developed normally during the embryogenesis and the early larval stages. However, 87% ($n = 23$) of *A3>BmCYP18A1* animals showed deleterious

effects on growth from L5D2 and died during the next few days and the other 13% *A3>BmCYP18A1* silkworms died during pupation. Similar lethal phenotypes were observed in 61% ($n = 64$) of the *Ser > BmCYP18A1* insects (Fig. 5A), although the lethality occurred from L5D4, at the late period of the fifth instar. The remaining 39% transgenic silkworms completed metamorphosis successfully. However, *Ser > BmCYP18A* moths showed severely deleterious effects on fecundity: *Ser > BmCYP18A1* females crossed with wild type males laid no or only a few eggs, and *Ser > BmCYP18A1* males silkworms could not mate with wild type females. Since endogenous *BmCYP18A1* showed an MSG-specific expression pattern, we focused our subsequent studies on *Ser > BmCYP18A1* lines. The size of MSG in *Ser > BmCYP18A1* silkworms was smaller compared to the control (Fig. 5B). Cross-sections of MSG from *UAS-BmCYP18A1* (Figs. 5C, D, E, F) and *Ser > BmCYP18A1* (Figs. 5G, H, I, J) animals showed that both the diameter of the gland and thickness of silk gland cell (SGCs) layer decreased in MSG from *Ser > BmCYP18A1* transgenic animals when compared to *UAS-BmCYP18A1* animals.

3.5. Altered expression levels of genes involved in 20E signaling pathway

To determine whether the phenotypes resulted from *BmCYP18A1* overexpression is due to decrease in 20E titer, we investigated the ecdysteroid titer in wandering larvae when the maximum effects of *BmCYP18A1* overexpression were observed. Compared to the ecdysteroid levels in the control (*UASBmCYP18A1*), a decrease in ecdysteroid levels of 40% in the haemolymph and 50% in MSG were detected in transgenic animals expressing *BmCYP18A1* (*Ser > BmCYP18A1*, Fig. 6). These data suggest that the overexpressed *BmCYP18A1* may have contributed to the inactivation of 20E and caused phenotypes observed.

Since 20E titers decreased in the *BmCYP18A1* transgenic silkworms, we hypothesized that the expression of genes in the 20E signaling pathway may have been affected in transgenic animals. To test this hypothesis, we investigated the expression of four representative 20E pathway genes in ASG, MSG and PSG of *Ser > BmCYP18A1* animals dissected at the wandering stage. The mRNA levels of ecdysone receptor heterodimer, *EcRB* and *USP* decreased by 90% and 50% respectively in the MSG of *Ser > BmCYP18A1* animals when compared to their levels in control ones (Figs. 7A, B). Transcription of *HR3* and *Ftz-F1* were decreased by 66% and 46% respectively in the transgenic animals (Figs. 7C, D). The mRNA levels of the four tested genes did not show much difference in ASG and PSG dissected from *Ser > BmCYP18A1* and *UASBmCYP18A1* transgenic animals except for a small decrease of *EcRB* and *Ftz-F1* mRNA levels in ASG of transgenic animals. These results confirmed that overexpressed *BmCYP18A1* functioned in the MSG specifically.

4. Discussion

The major contribution of the current study is the identification of *BmCYP18A1* as a tissue-specific 20E regulator in the silkworm. Two *BmCYP18* genes: *BmCYP18A1* and *BmCYP18B1* were identified in the silkworm genome. *BmCYP18A1* showed a predominantly MSG-specific expression in *Bombyx mori* (Figs. 1A, C), while *BmCYP18B1* expresses ubiquitously in all investigated tissues and developmental stages (Fig. 1E).

Interestingly, only one *CYP18* has been identified in *Drosophila melanogaster* (*DmCYP18A1*), which is mainly expressed in the fat body, epidermis and the salivary glands (Guittard et al., 2011). In another lepidopteran insect *Spodoptera littoralis*, *CYP18A1* mRNA has been detected in the midgut and fat body (Davies et al., 2006).

DmCYP18A1 has been reported to be induced by 20E and Broad-Complex and Ftz-F1 binding motifs have been found in the untranslated region (Crossgrove et al., 1996). In order to assess if both of the *BmCYP18* genes can be induced by 20E, we dissected and treated five main tissues (ASG, MSG, PSG, MG and FB) with 20E *in vitro*. Data included in this paper showed that *BmCYP18A1* gene is induced by 20E, confirming that *BmCYP18A1* is a 20E-inducible gene. However, no significant 20E induction of *BmCYP18B1* was observed (Fig. 2B). In a book chapter, Lafont et al. (2012) reported some unpublished data that showed no 26-hydroxylase activity for *BmCYP18B1* in the silkworm. Take together these data suggest that *BmCYP18B1* is not a significant player in ecdysteroid metabolism of silkworm. *BmCYP18A1* gene showed distinct 20E induction patterns in tissues including ASG, MSG, PSG, FB and MG (Fig. 2A). And it was induced as fast as 3 h and reached to the maximum induction of 240-fold within 9 h in MSG. However, the maximum induction in the other tissues was only 33-fold (24 h in ASG). This is reasonable since *BmCYP18A1* is predominantly expressed in MSG of *Bombyx mori* during wandering stage.

The biological significance of MSG-specific expression of *BmCYP18A1* is not clear at this time. However, we hypothesize that the fast and fierce 20E induction of *BmCYP18A1* by 20E is important for MSG degeneration after spinning. *Bombyx mori* silk glands are fully formed during embryogenesis but grow enormously in size without mitosis during the final larval stage (Dhawan and Gopinathan, 2003; Kimoto et al., 2012). At this time, massive accumulation of silk protein is provoked by activation of insulin pathway (Wang et al., 2009b), repressing *TOR* transcription and inhibition of 20E signal transduction (Delanoue et al., 2010). After spinning, silk gland is degenerated rapidly through programmed cell death and disappears by day 2 pupal stage (Zhang et al., 2009). High 20E titer and activated PCD signal are necessary for tissue degeneration and remodeling during larval-pupal transition (Liu et al., 2013; Tsuzuki et al., 2001). As we observed, *BmCYP18A1* expressed maximum at the late wandering stage (Figs. 1A, B), several hours after 20E titers reach their peak. An increase in 20E levels followed by a rapid decrease is required for transitioning MSG to programmed cell death and eventual destruction. It appears that tissue and stage specific expression of *CYP18A1* in MSG facilitates the tissue degeneration. We also examined the relative mRNA levels of 20E response genes and found most of them are expressed at lower levels in the MSG when compared to ASG and PSG during wandering stage, when *BmCYP18A1* gene showed the highest expression (Figs. 7A, B, C) in the control animals. Furthermore, significant down-regulation of all four investigated genes was observed in MSG but not in ASG and PSG in *Ser > BmCYP18A1* transgenic animals (Figs. 7A, B, C, D). These data support our hypothesis on the requirement of an increase followed by rapid fall in 20E titers for transitioning MSG to program cell death and destruction at the end of larval stage. Further research is also required to confirm this hypothesis.

Enzyme coded by *CYP18A1* may have metabolized 20E to 26-hydroxylated intermediate and ultimately to the completely inactive ecdysteroid (Rewitz et al., 2010; Guittard et al.,

2011) through an irreversible reaction. The MSG restriction of *BmCYP18A1* suggests that additional 20E inactivation pathways may function in the other tissues (Takeuchi et al., 2001). As shown previously, *ecdysone oxidase (EO)* converts 20E into 3-dehydroecdysteroid reversibly followed by the irreversible reduction with 3-dehydroecdysone 3 α -reductase (Takeuchi et al., 2001). Comparing with 3-dehydrogenation inactivation route, 26-hydroxylation route executed by *CYP18A1* is more efficient and irreversible (Guittard et al., 2011). For MSG, a rapid inactivation of 20E for tissue degeneration might be needed and the predominant expression of *BmCYP18A1* makes it possible. Our study provides a unique model for dissection of tissue specific *CYP18A1* biological functions in insects.

Overexpression of *BmCYP18A1* during the larval development by its ectopic expression in transgenic animals caused a decrease in 20E titers. This decrease in 20E titers may have caused stunted growth and larval lethality. However, no developmental abnormalities were detected during embryogenesis and early larval stages in transgenic animals when *BmCYP18A1* was overexpressed in a ubiquitous or MSG-specific manner. This is different from *Drosophila melanogaster*, in which 100% embryonic lethality was observed by ubiquitous overexpression of *CYP18A1* (Rewitz et al., 2010; Guittard et al., 2011). It is likely that the small amount of 20E secreted from PG is sufficient to complete embryogenesis and early larval development in *A3>BmCYP18A1* transgenic silkworms. However, we observed stunted growth and lethality in the final larval stage by MSG-specific overexpression of *BmCYP18A1* (Figs. 5A, B). One possible explanation for the lethality of *Ser > BmCYP18A1* silkworms is that overexpression of *BmCYP18A1* facilitates the accumulation of 20E metabolites in body cavity and cause “hyperecdysonism” such as melanotic tumors as reported by Platt et al. (2008). In fact, some 20E is required during the feeding stage for the reprogramming of larval-pupal transition as Dai et al. (1995) reported. And a small peak of 20E titer has been identified in the middle of the final instar larvae in silkworm (Mizoguchi et al., 2001), which has been described as the peak required for initiation of wandering and gut purge as well as cellular reprogramming. In the transgenic silkworms, at least 50-fold increase of *BmCYP18A1* was observed during the feeding stages of final instar larvae (Fig. 4B), resulting in 40% decrease of ecdysteroid titer in the haemocytes (Fig. 6). Thus it is possible that decrease of ecdysteroid titer during the feeding stages may have caused the developmental defects observed. We also investigated expression levels of genes involved in 20E signaling pathway including *EcrB*, *USP*, *HR3* and *Ftz-F1* and results showed these genes were down-regulated after *BmCYP18A1* up-regulation. Since these genes are directly regulated by 20E, these data provided additional evidence that *BmCYP18A1* up-regulation lowered 20E titer and subsequently derailed 20E-regulated gene expression.

Taken together, our results provide the first evidence that *CYP18A1* regulates tissue (MSG)-specific 20E inactivation in *Bombyx mori*. Such a case is the first report in insects, suggesting a wide diversity of steroid hormone metabolism in different insect species. Furthermore, MSG-specific 20E inactivation pathway offers an excellent model system for elucidating tissue-specific steroid hormone metabolism in insects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Spatial and temporal expression of *BmCYP18* genes. (A) Relative expression of *BmCYP18A1* in different tissues including PG (prothoracic gland), FB (fat body), MG (midgut), ASG (anterior silk gland), MSG (middle silk gland), PSG (posterior silk gland), MT (Malpighian tubules), Te (testis), Ov (ovary) and Epi (epidermis) isolated from three developmental stages: L5D3 (day three of the fifth instar larvae), W (wandering) and P1 (day 1 of pupae). (B) The relative mRNA levels of *BmCYP18A1* in MSG from L4D1 to P1. mRNA levels were normalized to the *B. mori* ribosomal protein 49 (*Bmrp49*). The data shown are mean $\pm$ S.E.M. ($n = 3$). (C) Translational level of BmCYP18A1 in five main tissues including H (haemocyte), MG, FB, MSG and PSG. (D) Expression pattern of BmCYP18A1 protein in MSG from the L5D1 to P1. α -Tubulin was used as the control for protein expression. (E) Relative expression of *BmCYP18B1* in different tissues at L5D3, W and P1.

Fig. 2.

20E induction of *BmCYP18* transcription. Five tissues- ASG, MSG, PSG, FB and MG dissected from L5D1 larvae were exposed to 20E (2 μ M) treatment. Samples were collected at 0, 3, 6, 9, 12 and 24 h after 20E treatment and used for RNA extraction and cDNA synthesis. qRT-PCR was carried out to determine the relative mRNA levels using *Bmrp49* for normalization. (A) Fold change in *BmCYP18A1* transcription was determined by dividing the *BmCYP18A1* mRNA levels in 20E treated tissues with those in DMSO treated tissues. (B) 20E induction of *BmCYP18B1* in three main tissues including MSG, MG and FB. The data shown are mean $\pm$ S.E.M. ($n = 3$).

Fig. 3.

Transformation schematics and fluorescence of transgenic silkworm embryos. (A) Schematics of *pBac[Actin3Gal4-3xp3-DsRed2]* (*A3-Gal4*), *pBac[Sericin1Gal4-3xp3-DsRed2]* (*Ser-Gal4*) and *pBac[UAS-BmCYP18A1-A3-EGFP]* (*UAS-BmCYP18A1*) vectors. (B–G) *DsRed* and *EGFP* positive G_1 late embryos. Embryos in B, C and D were photographed under bright field, E and F were photographed with UV light with RFP filter and G with EGFP filter (all scale bars stand for 250 μ m).

Fig. 4.

Ectopic over-expression of *BmCYP18A1* in transgenic animals. (A) *BmCYP18A1* relative expression in the tissues of *A3>BmCYP18A1* and control (*UAS-BmCYP18A1*) silkworms at L5D4. (B) *BmCYP18A1* transcript levels in tissues dissected from *Ser > BmCYP18A1* and the control (*UAS-BmCYP18A1*) animals on L5D4. (C) Temporal expression of *BmCYP18A1* in the control and *Ser > BmCYP18A1* transgenic animals in MSG from L5D1 to P1. The inset showed increased translation of BmCYP18A1 protein in MSG at wandering stage. (D) The relative mRNA levels of *sericin1* from L5D1 to P1. Four tissues – MG, MSG, FB and Epi determined by qRT-PCR in A and B and mRNA levels were normalized to *Bmrp49*.

Fig. 5.

Larval lethality and growth arrestment in *Ser > BmCYP18A1* transgenic silkworms. (A) Phenotypes of silkworm larvae resulted from *Ser > BmCYP18A1* at late period of the final instar larvae. Smaller larvae were from transgenic insects and larger larvae were from controls. (B) Decrease in growth of silk gland resulted from *BmCYP18A1* overexpression. Smaller silk gland shown in the top was from *Ser > BmCYP18A1* transgenic silkworm and the larger gland shown at the bottom was from control. (C–J) Cross sections of the middle silk glands (MSG) dissected from *UAS-BmCYP18A1* (C, D, E and F) and *Ser > BmCYP18A1* (G, H, I and J) transgenic animals. (C, D) *UAS-BmCYP18A1* MSG under bright field; (E, F) *UAS-BmCYP18A1* MSG with DAPI staining; (G, H) *Ser > BmCYP18A1* MSG under bright field; (I, J) *Ser > BmCYP18A1* MSG with DAPI staining. D, F, H and J ($\times 40$) are magnified sections of C, E, G and I, respectively. SGCs: silk gland cells; GC, gland cavity. Scale bars are indicated in the figures.

Fig. 6.

Decrease in ecdysteroid levels in haemolymph and MSG of transgenic insects. For each group, ecdysteroids were extracted from at least three individuals and used in enzyme immunoassay (EIA). Compared to the control (*UAS-BmCYP18A1*), ecdysteroid levels decrease to 60% in the haemolymph and 50% in MSG of *Ser > BmCYP18A1* silkworms. Ecdysteroid titer was normalized to the control with error bars representing S.E.M., and $p < 0.05$ was used to determine significance.

Fig. 7.

Relative expression of genes involved in 20E signaling pathway. qRT-PCR was used to determine relative mRNA levels of 20E pathway genes *-EcRB*, *USP*, *HR3* and *Ftz-F1*. The asterisk (*) represents the statistical significance ($p < 0.05$) and the data shown are mean $\pm$ S.E.M. ($n = 3$).