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Identification and dynamic expression profiling of circadian clock genes in *Spodoptera litura* provide new insights into the regulation of sex pheromone communication

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Research Paper

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Abstract

Spodoptera litura is an important pest that causes significant economic damage to numerous crops worldwide. Sex pheromones (SPs) mediate sexual communication in *S. litura* and show a characteristic degree of rhythmic activity, occurring mainly during the scotophase; however, the specific regulatory mechanisms remain unclear. Here, we employed a genome-wide analysis to identify eight candidate circadian clock genes in *S. litura*. Sequence characteristics and expression patterns were analyzed. Our results demonstrated that some circadian clock genes might regulate the biosynthesis and perception of SPs by regulating the rhythmic expression of SP biosynthesis-related genes and SP perception-related genes. Interestingly, all potential genes exhibited peak expression in the scotophase, consistent with the SP could mediate courtship and mating behavior in *S. litura*. Our findings are helpful in elucidating the molecular mechanism by which circadian clock genes regulate sexual communication in *S. litura*.

Introduction

Arthropods are invertebrates that have developed the largest insect populations in nature during their long-term evolutionary process. Consequently, because of the important position and vital role of insects in nature, such as pollination and maintaining a balance between animals and plants, daily physiological and behavioral characteristics of insects should be intensively studied. Previous studies have detected the characteristics of circadian or seasonal periodic changes in a series of insect life activities and behaviors, such as growth, metamorphosis, reproduction, and avoidance of harm (Rosén, 2002; Shakeel *et al.*, 2009; Seay and Thummel, 2011; Li *et al.*, 2018*a*). Therefore, it is believed that the biological clock is indispensable for 'temperature compensation' of fruit flies (Huang *et al.*, 1995), diapause of silkworms (Xu *et al.*, 2011; Tobback *et al.*, 2012), and photoperiod activity of other insects (Goto and Matsumoto, 2018). The circadian rhythm, a manifestation of the biological clock of insects, is a dynamic and endogenous oscillation that controls 24 h physiological and behavioral processes. Therefore, periodic activities of insects are the basis for unravelling the internal mechanisms of insect physiological behaviors and are subjects that require intensive study in integrated pest management.

Many studies have shown that there are obvious rhythmic phenomena in insect sexual behaviors, such as courtship and mating behaviors of *Spodoptera exigua* that occur during the first half of the scotophase (2–4 days after eclosion) (Luo *et al.*, 2003) and the sensitivity of male antennae (MA) to female sex pheromones (SPs), which is consistent with mating behavior in *Spodoptera littoralis* (Merlin *et al.*, 2007). Some studies have confirmed that light, temperature, humidity, and chemical signals can regulate the expression of circadian clock genes and the biosynthesis, release, and perception of SPs in moths (Merlin *et al.*, 2007; Tomioka and Matsumoto, 2015; Lu *et al.*, 2017; Chen *et al.*, 2018). Before mating, female moths can biosynthesize and release SPs rhythmically through the SP gland (Groot, 2014; Lu *et al.*, 2017). Age and mating could affect the courtship of male and female moths or other rhythmic behaviors (Groot, 2014). These lines of evidence suggest that circadian clock genes may regulate SP communication in moths, but the detailed molecular mechanisms underlying the function of these genes remain unknown.

The insect rhythmic behavior is controlled by the circadian clock consisting of the negative transcriptional-translational feedback loop. It includes various circadian clock genes, such as *Period* (*Per*), *Timeless* (*Tim*), *Cryptochrome* (*Cry*), *Cycle* (*Cyc*), *Clock* (*Clk*), *vrille* (*Vri*), and

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Doubletime (*Dbt*), identified in numerous insects (Rothenfluh *et al.*, 2000*b*; Tomioka and Matsumoto, 2015). The PAR domain protein gene (*Pdp1ε*) and the orange transcription factor gene (*cwo*) with the basic-helix-loop-helix (bHLH) domain might also be involved in the rhythmic expression process of circadian clock genes (Richier *et al.*, 2008; Benito *et al.*, 2010). A master protein, PER, encoded by *per* binds to TIM (encoded by *Tim*), to form a heterodimer that performs a feedback function and coordinates the circadian and supernal rhythms of *Drosophila melanogaster* (Crosby *et al.*, 2019) and the circadian rhythm activities of other moths, such as those involved in reproduction, mating, metamorphosis, and diapause (Beaver *et al.*, 2003; Tobback *et al.*, 2011). However, PER could also manifest heterotrophism by inhibiting dCLK/CYC-mediated transcription in *Drosophila* (Rothenfluh *et al.*, 2000*a*). Additionally, some researchers have found that *per* gene is expressed rhythmically and non-rhythmically in different insects (Sauman and Reppert, 1996; Lupien *et al.*, 2003) and has the same compensatory effect as *Tim* in expression regulation (Tobback *et al.*, 2012; Meuti *et al.*, 2015). Studies have shown that *Cry*, a photoreceptor gene, might participate in the regulation or metabolism of TIM expression, which implies that environmental conditions could drive the diversification of the basic clock and its mechanisms (Chen *et al.*, 2018), such as CRY1 with photoreceptors and mammalian-type CRY2 with repression (Ozturk, 2016). CLOCK (CLK) and CYCLE (CYC), transcription factors consisting of a core feedback loop, might bind to each other and enter the nucleus to activate the transcription of *period* and *timeless* (Zhu *et al.*, 2008; Tokuoka *et al.*, 2017). However, there are structural differences between them in various insect species (Rubin *et al.*, 2006). Additionally, the transcriptional–translational loop is regulated by genes, such as *Vrille* (*VRI*) and *Pdp1ε*. Therefore, to fully understand the rhythmic sexual communication of insects, it is necessary to understand the causes of this rhythmic behavior in insects, namely the regulatory role of circadian clock genes.

Spodoptera litura (Lepidoptera: Noctuidae) is an important polyphagous pest. Its larvae feed on over 100 crops, and this pest is widely distributed throughout tropical and subtropical areas of Asia, including China (Dinesh-Kumar *et al.*, 2018; Liu *et al.*, 2018; Wang *et al.*, 2018; Li *et al.*, 2018*b*). Currently, the management of *S. litura* larvae mainly depends on chemical insecticides. Because of various side effects of chemical insecticides, including pollution of the environment and resistance phenomena of the target insects (Shad *et al.*, 2012; Wang *et al.*, 2018), there is an urgent need to develop alternative strategies for modern pest management to control insect populations. Although there have been many studies on SP communication in *S. litura*, some revealed that two desaturase genes (*SlitDes5* and *SlitDes11*) (Xia *et al.*, 2019; Zhang *et al.*, 2019), three pheromone-binding proteins (SlitPBP1-3) (Liu *et al.*, 2012; Liu *et al.*, 2013), and two odorant receptors (SlitOR6 and SlitOR13) (Zhang *et al.*, 2015*a*) play important roles in SP biosynthesis and the perception processes; however, the specific regulatory mechanisms of the genes involved in this rhythmic behavior remain unknown. In the present study, sequences encoding putative candidate circadian clock genes in *S. litura* were identified through genome-wide transcriptome analysis. We performed a phylogenetic analysis of *S. litura* genes to establish the putative orthology of the different transcripts identified. Furthermore, we compared tissue specificity and temporal aspects of gene expression during development. The results could provide potential target genes to develop effective sexual communication disruptors to manage *S. litura* populations in the future.

Materials and methods

Insect rearing and tissue collection

Spodoptera litura larvae were reared at the College of Life Sciences, Huaibei Normal University, Huaibei, China, under laboratory conditions at a constant temperature of 28 ± 1°C, relative humidity of 60–70%, and a photoperiod of 14 h light: 10 h dark (LD). The eggs were incubated until hatching in an insect room, and various tissues were collected based on experimental purposes. To analyze the developmental expression levels of circadian clock genes, fresh eggs, 3- and 4-day-old larvae, pupae, and whole male and female adults were collected at 5 h after the beginning of the scotophase. To verify the tissue specificity of circadian clock genes expression, the following tissues were collected from virgin adults (3-day-old adults after eclosion and 5 h after the beginning of the scotophase) as follows: antennae (An), heads without antennae (He), thoraces (Th), abdomens (Ab), legs (Le), wings (Wi), hairpencils (Hp) of males, and SP glands of females (PG). To analyze the dynamic expression of circadian clock genes, SP glands and MA were collected from virgin female and male moths at 5–7 h after the beginning of the scotophase from different aged moths (0- to 5-day-old). Finally, to analyze the daily expression of circadian clock genes, we collected the SP glands and MA from virgin moths at different points from 2 to 3-day-old. The number of points was distributed according to the periods as 5 during the 14 h photophase, 6 during the 10 h scotophase, and 2 during the following 6 h photophase. At least three biological replicates were collected from each tissue and immediately stored in liquid nitrogen for later use.

RNA extraction and cDNA synthesis

According to experimental methods described previously (Zhang *et al.*, 2015*b*), total RNA was extracted from tissue samples using the TRIzol® reagent (Tiangen Biotech, Beijing, China) following the manufacturer's instructions. Agarose electrophoresis and spectrophotometry were used to verify RNA quality. Next, the corresponding cDNA was directly synthesized using the PrimeScript™RT kit (TaKaRa, Dalian, China) according to the manufacturer's instructions and stored at −80°C until use.

Sequence and phylogenetic analysis

The genomeand transcriptomes of *S. litura* were downloaded from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/genome/?term=spodoptera+litura) (Cheng *et al.*, 2017). We first collected the circadian clock gene sequence of other insects from GenBank (https://www.ncbi.nlm.nih.gov/genbank/), and then, these genes were used as queries to screen the stand-alone genome of *S. litura* using NCBI-TBLASTN, with an E-value cut-off of e^{-5} for each query. The identified clock genes of *S. litura* were verified using NCBI BLASTX. The number and location of exons and introns of the circadian clock genes were generated using the Exon-Intron Graphic Marker (http://www.wormweb.org/exonintron). Open reading frames (ORFs) of all circadian clock genes were predicted using the ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Similarity searches were performed using the NCBI BLASTX

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network server (http://blast.ncbi.nlm.nih.gov/). The amino acid sequences of all clock genes of *S. litura* and other insects (table S1) were aligned using ClustalX 2.0, and phylogenetic neighbor-joining trees (1000 sampling analysis) were constructed using MEGA6.0 (Tamura *et al.*, 2013).

Measurement of mRNA levels

We used real-time quantitative PCR (qRT-PCR) to determine the specific expression patterns of clock genes and other genes related to SP communication in *S. litura*. First, the sample extraction mixture was used to detect the specificity of primers designed using Beacon Designer 7.9 (PREMIER Biosoft International, CA, USA) or referenced from other studies (Zhang *et al.*, 2015*b*; Lin *et al.*, 2017) (table S2). All PCRs were conducted in a 10 µl reaction volume containing 5 µl 2X SYBR Green PCR Master Mix (Vazyme Biotech, Nanjing, China), 0.25 µl of paired primers, 1 µl of cDNA, and increased to 10 µl with nuclease-free water. At least three technical replicates were performed for each biological sample. PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. The final extension step was performed at 72°C for 10 min, and suitable aliquots were loaded onto a 2% agarose gel for visualization. Following the manufacturer's instructions, the melting curve was determined using the LightCycler®96 (Roche Diagnostics, Basel, Switzerland). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the elongation factor (EF) of *S. litura* were used as internal reference genes (Zhang *et al.*, 2015*b*; Zhang *et al.*, 2016) to calculate the relative expression levels of each gene. The normalized expression of each gene was analyzed using Q-Gene (Muller *et al.*, 2002; Simon, 2003). Each biological sample had three technical replicates.

Statistical analysis

SPSS software (version 21.0; SPSS Inc., Chicago, IL, USA) was used for data processing and significance analysis ($P < 0.05$) using a one-way analysis of variance (ANOVA).

Results

Identification of candidate circadian clock genes

Based on the genome-wide annotation and comparative analysis in *S. litura*, we identified eight putative candidate circadian clock genes, including *Per*, *Tim1*, *Tim2*, *Cry1*, *Cry2*, *Cyc*, *Clk*, and *Vri*. The number of identified genes was more than *Helicoverpa armigera*, *Bombyx mori*, and *Apis mellifera*, and less than *Danaus plexippus*, *D. melanogaster*, *Tribolium castaneum*, and *Acyrthosiphon pisum* (fig. 1a). The corresponding sequence information of the eight circadian clock genes in *S. litura* is presented in table 1. According to the results of BLASTX match analysis, all clock genes were predicted to have full-length ORFs that encode 417–1284 amino acids and have a high level of identity among other noctuid pests (approximately 86–99%). The size and position of exons and introns were analyzed based on genome and cDNA sequences, and the results showed that *SlitPer* had the greatest number of exons and introns, whereas *SlitCry2* had the least (fig. 1b). Next, six phylogenetic trees of insect circadian clock genes were constructed using various genes of *S. litura* and other insects (fig. 2). The results showed that each clock gene of *S. litura* formed a cluster on the same branch as the Lepidopteran species, and all branches were highly supported (bootstrap values greater than 60%).

Developmental spectrum and tissue expression of clock genes in S. litura

To investigate the relationship between the expression of circadian clock genes relative to developmental stage, we analyzed the expression pattern of circadian clock genes at different developmental stages (from egg to adult) of *S. litura* using qRT-PCR (fig. 3). The results showed that all clock genes could be detected during each developmental stage of *S. litura*. Among these genes, the expression of *SlitCry1* in eggs was significantly higher than that in the other developmental stages, and four genes (*SlitPer*, *SlitCry2*, *SlitTim1*, and *SlitCyc*) were significantly expressed in adults, three genes (*SlitCry2*, *SlitTim2*, and *SlitCyc*) were differentially expressed between the sexes in adults. Interestingly, we found that some genes in the same family showed different expression characteristics, such as *SlitCry1*, *SlitCry2*, *SlitTim1*, and *SlitTim2*. We further analyzed the expression levels of all clock genes in different tissues of unmated adults. The results showed that all the genes displayed significant sex differences (fig. 4). There was one highly expressed gene in the female antennae (*SlitPer*), heads (*SlitCry2*), and abdomens (*SlitTim2*), and two in the wings (*SlitCyc* and *SlitVri*), whereas there was no significant difference in expression in different tissues of *SlitCry1*. In contrast to females, five genes (*SlitPer*, *SlitCry2*, *SlitTim2*, *SlitCyc*, and *SlitVri*) in males exhibited significantly higher expression in the thoraxes and two genes (*SlitCry1* and *SlitTim1*) were highly expressed in the MA and hair-pencils.

Expression profiles of genes related to sex pheromone communication or circadian clock in adult S. litura

To determine whether there was a relationship between the dynamic distribution of SP communication-related genes and circadian clock genes in *S. litura*, we analyzed the dynamic expression of these genes using qRT-PCR. Based on previous studies (Liu *et al.*, 2013; Zhang *et al.*, 2015*a*; Xia *et al.*, 2019; Zhang *et al.*, 2019), some SP communication-related genes with confirmed functions were selected for the analysis, including SP biosynthesis-related genes (*SlitDes5* and *SlitDes11*) in the female SP gland and SP perception-related genes (*SlitOR6*, *SlitOR11*, *SlitOR13*, *SlitOR16*, and *SlitPBP1-3*) in the MA. The results showed that the expression of *SlitDes5* and *SlitDes11* reached the highest level in 3-day-old PGs of *S. litura* (fig. S1), whereas other SP perception-related genes reached a peak in 4- or 5-day-old MA and six genes (*SlitOR6*, *SlitOR11*, *SlitOR13*, *SlitOR16*, *SlitPBP2*, and *SlitPBP3*) exhibited a rapid increase after 3- or 4-day-old. Regarding the circadian clock genes of *S. litura*, the qRT-PCR results showed that in female PGs, *SlitPer* and *SlitCry2* had a similar dynamic expression pattern as that of *SlitDes5* and *SlitDes11* (fig. 5). However, in MA, all clock genes reached their peak levels at 2 or 3 days after eclosion, showing different expression patterns from those of SP perception-related genes (fig. 5).

Daily expression profiles of genes related to sex pheromone communication or circadian clock in S. litura

Similar to other nocturnal moths, SP-mediated sexual communication of *S. litura* occurred mainly during the scotophase in 2- to

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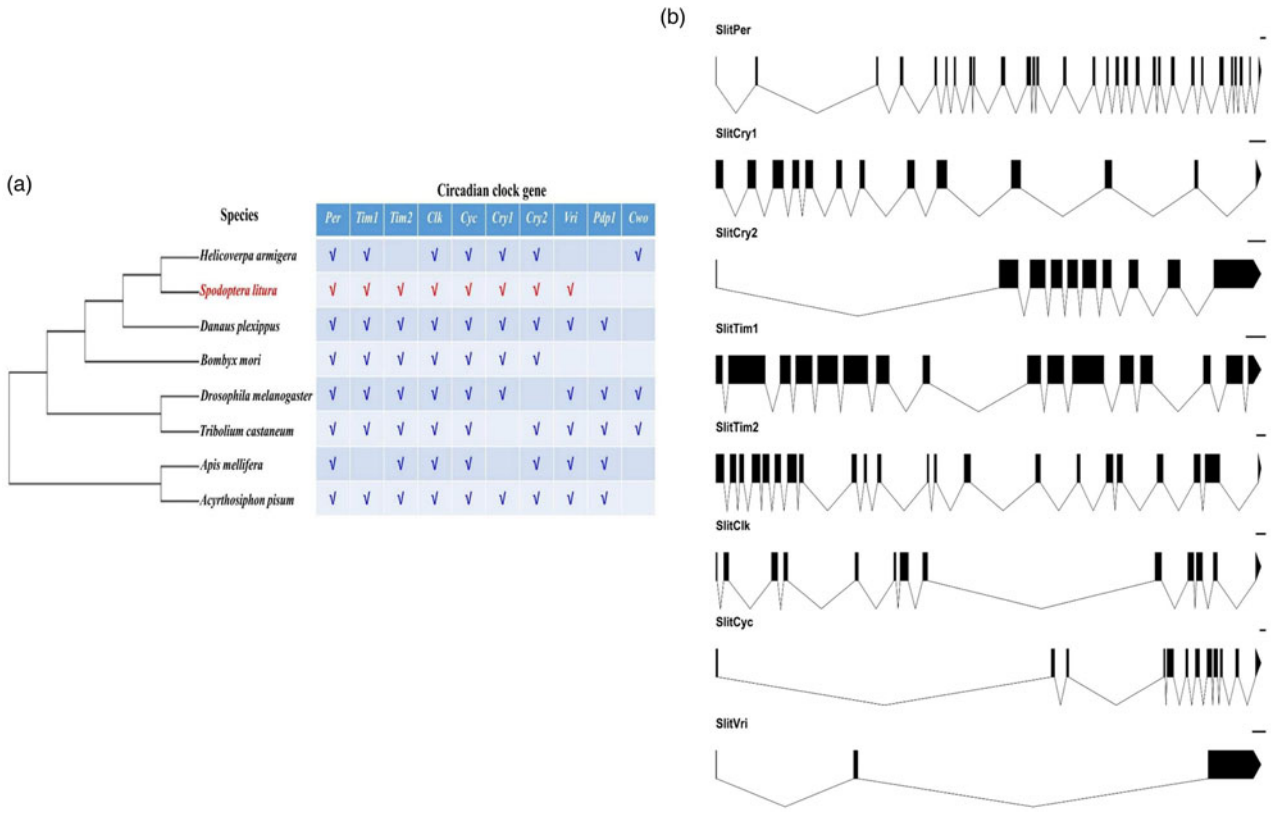

Figure 1. Sequence information and structural characteristics of circadian clock genes in *S. litura*. (a) Distribution and comparison of circadian clock genes between *S. litura* and other insect species. (b) Intron and exon distribution of circadian clock genes in *S. litura*, scale bars represent a size of 500 bases.

Table 1. The Blastx match of *S. litura* circadian clock genes.

				Best Blastx match		
Gene name	ORF (aa)	MW (KDa)	IEP	Identity (%)	Species	Acc. No.
SlitPer	1240	137.48	6.80	86	*H. armigera*	XP_021187927.1
SlitTim1	1276	144.11	5.23	88	*H. armigera*	XP_021189241.1
SlitTim2	1284	149.04	5.40	88	*H. armigera*	XP_021188135.1
SlitClk	619	69.51	5.64	92	*M. separata*	ARB18436.1
SlitCyc	702	75.47	7.13	86	*H. armigera*	XP_021194686.1
SlitCry1	548	62.86	6.51	99	*S. exigua*	ADY17887.1
SlitCry2	814	93.09	7.80	90	*H. armigera*	XP_021201222.1
SlitVri	417	46.59	6.37	99	*S. frugiperda*	AJB84657.1

H. armigera, *Helicoverpa armigera*; *M. separata*, *Mythimna separata*; *S. exigua*, *Spodoptera exigua*; *S. frugiperda*, *Spodoptera frugiperda*; ORF, open reading frame; aa, amino acid; MW, molecular weight; IEP, isoelectric point.

4-day-old adults (Sun *et al.*, 2002). To explore whether the expression of SP communication-related and clock genes was related to the alternation of the photophase and scotophase, we analyzed daily expression of genes with samples collected during 14 h photophase (5 points), 10 h scotophase (6 points), and the ensuing 6 h photophase (2 points).

The qRT-PCR results showed that *SlitDes5* and *SlitDes11* in female PGs reached their expression peaks 6 and 2 h after the onset of scotophase, respectively (fig. S2). In MA, three SP perception-related genes (*SlitOR6*, *SlitOR13*, and *SlitPBP1*) peaked at the first point of the photoperiod, decreased gradually, peaked in the second hour of scotophase, and then decreased rapidly. However, *SlitPBP2* reached its expression peak during the first hour of scotophase, and the expression peak for other SP perception-related genes (*SlitOR11*, *SlitOR16*, and *SlitPBP3*) did not occur during the scotophase; rather, a peak was reached during the photoperiod. The rise and decrease of the peak were not as rapid as that of other genes, suggesting the expression trend of the three genes in the entire LD cycle was different from that of the other genes (fig. 5).

We then measured the daily expression of all the *S. litura* clock genes in PGs and MA. Among all clock genes, *SlitPer* had the

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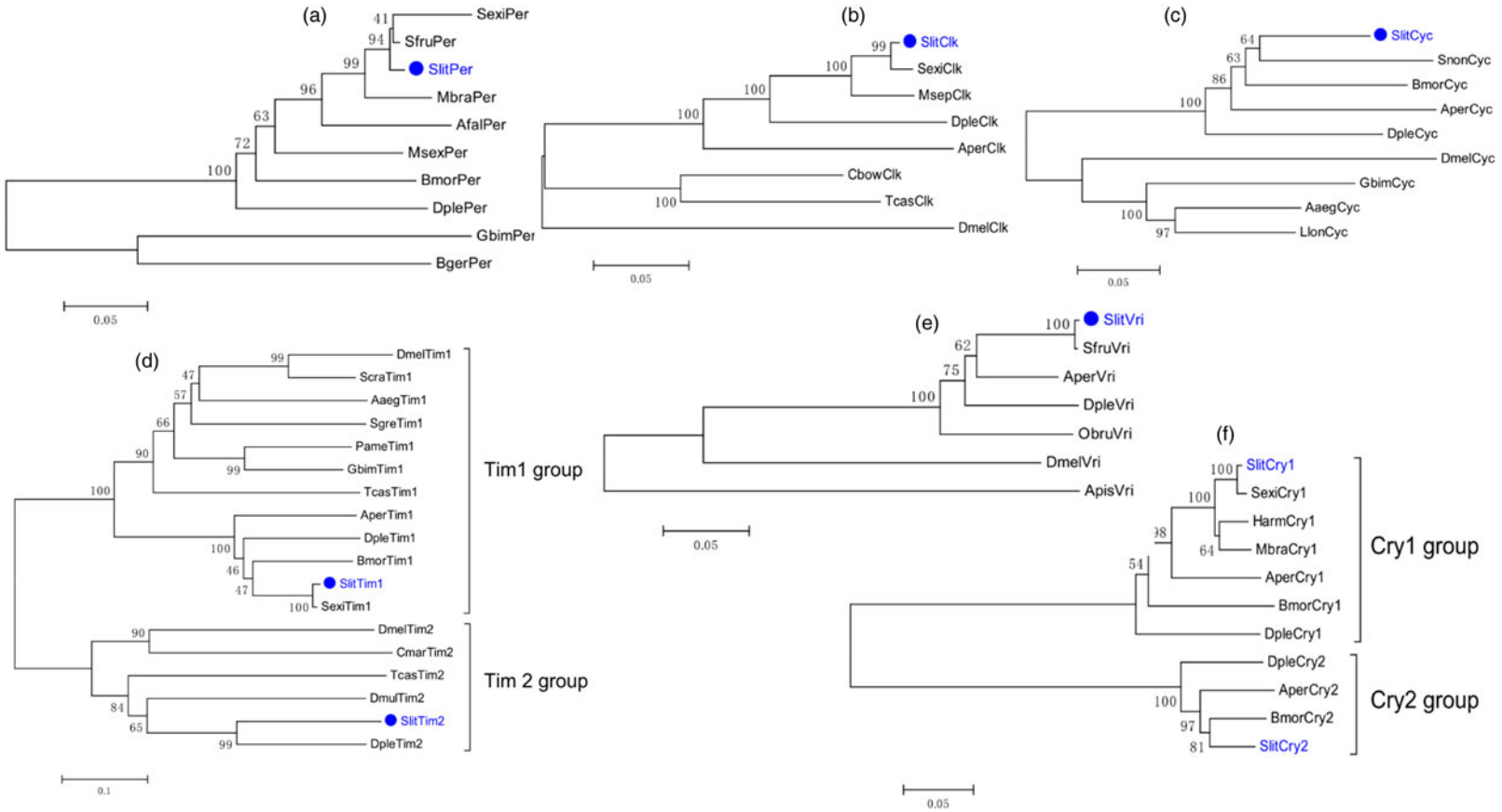

Figure 2. Phylogenetic trees of insect circadian clock genes. The *S. litura* translated genes are shown in blue. Other insects are as follows: *Spodoptera exigua*, Sexi; *Spodoptera frugiperda*, Sfru; *Mamestra brassicae*, Mbra; *Anagrapha falcifera*, Afal; *Manduca sexta*, Msex; *Bombyx mori*, Bmor; *Danaus plexippus*, Dple; *Gryllus bimaculatus*, Gbim; *Blattella germanica*, Bger; *Antheraea pernyi*, Aper; *Mythimna separata*, Msep; *Colaphellus bowringi*, Cbow; *Tribolium castaneum*, Tcas; *Drosophila melanogaster*, Dmel; *Sesamia nonagrioides*, Snon; *Aedes aegypti*, Aaeg; *Lutzomyia longipalpis*, Llon; *Sarcophaga crassipalpis*, Scra; *Schistocerca gregaria*, Sgre; *Periplaneta Americana*, Pame; *Clunio marinus*, Cmar; *Diachasma muliebre*, Dmul; *Operophtera brumata*, Obru; *Acyrthosiphon pisum*, Apis; *Helicoverpa armigera*, Harm. These trees were constructed using MEGA6.0 based on the alignment results of ClustalX2.0.

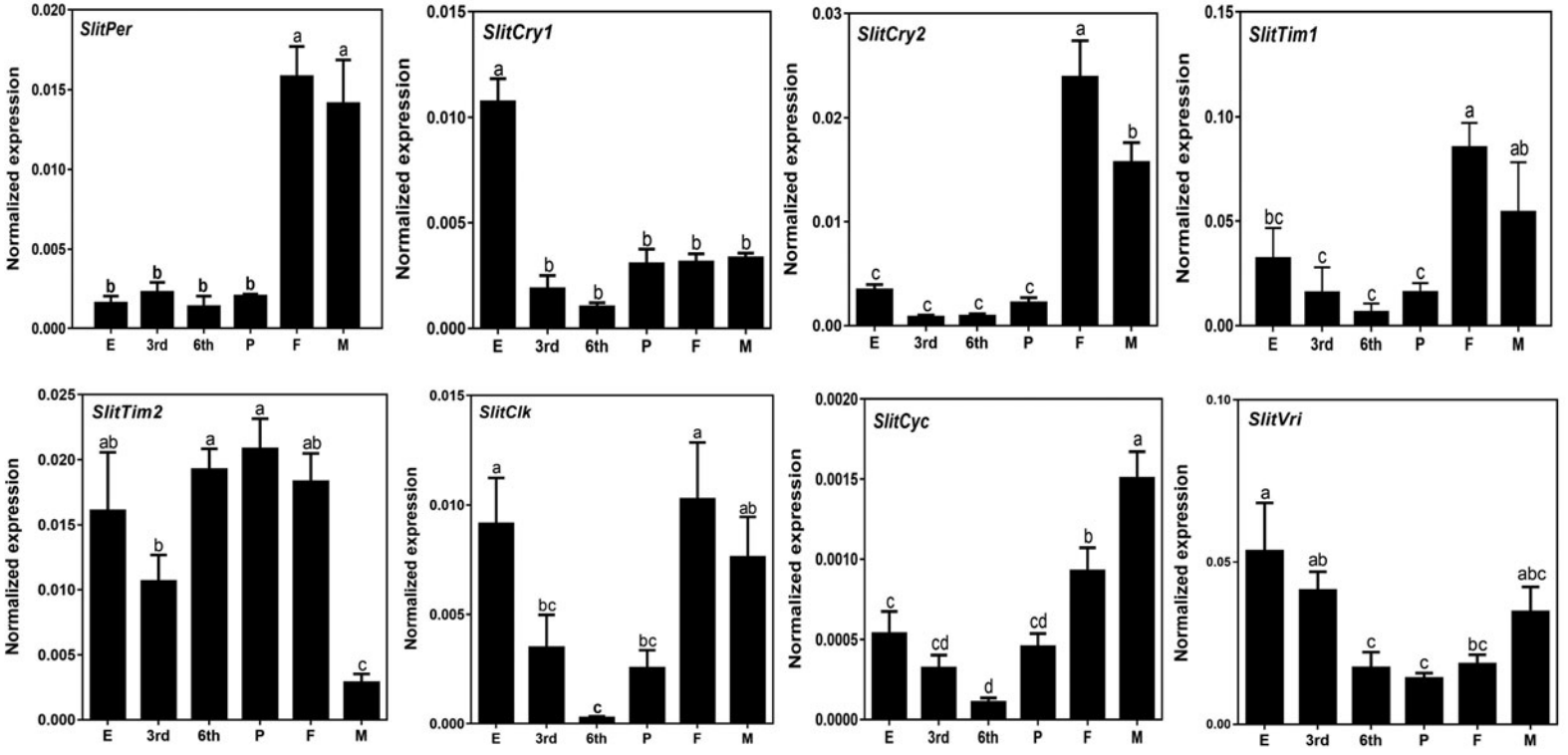

Figure 3. The expression analysis of circadian clock genes at different developmental stages of *S. litura* or others. The different lowercase letters mean significance between samples ($P < 0.05$, ANOVA, LSD). E, egg; 3rd, third larva; 6th, sixth larva; P, pupa; F, female; M, male. Horizontal axis: the different developmental stages of *S. litura*; vertical axis: the normalized expression of target gene was calculated by Q-gene software.

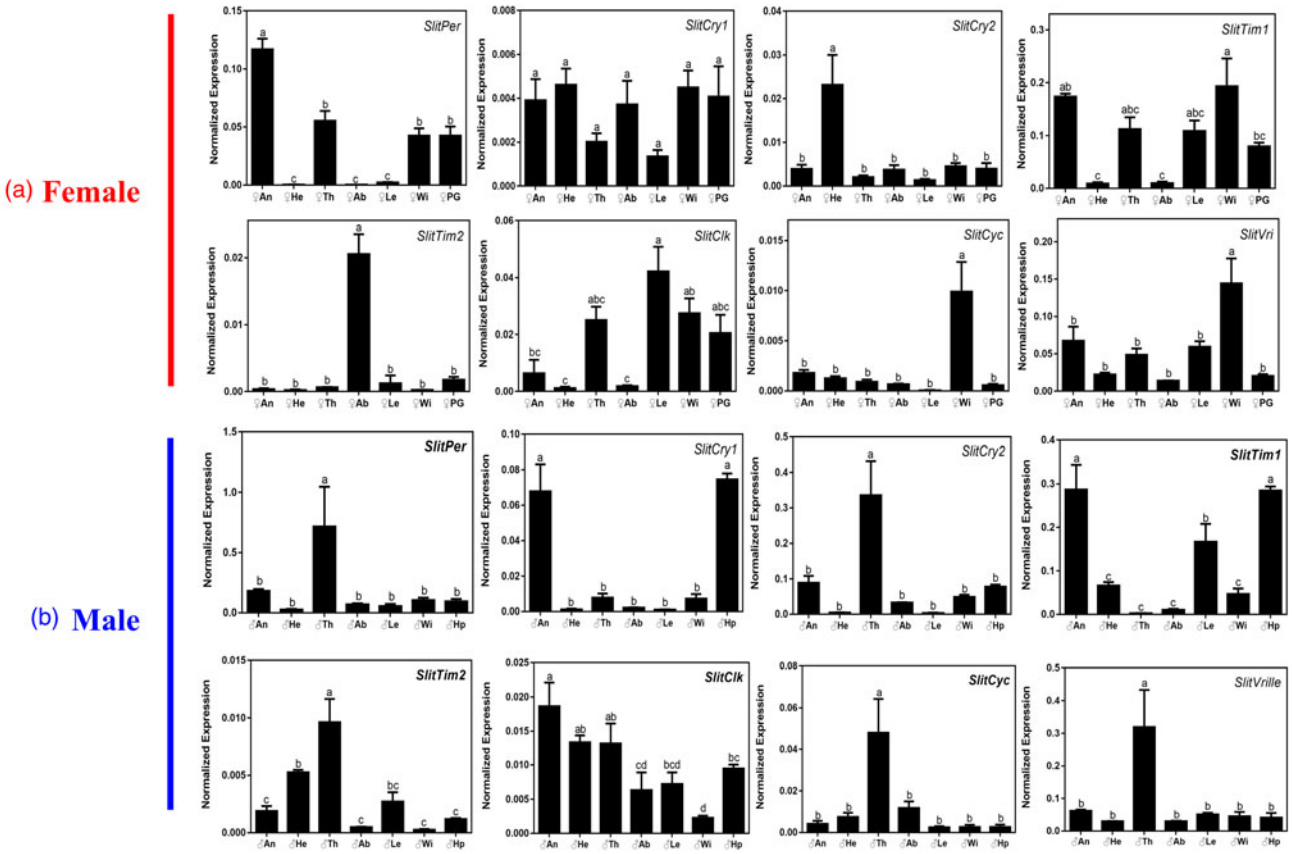

Figure 4. Tissue expression of circadian clock genes in *S. litura*. The different lowercase letters mean significance between tissues ($P < 0.05$, ANOVA, LSD). An, antenna; He, head without antenna; Th, thorax; Ab, abdomen; Le, leg; Wi, wing; Hp, hairpencil; PG, sex pheromone gland. (A) Female tissues, (B) Male tissues. Horizontal axis: the different tissue of adults *S. litura*; vertical axis: the normalized expression of target gene was calculated by Q-gene software.

most similar daily expression pattern to *SlitDes5* and *SlitDes11* in PGs of *S. litura*. In the female PGs, the expression characteristics of *SlitPer* were that peak expression occurred at the sixth hour of the scotophase and the expression level increased rapidly after entering the scotophase and then decreased rapidly at the end of the scotophase (fig. 6). Although the expression peak of *SlitCry2* appeared once in the photophase and once in the scotophase, the expression of *SlitCry2* rapidly increased and then decreased after entering the scotophase, which was similar to the expression patterns of *SlitDes5* and *SlitDes11* in the scotophase (fig. S2). In the MA of *S. litura*, the qRT-PCR results showed that four clock genes (*SlitPer*, *SlitTim2*, *SlitClk*, and *SlitCry2*) and three SP perception-related genes (*SlitOR6*, *SlitOR13*, and *SlitPBP1*) had similar expression features; they decreased from the peak value in the first photoperiod and then reached the peak value at 14:00 after entering the scotophase (figs 7 and S2). There was no significant difference between the peak values in photoperiod and scotophase in the four clock genes. However, the peaks of *SlitTim1* and *SlitVri* only appeared in the scotophase (14:00), but not in the photophase, and the peaks of *SlitCyc* and *SlitCry1* appeared only in the photophase (1:00) (fig. 7).

Discussion

To determine the possible involvement of the circadian clock machinery of sexual communication based on the SPs in *S. litura*, it is necessary first to understand the classification, molecular structure, and expression characteristics of circadian clock genes in key sexual communication tissues (PG and MA). In the present study, we obtained eight candidate circadian clock genes in *S. litura* based on genomic data (Cheng *et al.*, 2017), including *SlitPer*, *SlitTim1*, *SlitTim2*, *SlitCry1*, *SlitCry2*, *SlitCyc*, *SlitClk*, and *SlitVri*. Comparison with other Lepidopteran species whose genome sizes are presently known (Tomioka and Matsumoto, 2015) showed that the number of clock genes in *S. litura* is higher than that of *H. armigera* (7 clock genes) and *B. mori* (7 clock genes), but less than *D. plexippus* (9 clock genes). The putative amino acid sequences and phylogenetic tree analyses of clock genes of *S. litura* were highly similar to their homologs obtained from other moths examined. The data suggested that the clock genes may evolve species-specific categories based on conserved functions, such as between moths or between moths and butterflies.

Many studies on different insects have shown that circadian clock genes are involved in the regulation of some important physiological activities at various developmental stages (Groot, 2014; Tomioka and Matsumoto, 2015). For example, *Per* mutations could affect the basic oscillator, such as the pupal eclosion rhythm and adult activity rhythm (Konopka and Benzer, 1971); *Vri* could affect wing morphology by interacting with the biological skin growth factor decapentaplegic (dpp) signal transduction pathway (George and Terracol, 1997); *Cry* could regulate the feeding and metabolism of *Drosophila* (Seay and Thummel, 2011); eggshells could also be related to the regulation of insect biological clocks (Cuesta *et al.*, 2014); *Per*, *Tim*, and *Cry2* could

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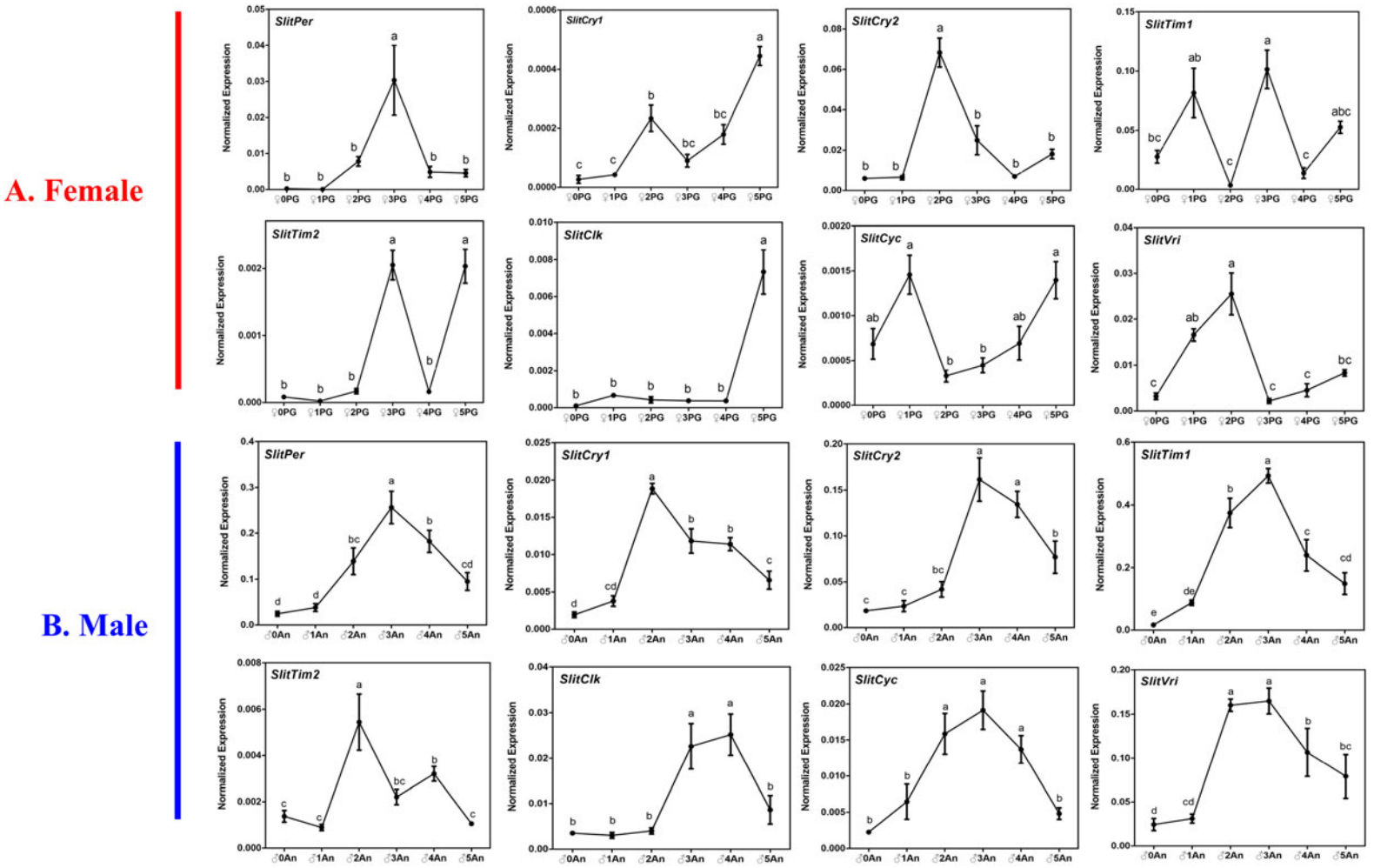

Figure 5. Dynamic expression of circadian clock genes in adult female sex pheromone gland (PG) and male antennae (MA) of *S. litura*. The different lowercase letters mean significance between tissues ($P < 0.05$, ANOVA, LSD). ♀, Female adult; ♂, male adult; PG, sex pheromone gland; An, antenna. Horizontal axis: the PG or An from virgin female or male moths at 5–7 h of the scotophase at different ages (0- to 5-day-old); vertical axis: the normalized expression of target gene was calculated by Q-gene software.

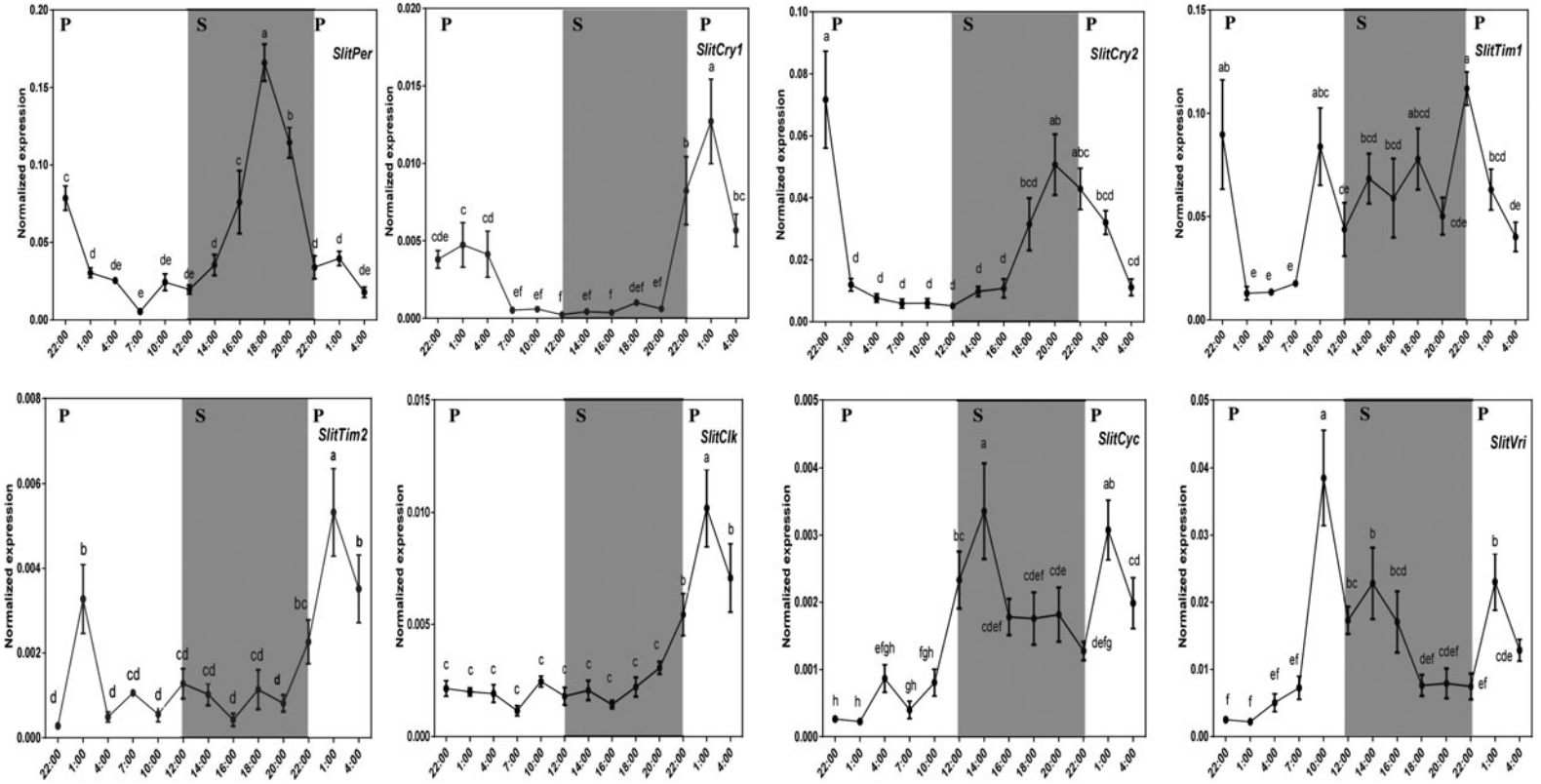

Figure 6. Daily expression of circadian clock genes in adult female sex pheromone gland (PG) of *S. litura*. The different lowercase letters mean significance between tissues ($P < 0.05$, ANOVA, LSD). P, photophase; S, scotophase. Horizontal axis: the different points of samples were collected; vertical axis: the normalized expression of target gene was calculated by Q-gene software.

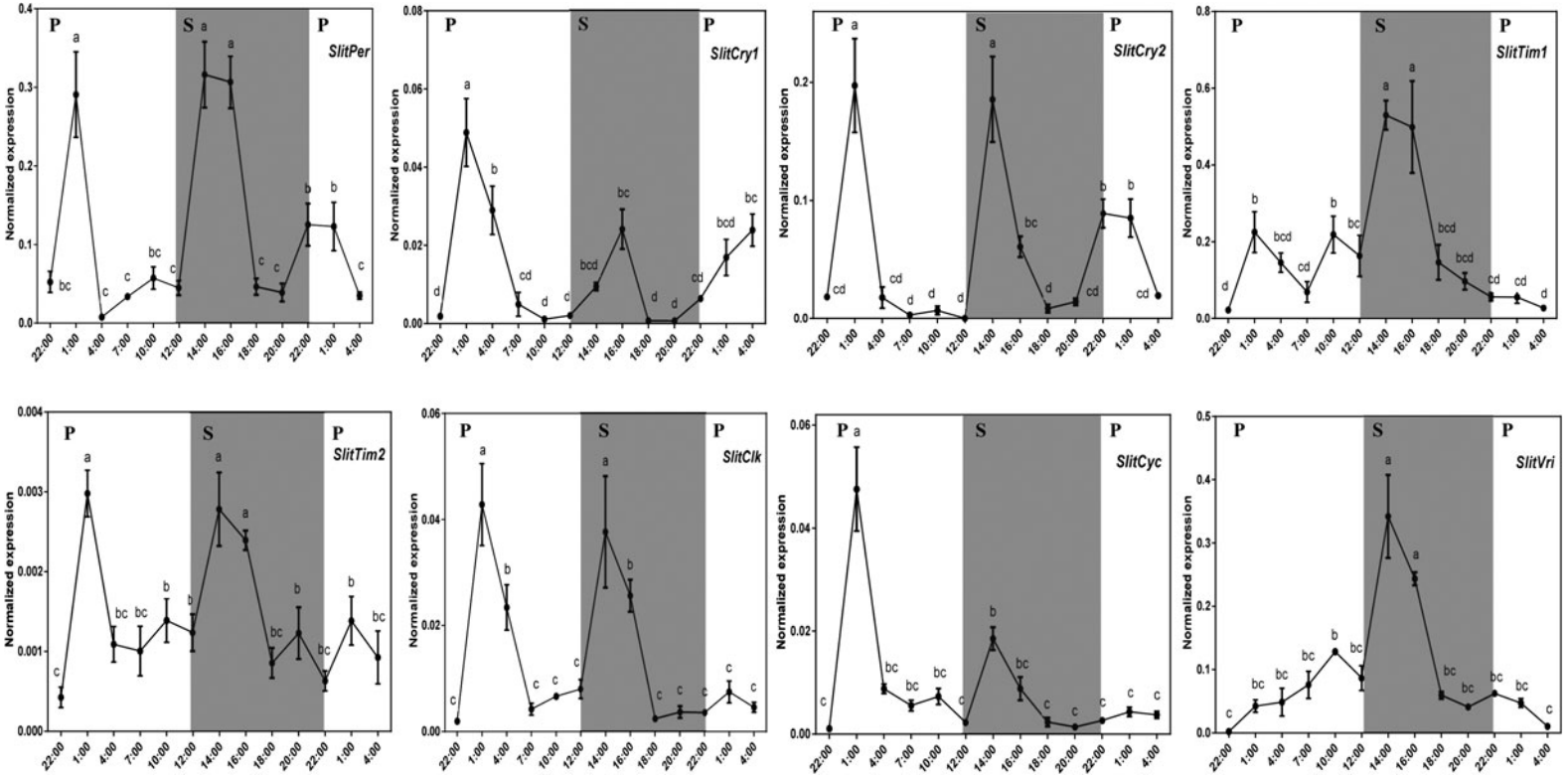

Figure 7. Daily expression of circadian clock genes in adult male antennae (MA) of *S. litura*. The different lowercase letters mean significance between tissues ($P < 0.05$, ANOVA, LSD). P, photophase; S, scotophase. Horizontal axis: the different points of samples were collected; vertical axis: the normalized expression of target gene was calculated by Q-gene software.

be related to the diapause of *Culex pipiens* (Meuti *et al*., 2015); and the *Tim* gene knockout could disrupt eclosion behavior in *Drosophila* and affect metamorphosis in *T. castaneum* (Jiang *et al*., 2018; Li *et al*., 2018*a*). Our results showed that all clock genes could be detected at each developmental stage of *S. litura*, and some circadian clock genes were highly expressed in specific developmental stages, different sexes, and tissues of adults, indicating that circadian clock genes of *S. litura* may have similar functions to those reported above; that is, they may be involved in regulating the physiological activities of *S. litura* at different developmental stages from egg to adult, such as *SlitCry1*, four clock genes (*SlitPer*, *SlitCry2*, *SlitTim1*, and *SlitCyc*), and three genes (*SlitCry2*, *SlitTim2*, and *SlitCyc*) may regulate the development of eggs, adults, and some sex-related behaviors, respectively.

It is well known that the precise and complex SP communication system plays an important role in sexual communication and species differentiation in insects, especially moths (Vogt, 2005). This system ensures that moths can perform courtship and mating rhythmically (Groot, 2014). Previous studies have shown that female moths can use specific SP biosynthesis-related genes to precisely biosynthesize SPs at different ratios, such as the desaturase gene (*Des*) and fatty acyl reductase gene (*FAR*) (Roelofs, 1995; Roelofs and Jurenka, 1996; Merlin *et al*., 2007). Male moths usually use specific SP perception-related genes to accurately recognize the conspecific female SPs, such as PBPs and pheromone receptors (PRs) (Vogt, 2005; Leal 2013). However, whether the expression of these SP communication-related genes is rhythmic and whether they are regulated by the circadian clock is still unclear. According to previous studies (Liu *et al*., 2013; Zhang *et al*., 2015*a*; Xia *et al*., 2019; Zhang *et al*., 2019), nine genes (two *Dess*, four *PRs*, and three *PBPs*) related to SP communication in *S. litura* have been functionally confirmed; thus, we chose these genes and all the clock genes of *S. litura* for dynamic expression analysis in this study.

Previous studies confirmed that the peak of courtship and mating of *S. litura* adults is at 2- to 4-day-old of age (Sun *et al*., 2002), and our results showed that two SP biosynthesis-related genes (*SlitDes5* and *SlitDes11*) and two clock genes (*SlitPer* and *SlitCry2*) have similar dynamic expression trends, with their peak expression levels at 2- to 4-day-old of age. Furthermore, the daily dynamic analysis of these four genes showed similar expression trends in the scotophase, and all had a peak expression before the 8th hour of the scotophase, which is just before the peak of female courtship (Sun *et al*., 2002). The courtship and mating of moths is a behavioral response mediated by SP signals (Ando and Yamakawa, 2011), which supports the notion that *SlitDes5* and *SlitDes11* are the key genes in the biosynthesis process of *S. litura* SPs (Zhang *et al*., 2015*b*; Xia *et al*., 2019; Zhang *et al*., 2019). Therefore, we infer that *SlitDes5* and *SlitDes11* can promote the content of SlitDes5 and SlitDes11 by increasing the mRNA expression level before the female moths enter the courtship peak to ensure the biosynthesis of SPs. Given that the expression of *Per* and *Cry2* is usually regulated by CLK and CYC in insects (Tomioka and Matsumoto, 2015), we presume that CLK and CYC may be the transcription factors for the two *SlitDess*, and *SlitPer* and *SlitCry2* may indirectly regulate the expression of *SlitDes5* and *SlitDes11*.

Perret *et al*. (2003) confirmed that the olfactory bulb shows significant circadian changes in nocturnal animals. In insects, studies have shown that olfactory behavior and the expression of some olfactory genes have circadian rhythm characteristics. For example, Merlin *et al*. (2007) found that the transcripts of some clock and olfactory genes cycled circadianly in the antennae of *S. littoralis* (Merlin *et al*., 2007). These also had a circadian rhythm in the expression profiles of some olfactory genes in the antennae of *S. exigua*, similar to their behavioral rhythm (Wan *et al*., 2015); Nagari *et al*. (2017) provided evidence for circadian regulation of antennal olfactory responsiveness and odorant pulse-tracking capacity in bees or other hymenopteran insects (Nagari *et al*., 2017). According to our results, there is no similarity in the dynamic expression between SP perception-related and circadian clock genes in the MA of *S. litura*; however, three SP perception-related genes (*SlitOR6*, *SlitOR13*, and *SlitPBP1*) and four clock genes (*SlitPer*, *SlitTim2*, *SlitClk*, and *SlitCry2*) had similar daily expressions, suggesting the regulation of clock genes based on SP perception of *S. litura* may only occur at the male age and time of the SP perception peak. Clock genes may also participate in other physiological behaviors involving non-SP perception in MA during other periods. We also noticed that these three genes (*SlitOR6*, *SlitOR13*, and *SlitPBP1*) in *S. litura* were MA-specific or highly expressed and played a leading role in the process of SP perception (Liu *et al*., 2013; Zhang *et al*., 2015*a*). These findings indicate that the expression characteristics of these genes were consistent with the rhythm of courtship and mating behaviors of *S. litura* and may also be regulated by the clock genes (*SlitPer*, *SlitTim2*, *SlitClk*, and *SlitCry2*). Furthermore, we found that the expression profiles of some genes (*SlitCry1*, *SlitCry2*, and *SlitClk* in the female PG, and *SlitOR6*, *SlitOR13*, *SlitPer*, *SlitTim2*, *SlitClk*, and *SlitCyc* in the MA) were quite different between the two photophases. Generally, moths will increase 1 day (age) after a complete scotophase, whereas adult *S. litura* live for only 5 or 6 days, and the changes occurring at each age may affect physiological activities *in vivo*. Therefore, we infer that the reason for this phenomenon may be that the increase in age leads to a change in the physiological response of *S. litura in vivo*. Of course, this may also be caused by other factors. We plan to use different approaches to focus on the molecular mechanisms of this difference in the future.

In conclusion, we identified and analyzed the characteristics of the sequence, genome structure, and phylogeny of all candidate circadian clock genes from *S. litura* based on genomic data analysis. We comprehensively compared the developmental spectrum, tissue, and temporal expression as a first step toward understanding their potential role in regulating SP communication behavior. We demonstrated that some clock genes might regulate the biosynthesis and perception of SPs by regulating the rhythmic expression of SP communication-related genes. These findings will help us elucidate the molecular mechanism of circadian clock genes involved in the regulation of sexual communication behavior and provide potential target genes for developing effective sexual communication disruptors in the future.

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