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Full Length Research Paper

Timeless is a critical gene in the diapause of silkworm, *Bombyx mori*

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The diapause is a specific physiological phenomenon induced by circadian zeitgebers of temperature and photoperiod at the stage of maternal embryonic development. In this research, the authors investigated the linkage of diapause and the regulation of circadian rhythm by inspecting the expression profiles of a circadian clock gene timeless *(tim)* under diapause-inducing temperature and photoperiod in *Bombyx mori*. The results show that the level of *tim* mRNA was higher in 25LL than in 20LD or in 15DD during the late embryonic stages (EAT 2520-3240°C·h), which is the most effective inducing period of temperature and photoperiod on diapause in the progeny. The *tim* transcription level was significantly higher in darkness than in light during postembryonic development, which contradicted the results found in the pupa and adult stages at 25°C. Exposure of maternal embryos to a high temperature (25°C) resulted in up-regulated levels of *tim* mRNA in egg stage of progeny. However, this up-regulation only occurred before egg-age at 72 h, which was a pivot for diapause and diapause termination. In conclusion, diapause-inducing temperature and photoperiod of incubation directly affect *tim* gene expression throughout the life cycle of *B. mori*.

Key words: Circadian clock gene, timless gene, diapause, temperature, photoperiod, *Bombyx mori*.

INTRODUCTION

Most organisms demonstrate circadian rhythms in gene expression and behavior controlled by endogenous clocks (Kaushik et al., 2007). Circadian rhythms represent adaptations of living organisms to daily changes in environmental conditions (Saunders, 2002). Diapause is a physiological phenomenon in which growth and activity become temporarily suspended at a specific developmental stage. Many insects make effective use of diapause in their life cycles (Isobe et al., 2006).In order to survive during winter, many insects would cease development or initiate diapause at species- specific stages (Denlinger, 2002). The silkworm, *Bombyx mori*, is an egg-diapause species. The nature of the silkworm diapause is primarily determined by environmental and genetic factors. As is in many other insects, the *B. mori* diapause is not induced by the temporal environment, neither is it immediately broken after a return to favorable conditions. Indeed, it is induced by parental exposure to particular temperature and photoperiod conditions during the incubation stage. In typical bivoltine *B. mori* strains, maternal exposure to a high temperature (25°C) induces embryonic diapause in the progeny, and incubation at a low temperature (15°C) leads to non-diapause progeny eggs. However, entry of the eggs into diapause is dependent on the temperature after oviposition (Isobe et al., 2006). When the eggs are incubated at an intermediate temperature of 20°C, long-day illumination will stimulate diapause, while short-day illumination prevents diapause, although the precise mechanism of this switch remains yet unknown (Kitagawa et al., 2005; Isobe et al., 2006).

In *B. mori* diapause eggs, the metallo-glycoprotein time-interval measuring enzyme-esterase A4 (TIME-EA4)

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Abbreviations: tim, Timeless; **EAT,** effective accumulated temperature; **DDE,** diapause-destined eggs; **NDE,** non-diapause eggs; **DTE,** diapause-terminated eggs; **TIME-EA4,** time-interval measuring enzyme-esterase A4.

is supposed to be a diapause development-duration clock, because its ATPase activity is transiently elevated at the end of the required 5°C cold period (Kai et al., 1984, 1995; Ti et al., 2004), after hydrochloric acid treatment (Kai and Nishi, 1976; Xu et al., 1998), or hot-water stimulation (Xu et al., 2003).

Some studies reported that degradation of TIM protein by light in *Drosophila melanogaster* which resulted in resetting of the molecular oscillations. Many clock genes identified in *Drosophila*, such as period (*per*), timeless (*tim*), clock (*cyc*), double-time (*dbt*), vrille (*vri*) and cryptochrome (*cry*) are conserved in mammals and *tim* is the second clock gene identified in *Drosophila.* Sehgal et al. (1994) showed that *tim* mutants failed to express circadian rhythms in eclosion and locomotor activity. In addition, the nuclear localization of PER protein was blocked in *tim* mutants (Vosshall et al., 1994). This suggests that nuclear localization of some PER sequences is inhibited in the absence of *tim*. The *tim* gene product may interact with PER either directly or indirectly to facilitate nuclear entry of PER, or it may regulate factors that promote the cytoplasmic retention of PER (Takahashi, 1995). Iwai et al. (2006) cloned the silkworm circadian clock gene *tim* and studied the oscillation rhythms in a range of adult tissues under different photoperiods. However, no subsequent studies have been published pinpointing at the relationships between the zeitgebers of circadian rhythm and diapause. Therefore, we studied the effects of diapause-inducing temperature and photoperiod on the expression patterns of *tim* gene.

MATERIALS AND METHODS

Insects and diapause-inducing treatments

A pure bivoltine strain C108 of *B. mori* was used. Eggs were incubated under constant illumination at 25°C (25LL) for diapause eggs, in darkness at 15°C (15DD) for non-diapause eggs, and in 12-h light-dark cycles (LD = 12:12 h) at 20°C (20LD) for mixed populations. Illumination was provided by incandescent lamps at the level of the sample during the day (150 to 200 lux), while darkness was provided by dim red light (0.1 lux). Eggs from 25LL, 20LD and 15DD were sampled at 24, 36 and 72 h intervals. Samples were processed according to the same effective accumulated temperature (EAT), using the formula:

$$EAT = (T-10) \times t$$

Where, EAT is in °C·h, T represents the average temperature in 1 h (°C), and t represents protected hours (h).

Sampling of postembryonic stage and progeny eggs

Larvae from eggs incubated under 25LL, in darkness at 25°C (25DD), under constant illumination at 20°C (20LL), in darkness at 20°C (20DD), under constant illumination at 15°C (15LL) and under 15DD conditions were fed fresh mulberry leaves, and the developmental stages were synchronized at the molting stage. Larvae, pupae and adults were kept under the same conditions as their incubation stages. Whole body from first to third instar larvae, and tissues (organs) of gonads, head and fat body from fourth instar larvae to adults were collected at the indicated times and dissected in ice cold 0.75% NaCl.

After 5 h copulation, female adults from 25LL and 15DD were allowed to lay eggs at 25°C and under weak light to generate diapause-destined eggs (DDE) and non-diapause eggs (NDE), respectively. Eggs laid within 10 min were collected to obtain batches of progeny eggs for synchronous development. Progeny eggs were also collected from virgin adults. DDE were subjected to acid treatment to terminate diapauses and the eggs were named diapause-terminated eggs (DTE). Acid-treatment was performed at 24 h after oviposition, using 15% hydrochloric acid (w/v) at 46°C for 5 min.

RNA extraction and reverse transcription-polymerase chain reaction analysis

Samples were immediately frozen in liquid nitrogen, and stored at -80°C. Total RNA was extracted using RNAiso reagent and analyzed by electrophoresis in a nucleic acid analyzer (DU-730, Beckman Coulter, USA). After extraction, RNAs were treated with RNase-free DNaseI (TaKaRa) and semi-quantitative reverse transcription PCR (RT-PCR) was performed to determine the level of *tim* transcripts as compared to the reference, *Actin*3.

Total RNA (3 µg) was reversely transcribed using an M-MLV RT Kit (Fermentas, EU) according to the manufacturer's instructions. The method was as follow: PCR amplification was performed using specific primers (5'-TGACCATTGTGGCGAGAT-3' and 5'-ATTAGGCTTGACACGGAGAA-3'), which amplified 979 bp of the *tim* coding sequence. A 411 bp fragment of *B. mori Actin*3 was amplified in parallel using 5'-CCCCATCGAACACGGAATCG-3' and 5'-CGCTCGGCAGTGGTAGTGAA-3' primers. PCR conditions were 94°C for 3 min, 30 cycles of 94°C for 50 s, 55°C for 50 s and 72ºC for 1 min, and final extension at 72°C for 10 min. Products were detected by agarose gel electrophoresis and analyzed by a gel image system (GIS) software package (Tanon Company, China).

Electronic analysis of expression profile

The expression profile of *tim* was based on its cDNA sequence. A blast search using *tim* identified a probe ID for microarray-based gene expression profiles in 10 tissues from day-3 fifth-instar larvae in the SilkDB database http://silkworm.genomics.org.cn/ (Wang et al., 2005).

RESULTS

Effects of diapause-inducing temperature and photoperiod on *tim* expression during embryonic development

More than 99% of progeny eggs were in diapause under constant illumination at 25°C (25LL). A low temperature resulted in less than 2% of progeny eggs in diapause, while eggs incubated at an intermediate temperature (20°C) with natural-day illumination (LD = 12:12h) (20LD), gave 30 to 70% of diapause progeny eggs mixed with non-diapause progeny eggs. It demonstrates that classic diapause-inducing incubation conditions caused remarkable differences in the numbers of eggs entering diapause. We found that C108 eggs hatched at about

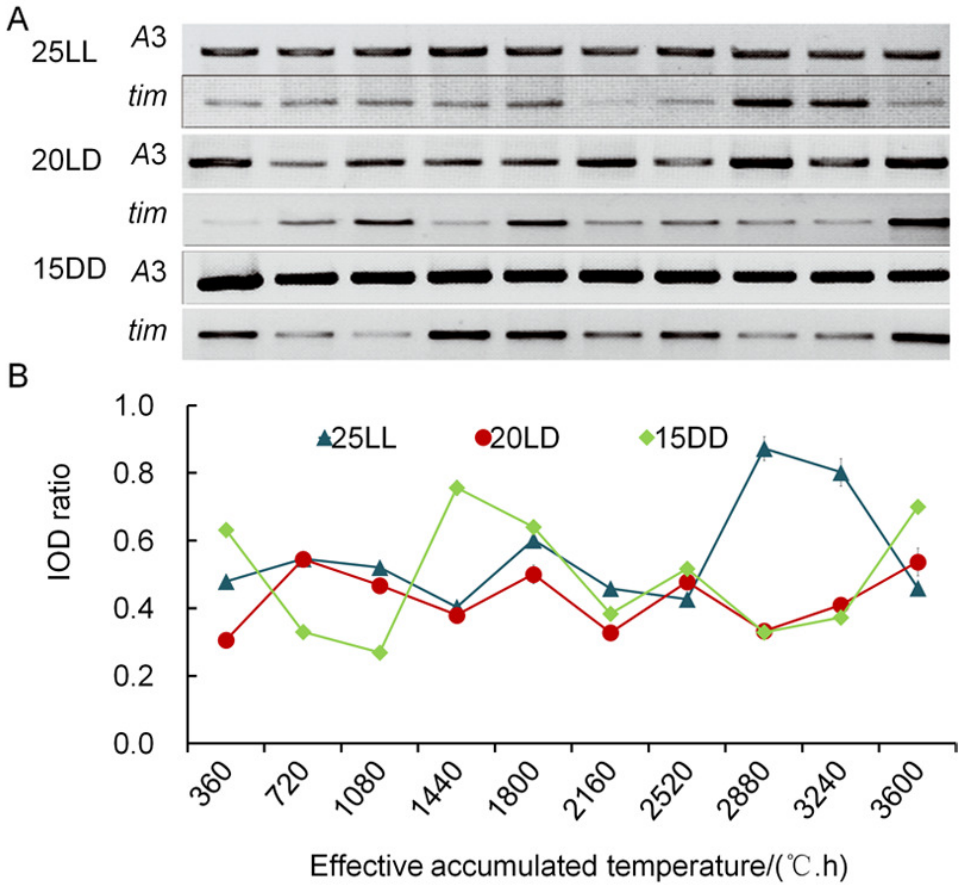

Figure 1. Effects of diapause-inducing temperature and photoperiod on *tim* expression in *B. mori* eggs. (A) Eggs were incubated under certain conditions of photoperiod and temperature, and the mRNA was measured by semi-quantitative RT-PCR; (B) the y-axis indicates the ratio of integrated optical density (IOD) of *tim* and *Actin*3. The data are from an average of three independent RT-PCR experiments.

3600℃·h EAT in all incubation conditions of 15DD, 15LL, 20LD and 25LL, although they developed at different rates. As a result, one embryonic developmental stage could be generated from the same EAT, but under different incubation conditions.

The level of *tim* mRNA was higher in 25LL than in 20LD or in 15DD after EAT 2520℃·h. During the early embryonic developmental stages (EAT 720 to 1080℃·h), the *tim* mRNA levels in 20LD were higher than in 15DD; however, this situation reversed after the middle embryonic development (1440 to 3600℃·h EAT) (Figure 1).

Effects of diapause-inducing temperature and photoperiod on *tim* expression during postembryonic development

After reversal of diapause by alterations in temperature and photoperiod, the changes associated with diapause at later stages of *B. mori* development, that is, larval, pupal and adult eclosion phases, were studied. To investigate this phenomenon, generations of *Bombyx* were reared under 25LL, 25DD, 20LL, 20DD, 15LL and 15DD conditions. We also analyzed the *tim* expression profiles during postembryonic development. The results show that *tim* transcription was higher in darkness than in the light, except in the pupa and adult stages at 25℃ (Figure 2).

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis showed that *tim* transcript levels in darkness were higher than in light, except in the pupa and adult stages at 25℃ (Figure 2). We therefore assumed that *tim* mRNA was strongly sensitive to light at high temperatures during the pupa and adult stages. The level of transcription at high temperature was significantly higher than at low temperature under the same light conditions (Figure 2). It was notable that the levels of *tim* transcription under constant illumination were lower at 20℃ than at 25℃, while the levels were up-regulated at

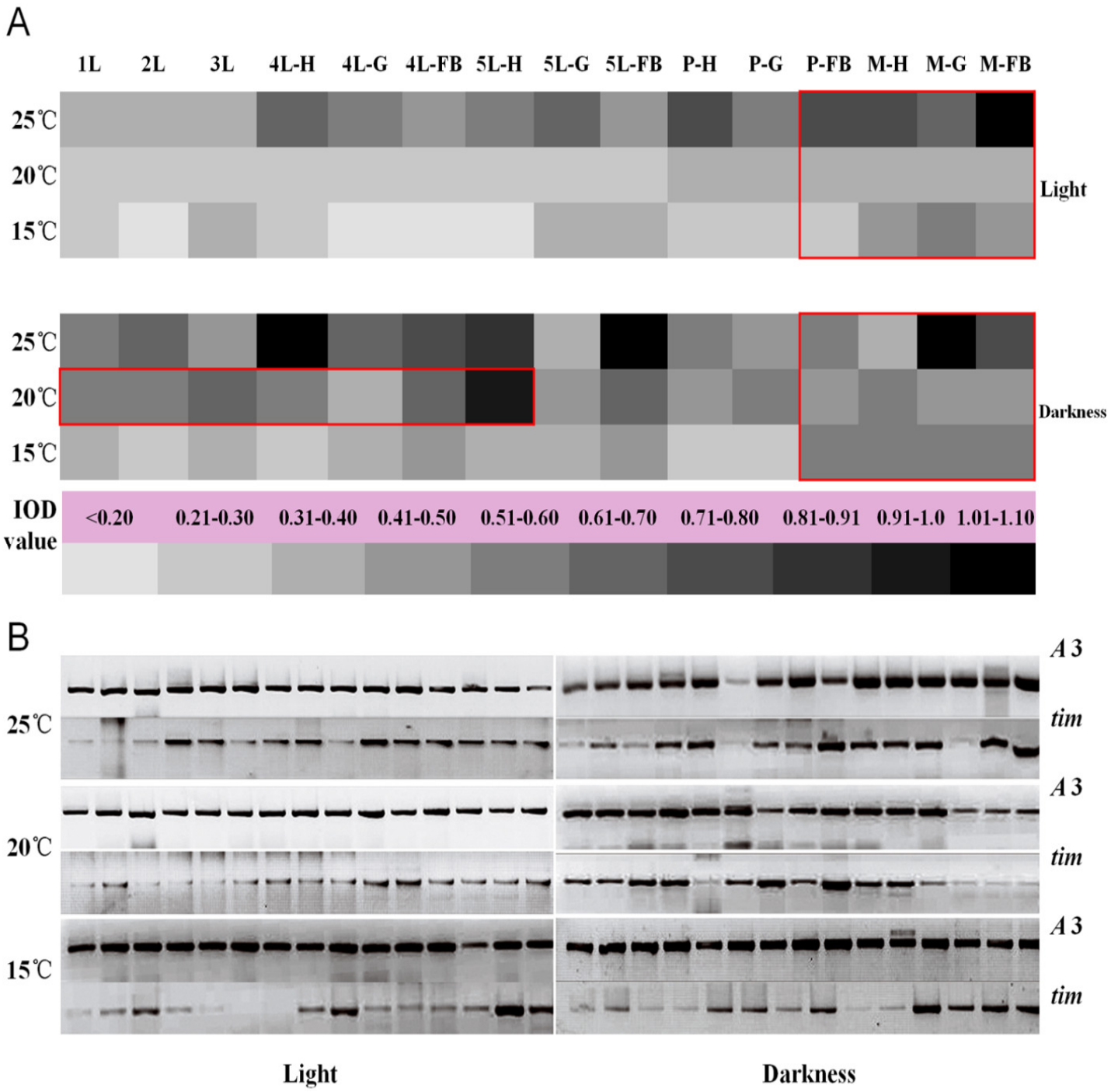

Figure 2. Effects of diapause-inducing temperature and photoperiod on *B. mori tim* during postembryonic development. (A) *tim* gene expression level detected by RT-PCR; (B) quantitative analysis of *tim* gene expression products; H, head; G, gonad; FB, fat body; 1L-5L,1st to 5th instar larval stage; P, pupal stage; M, moth stage. PCR amplification was run with 34 cycles, IOD value is the ratio of average integrated optical density (IOD) of products of genes *tim* and *Actin*3. Entire generation (eggs, larvae, pupae and adults) under 25LL, 25DD, 20LL, 20DD, 15LL or 15DD. 1L, first instar day-1 larvae; 2L, second instar day-2 larvae; 3L, third instar day-1 larvae; 4L, fourth instar day-3 larvae; 5L, fifth instar day-3 larvae; P, pupal stage day-1; M, moth stage day-1. Whole bodies from first to third instar larvae, tissues from fourth instar larvae to adult stage, were collected for *tim* mRNA.

20℃ under constant darkness. The levels were higher at 20℃ than at 25℃ in some periods, suggesting that 20℃ is a sensitive temperature.Using the online databases in SilkDB, we obtained microarray-based gene expression

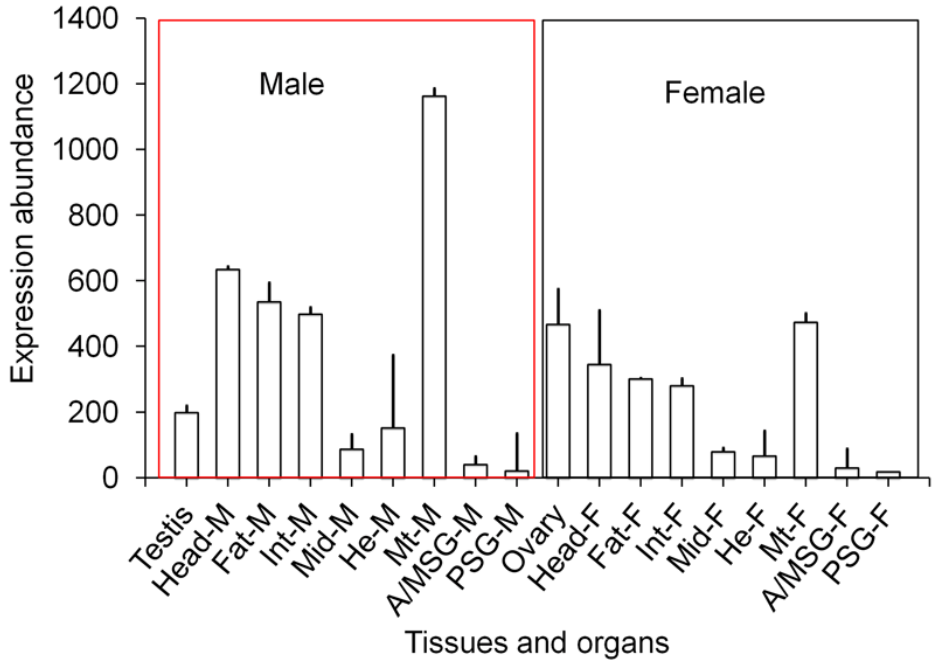

Figure 3. The microarray-based *tim* gene electronic expression profile from multiple tissues on day 3 fifth-instar larvae. T, Testis; O, ovary; H, head; FB, fat body; Int, integument; MG, midgut; He, hemolymph (blood); MT, malpighian tubule; A/MSG, anterior/middle silk gland; PSG, posterior silk gland; F, female; M, male.

profiles in multiple tissues on day 3 of the fifth instar larva. The *tim* gene was expressed in ten tissues, and the mRNA levels were relatively high in malpighian tubules, head and fat body, but low in silk gland, midgut and hemocytes. The *tim* mRNA level in the testis was significantly higher than that in the ovary (Figure 3).

Differential expression of *tim* during diapause and after diapause termination

DTE were generated by treatment of DDE with hydrochloric acid at 24 h of egg-age. Therefore, both DTE and DDE had the same gene expression profiles ranging from 0 to 24 h of egg-age. In this study, no significant differences in *tim* expression levels could be detected by RT-PCR between DTE and DDE within 72 h after oviposition (Figure 4A and B). However, the *tim* gene transcription level in DTE was significantly higher than that in DDE afterwards (Figure 4A and B).

In the non-diapause eggs (NDE), the *tim* mRNA level was slightly higher than that in DDE from egg-age of 120 to 168 h, while it was significantly lower at 0 h (Figure 4C).

The gene transcription level was up-regulated drastically less than that in diapause-terminated eggs at the comparative stages. Maternal exposure under certain photopeiod conditions at 15°C resulted in different transcription levels of the *tim* gene in non-diapause eggs of their progeny. Specifically, the *tim* mRNA level in progeny eggs was drastically up-regulated from 0.5 to 72 h of egg-age by maternal exposure to darkness (15DD), while it was almost consistent with that in 15LL after 96 h (Figure 4D).The levels of *tim* mRNA were different among 25LL, 20LD and 15DD within 72 h after oviposition, while there was no significant difference after egg-age at 96 h (Figure 4E). It suggests that the transcript levels of *tim* before egg-age at 72 h could be affected by both the diapause-inducing temperature and photoperiod, either individually or corporately.

In *B. mori*, sperm inseminates eggs at the moment of laying, but fertilization takes place at about egg-age at 2 h. In this study, high *tim* mRNA levels were detected in NDE and DDE of virgin adults (0 h eggs). These results reveal that *tim* mRNA was transcribed in the maternal generation and stored in eggs.

DISCUSSION

Expression of the circadian rhythm gene *tim* was directly related to diapause-inducing temperature and photoperiod

Studies with *D. melanogaster* have provided numerous insights into the time-keeping mechanisms governing circadian rhythms. Four circadian clock proteins in *D. melanogaster*, termed PERIOD (PER), TIMELESS (TIM),

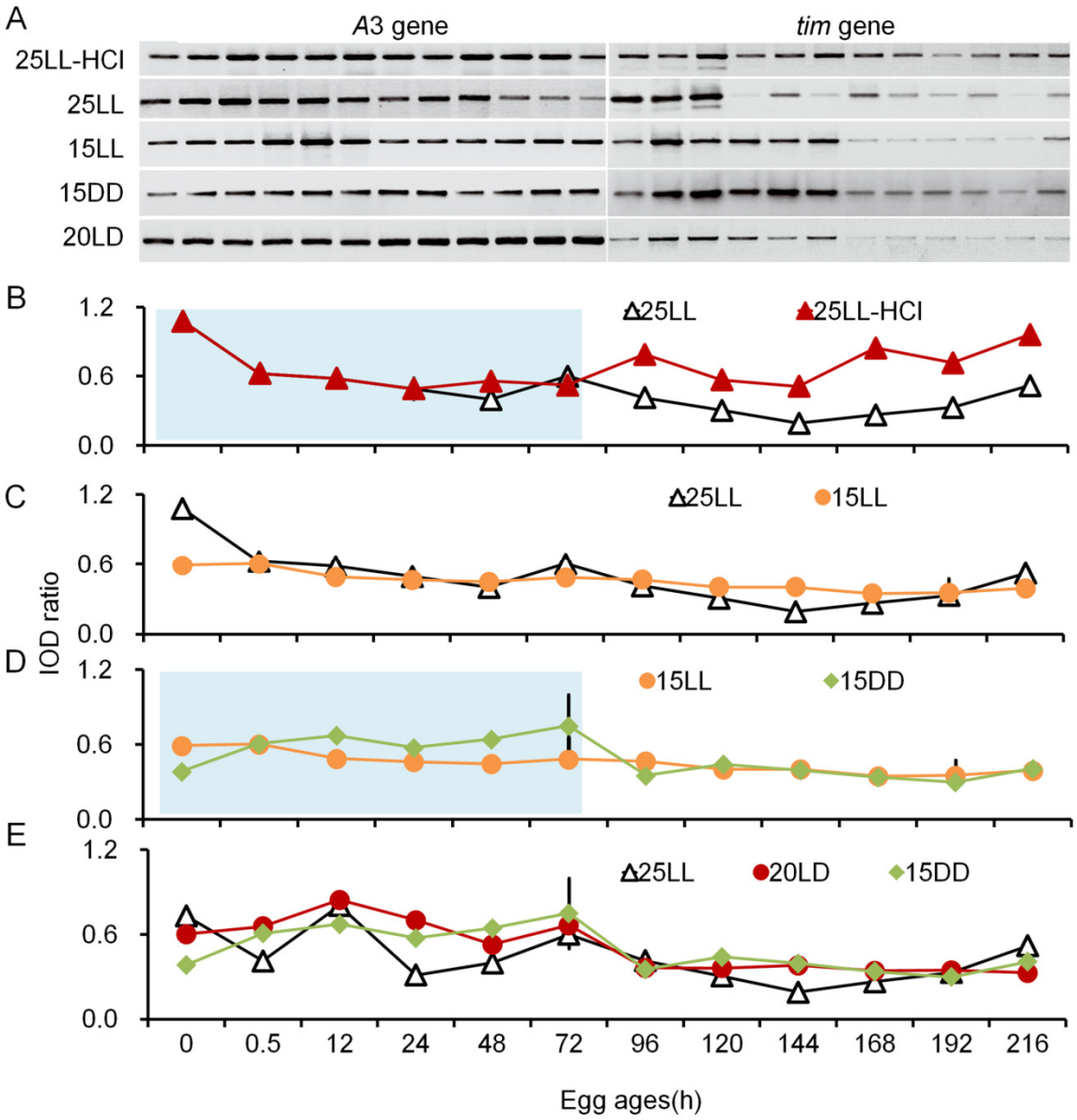

Figure 4. Differential expression of *tim* in diapause incidence and termination in *B. mori* eggs. (A) *tim* expression was detected by RT-PCR assay and electrophoresis; (B) to (E) quantitative analysis of *tim* gene mRNA. Numbers on Y-axis in the graph indicate the ratio of average integrated optical density (IOD) of RT-PCR products of genes *tim* and *Actin*3; AT, represents artificial hatching treatment with hydrochloric acid.

dCLOCK (dCLK) and CYCLE (CYC/dBMAL1) function in a transcriptional-translational feedback loop that constitutes a core element of the oscillator mechanism in this species. In *B. mori*, temperature and photoperiod are the two main *zeitgebers* that directly affect the oscillation rhythms in mRNA and protein expression levels (Qiu and Hardin, 1996; Kaushik et al., 2007; Boothroyd et al., 2007; Fan et al., 2007; Yoshii et al., 2007). In this study, temperature and photoperiod, which are closely related to diapause induction and decision during incubation, were used to investigate the *tim* expression profile during embryonic development. *tim* mRNA levels were higher in darkness than in light during postembryonic development, except in the pupa and adult stages at 25℃. Silkworm diapause is a heterogamous rhythm. It is closely related to circadian rhythm, but not completely explained yet. The gene regulatory network responsible for diapause induction in *B. mori* has not yet been determined, and it is likely to overlap with other gene regulatory networks, such as the circadian rhythm-related gene network and the endocrine regulation-related gene network. It has been suggested that more than 13 genes might be involved in diapause induction. The results of this study show that *tim* mRNA levels were higher in

darkness than in light throughout the postembryonic period, except in the pupal and adult stages at 25°C. The *tim* mRNA transcript levels under the same photoperiod conditions were significantly higher at high temperatures than at low temperatures, though the levels were augmented under constant darkness at 20°C. This suggests that the effect of darkness on *tim* expression was stronger than that of temperature.In addition, the expression levels of *tim* were higher in 15DD than in 25LL. These results suggest that *tim* might be the darkness-sensitivity gene in the diapause-induction gene network. Quantitative results indicate that *tim* gene expression was directly related to embryonic diapause-inducing temperature and photoperiod in *B. mori*.

In *B. mori*, sperm inseminates eggs at the moment of laying, but fertilization takes place at about egg-age 2 h. High *tim* mRNA levels were seen in NDE and DDE from virgin adults (0 h eggs), suggesting that *tim* mRNA was transcribed in the maternal generation, and stored in the eggs. Further studies are acquired to investigate the influence of *tim* on fertilization and early embryonic development. In our study, there was no difference in *tim* mRNA expression between DDE and DTE within 72 h after oviposition, though levels were significantly higher in DTE than DDE after egg-age at 72 h (Figure 4B).

Bombyx embryos that developed during maternal exposure to high-temperature (25°C), darkness or 20LD, produced progeny with high *tim* mRNA levels. However, this change only occurred before egg-age at 72 h. These results suggest that *tim* expression is closely related to embryonic diapause in *B. mori.*

B. mori diapause can be used to investigate the molecular mechanism of temperature as a zeitgeber

Despite the obvious importance of photoperiod, ambient temperature is also a key environmental factor that regulates circadian rhythm. This is not surprising because seasonal changes in day length at temperate latitudes are also accompanied by predictable changes in average daily temperature.

Circadian clocks can be highly sensitive to temperature changes; clocks in some insects, lizards and vertebrates can be entrained by a regular temperature cycle that oscillates by as little as 1 to 2°C. Light and temperature are the only periodic or quasi-periodic environmental variables to which the endogenous oscillation can be coupled: in nature, they entrain the endogenous oscillation, by controlling period and establishing the appropriate phase. According to Pittendrigh et al. (1958), biological clock oscillations might be both light and temperature sensitive.All circadian systems contain at least three elements: (a) an input pathway or set of input pathways that convey environmental information to the circadian pacemaker for entrainment; (b) a circadian pacemaker that generates the oscillation; (c) an output pathway or set of output pathways through which the pacemaker regulates its various output rhythms. All systems include a photic entrainment pathway as an input. Photic entrainment pathways differ remarkably among organisms, and these differences exist at both the receptor and signal-transduction levels. Diversity is even more notable at the output level, and clock-regulated outputs span the molecular to behavioral levels. As discussed earlier, the input and output pathways of the circadian system are organism-specific (Takahashi, 1991). Despite these differences in coupling pathways (inputs and outputs) of circadian clocks, the core mechanism of the pacemaker appears to be fundamentally similar in all organisms. Whether these similarities in pacemaker mechanisms are functionally analogous or phylogenetically homologous remain to be determined.

In nature, temperature and photoperiod are the two main zeitgebers affecting the expression of circadian genes. The results of this study demonstrate that *tim* gene expression was closely related to embryonic diapause in *B. mori*. Embryonic diapause in progeny can be induced by maternal exposure of the late embryo to specific incubation temperatures and photoperiods. Diapause is thus a useful character for studying the mechanism behind the thermal regulation of physiological processes. The results also provide a basis for further studies of diapause.

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