EUROPEAN JOURNAL OF ENTOMOLOGY
ISSN (online): 1802-8829
http://www.eje.cz
*Eur. J. Entomol.* **119**: 405–412, 2022
doi: 10.14411/eje.2022.042
ORIGINAL ARTICLE

# Genome-wide screening of genes involved in programming diapause in the next generation in silkworm, *Bombyx mori* (Lepidoptera: Bombycidae)

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**Key words.** *Bombyx mori*, diapause programming, embryonic diapause, folate, juvenile hormone

**Abstract.** Maternal silkworms (*Bombyx mori*) of bivoltine strains are destined to produce either diapause or non-diapause eggs depending on environmental factors, such as, temperature and photoperiod experienced during the egg and larval stages. However, the molecular mechanisms that program diapause, which depend on information about the environment, remain unclear. We aimed to identify genes that are involved in programming diapause in the next generation in bivoltine silkworms. We therefore screened differentially expressed genes (DEGs) in the larval brains of diapause- and non-diapause-egg producers kept under three different diapause-inducing conditions using cap analysis of gene expression. Under each condition, only temperature, illumination or photoperiod was changed during the egg or larval stage as a diapause-controlling stimulus to induce the production of diapause or non-diapause eggs. We then verified the expression of DEGs that were common to all the three conditions using real-time quantitative PCR. We investigated the functional involvement of candidate genes in programming diapause using double-stranded RNA interference (RNAi) for gene knockdown. The results showed more abundant *juvenile hormone acid methyltransferase* (*Jhamt*) and *proton-coupled folate transporter* (*Pcft*) gene expression in the brains of fifth instar larvae of producers of diapause eggs than those of non-diapause eggs under the three conditions. Furthermore, RNAi against either of these genes significantly decreased the incidence of diapause in the next generation. These findings indicate that both *Jhamt* and *Pcft* are involved in the programming of diapause in the silkworm brain. These genes could function in retaining information that leads to diapause in the next generation.

## INTRODUCTION

A dormant state of hormonally mediated developmental arrest is called diapause. Insects enter diapause at a species-specific developmental stage in order to survive adverse environmental conditions. Diapause is a phenotypic plastic response that is often programmed in response to environmental factors such as temperature and photoperiod (day-length or night-length) that are perceived long before the actual start of diapause (Tauber et al., 1986; Denlinger, 2022).

The mechanism of diapause has been investigated in *Bombyx mori* L. (Lepidoptera: Bombycidae) silkworms for several decades (Watanabe, 1924; Kogure, 1933; Fukuda, 1951, 1952; Hasegawa, 1951). Embryonic diapause in this species is under maternal control. In bivoltine strains, maternal silkworms are destined to produce either diapause or non-diapause eggs depending on environmental conditions during the egg and larval stages (Kogure, 1933; Yamashita & Hasegawa, 1966). The sub-oesophageal ganglion of silkworms is stimulated at an early stage in the development of the pupae to secrete the hormone that induces the production of diapause eggs, but the hormone secretion is programmed before pupation (Fukuda, 1951, 1952; Hasegawa, 1951). The transplantation of cultured brains from silkworm larvae indicates that the diapause program is created and memorized in the brain (Hasegawa & Shimizu, 1987). However, it is unclear how information on environmental conditions affects the molecular mechanisms that determine diapause status.

The present study aimed to identify genes that are involved in the programming of diapause in next generation eggs in bivoltine silkworms. We postulated that key genes involved in silkworm programming of diapause are differentially expressed in diapause-egg (DEPs) and non-

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diapause-egg producers (NDEPs) regardless of the type of stimulus that induces diapause (Akitomo et al., 2017). Therefore, we generated DEPs and NDEPs using three different diapause-inducing conditions in which silkworms were kept at either different temperatures or different intensities of illumination during the egg stage, or different photoperiods during the larval stage.

We then compared genome-wide transcriptome profiles of larval brains of DEPs and NDEPs using cap analysis of gene expression (CAGE) and screened them for differentially expressed genes (DEGs). The CAGE method enumerates short reads at the 5'-end of capped transcripts using next-generation sequencing, which quantifies the number of transcripts expressed at low levels and allows data analysis without bias due to transcript length (Carninci et al., 1997; Kodzius et al., 2006; Kanamori-Katayama et al., 2011; Itoh et al., 2012; Murata et al., 2014). In the present study, we focused on the brains of the fifth instar. This is because our previous study using DNA microarray analysis indicates that genes related to juvenile hormone or ecdysteroids are differentially expressed in the brains of fifth instar larvae of DEPs and NDEPs (Akitomo et al., 2017).

The CAGE screening identified DEGs that were common to all three diapause-inducing conditions. We believe that multiple screening using distinct diapause-controlling stimuli (temperature, illumination and photoperiod) detected candidate genes. We then verified DEG expression by real-time quantitative PCR (RT-qPCR). In addition, the functional involvement of candidate genes was investigated in the programming of diapause in silkworms using double-stranded RNA interference (RNAi) for gene knockdown.

## MATERIALS AND METHODS

### Animals

We used the bivoltine silkworm strain p50 provided by the National Bio-Resource Project (NBRP) of the Ministry of Education, Science, Sports and Culture of Japan (http://www.shigen.nig.ac.jp/silkwormbase/index.jsp). This strain can be induced to become either diapause-egg producers (DEPs, diapause incidence = 100%) or non-diapause-eggs producers (NDEPs, diapause incidence = 0%) by adjusting temperature, illumination or photoperiod during the egg or larval stages (Egi et al., 2014). We incubated eggs at 25°C or 18°C under continuous light or continuous darkness. Newly hatched larvae were reared at 25°C under a 12L : 12D cycle (lights on 8:00–20:00 h) or 20L : 4D (lights on 4:00–24:00 h). We fed the larvae Silkmate PS Artificial Diet (Nihonnousan Kogyo Co. Ltd., Yokohama, Japan). The illumination source was a white fluorescent lamp emitting 100–150 lux at the level of the insects.

The diapause status of eggs laid by resultant female moths (n = 27–65) in each experimental group was confirmed and the diapause incidence was defined as the percentage (%) of female moths that produced diapause eggs (Egi et al., 2014). Briefly, 3–5 h after eclosion each female copulated with a male for 2 h and laid eggs for 24 h. We stored eggs at 25°C for 14 days and then recorded their diapause status. Diapause eggs produce a characteristically ommochrome-pigmented serosal membrane, but non-diapause eggs develop without pigmentation and hatch in about 10 days.

### Experimental conditions

We generated DEPs and NDEPs under the conditions described below based on our previous findings for the p50 strain (Egi et al., 2014).

Egg thermal stimulation: eggs were incubated at 25°C or 18°C in the dark in order to generate DEPs or NDEPs, respectively. We reared hatched larvae at 25°C under 12L : 12D.

Egg illumination stimulation: eggs were kept under continuous light or continuous darkness at 18°C to generate DEPs or NDEPs, respectively. We reared hatched larvae at 25°C under 12L : 12D. Because rearing conditions during the egg and larval stages were identical for the NDEPs, the same samples were used for both egg thermal and illumination stimulation experiments.

Larval photoperiod stimulation: eggs were kept at 25°C under continuous darkness. We reared hatched larvae under 12L : 12D or 20L : 4D to generate DEPs or NDEPs, respectively, at 25°C. Larvae of diapause-egg producers reared under 12L : 12D were transferred to 20L : 4D on the day of the fourth larval moult or on day 0 of the fifth (last) instar, in order to collect tissue samples from both diapause and non-diapause producers under the same photoperiod of 20L : 4D on day 2 of the fifth instar.

### Tissue collection and RNA extraction

The brains of fifth instar female larvae were obtained at midday (14:00 h) on day 2 of the instar. The samples for RT-qPCR to evaluate RNAi efficiency were obtained on day 3 of the fifth instar. The brains were carefully dissected and the corpora allata and corpora cardiaca were removed. We collected one tissue pool containing 50 brains for CAGE analysis and tissue pools containing 10–20 brains (n = 3–6) for RT-qPCR for each experimental group. Total RNA was extracted using RNeasy Plus Micro Kits (Qiagen GmbH, Hilden, Germany).

### CAGE analysis

CAGE library preparation, sequencing, mapping and gene expression analysis were carried out by DNAFORM (Yokohama, Kanagawa, Japan). First strand cDNAs were transcribed to the 5' end of capped RNAs and attached to CAGE bar code tags (Murata et al., 2014). The generated libraries were sequenced using an Illumina HiSeq 2500 system (Illumina Inc., San Diego, CA, USA). The sequenced CAGE tags were mapped to the silkworm genome (https://sgp.dna.affrc.go.jp/KAIKObase/) using Burrows-Wheeler Aligner (BWA) v. 0.5.9 after discarding ribosomal RNAs or RNAs containing non-A/C/G/T bases (Li & Durbin, 2009). The CAGE-tag 5' coordinates were provided as input for CAGEr tag clustering with 20 bases as the maximal allowed distance between neighbouring tags and the minimum counts per million (CPM) was set at 1 (Haberle et al., 2015).

### Real-time quantitative PCR

We reverse-transcribed RNA into cDNA using ReverTra Ace qPCR RT Kits (Toyobo, Osaka, Japan). The RT-qPCR reaction proceeded using THUNDERBIRD SYBR qPCR Mix (Toyobo), the primers listed in Table S1 and a Thermal Cycler Dice Real Time System TP800 (Takara Bio Inc., Kusatsu, Japan). The reaction parameters were 95°C for 60 s followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Expression of the target gene was normalized to that of the *rp49* transcript.

### Preparation and injection of dsRNA

Double-stranded RNAs for silkworm genes were prepared using MEGAscript RNAi Kits (Applied Biosystems, Foster City, CA, USA). We produced PCR fragments using gene-specific primers with a T7 promoter sequence appended to the 5' end (Table S2) to serve as DNA templates for dsRNA transcription. We mixed the dsRNA with Metafectene PRO (Biontex GmbH,

Planegg, Germany) 1 : 1 (v/v) then injected 3 µL containing 600 ng of dsRNA into the thorax of each larva using a Hamilton syringe on day 0 of the fifth instar. Control dsRNA was generated from the jellyfish *GFP* gene (Tschuch et al., 2008). We assessed RNAi efficiency 3 days later by measuring the target gene expression in the larval brain using RT-qPCR. The diapause incidence was determined for the eggs laid by the resultant female moths.

Larvae from diapause-egg producers incubated at 25°C under continuous darkness during the egg stage and reared at 25°C under 12L : 12D during the larval stage, were injected with dsRNA on day 0 of the fifth instar. The silkworms were then transferred from 12L : 12D to 20L : 4D, which induces the production of non-diapause eggs in order to assess the effects of dsRNA in the absence of diapause inducing stimulation.

### Statistical analysis

The abundances of mRNA measured using RT-qPCR are expressed as means ± S.E.M. (n = 3–6 independent samples). The data were statistically analysed using Welch's *t*-test. The diapause incidence was compared among different groups using Fisher's exact test with Benjamini-Hochberg multiple testing correction. Values with $P < 0.05$ were considered significant.

## RESULTS

### Identification of DEGs in DEPs and NDEPs using CAGE

We obtained 33–70 million reads (sequenced CAGE tags) from each sample. The reads were mapped against the silkworm genome and counted at the transcription start sites for each gene. The expressions of ~ 6,500 genes were quantified in larval brains and gene expression profiles of DEPs and NDEPs compared for each experimental condition in order to isolate genes that were differentially expressed ≥ 1.5-fold and at a CPM (count per million) > 1.

### Egg thermal stimulation experiment

We generated DEPs and NDEPs by incubating eggs at 25°C and 18°C, respectively. The CAGE findings showed that 190 genes (2.96% of the total examined) were expressed more abundantly in DEPs than NDEPs, and 272 genes (4.23% of the total examined) were expressed more abundantly in NDEPs than DEPs.

### Egg illumination stimulation experiment

We generated DEPs and NDEPs by incubating eggs under continuous light and continuous darkness, respectively. The CAGE findings showed that 152 genes (2.37% of the total examined) were expressed more abundantly in DEPs than NDEPs, and 172 genes (2.68% of the total examined) were expressed more abundantly in NDEPs than DEPs.

### Larval photoperiod stimulation experiment

We generated DEPs and NDEPs by rearing larvae under 12L : 12D and 20L : 4D, respectively. The CAGE findings showed that 222 genes (3.46% of the total examined) were expressed more abundantly in DEPs than NDEPs, and 114 genes (1.77% of the total examined) were expressed more abundantly in NDEPs than DEPs.

We analysed the DEGs that were common to all three conditions and found that 9 genes were expressed more abundantly in DEPs than NDEPs (Table S3), and 20 genes were expressed more abundantly in NDEPs than DEPs (Table S4).

However, most transcripts detected by CAGE are non-coding RNAs (Carninci et al., 2005; Katayama et al., 2005; Forrest et al., 2014; Arner et al., 2015) and CAGE reads of low numbers do not always accurately reflect amounts expressed. Therefore, we initially verified the expression of DEGs screened by CAGE using RT-qPCR before investigating further.

### Verification of CAGE data using RT-qPCR

The expression of only 4 of the genes screened by CAGE coincided with that assessed by RT-qPCR (Figs 1, S1; Table 1). These genes were identified as juvenile hormone acid methyltransferase (*Jhamt*), Krüppel homolog 1 (*Kr-h1*), proton-coupled folate transporter (*Pcft*) and uncharacterized LOC110385080 (*LOC-80*) with unknown function. The expression of these genes was more abundant in DEPs than NDEPs under all three conditions. Therefore, we further investigated them as candidate genes. We did not find any common genes that were expressed more abundantly in NDEPs than DEPs.

**Table 1.** Comparison of gene expression in larval brains of diapause- and non-diapause-egg producers using CAGE and three experimental conditions.

| Gene name | Reference sequence | Egg thermal stimulation | | | Egg illumination stimulation | | | Larval photoperiod stimulation | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 25°C (Diapause) | 18°C (Non-diapause) | Fold change 25°C/18°C | Light (Diapause) | Darkness (Non-diapause) | Fold change Light/Darkness | 12L : 12D (Diapause) | 20L : 4D (Non-diapause) | Fold change 12L : 12D/20L : 4D |
| *Juvenile hormone acid methyltransferase* | NM_001043436.1 | 2.73 | 0.72 | 3.79 | 4.34 | 0.72 | 6.03 | 1.53 | 0.24 | 6.38 |
| *Krüppel homolog 1* | NM_001177861.1 | 15.25 | 3.32 | 4.59 | 15.63 | 3.32 | 4.71 | 15.77 | 8.07 | 1.95 |
| *Proton-coupled folate transporter* | XM_004933326.3 | 2.73 | 1.40 | 1.95 | 3.88 | 1.40 | 2.77 | 3.45 | 0.80 | 4.31 |
| *Uncharacterized LOC110385080* | XM_021347590.2 | 2.90 | 0.87 | 3.33 | 4.11 | 0.87 | 4.72 | 1.81 | 0.56 | 3.23 |

Findings of cap analysis of gene expression (CAGE) of listed genes were confirmed by real-time quantitative PCR (Fig. 1). Expression of all four genes was ≥ 1.5-fold higher in producers of diapause eggs than non-diapause eggs under the three conditions. In the egg thermal stimulation experiment, diapause- and non-diapause-egg producers were induced by incubating eggs at 25°C and 18°C, respectively. In the egg illumination stimulation experiment, diapause- and non-diapause-egg producers were induced by incubating eggs under continuous light and continuous darkness, respectively. In the larval photoperiod stimulation experiment, diapause- and non-diapause-egg producers were induced by rearing larvae under 12L : 12D and 20L : 4D, respectively. Values quantified by CAGE are numbers of reads (counts) per million counted at transcription start sites for each gene. LD – light/dark.

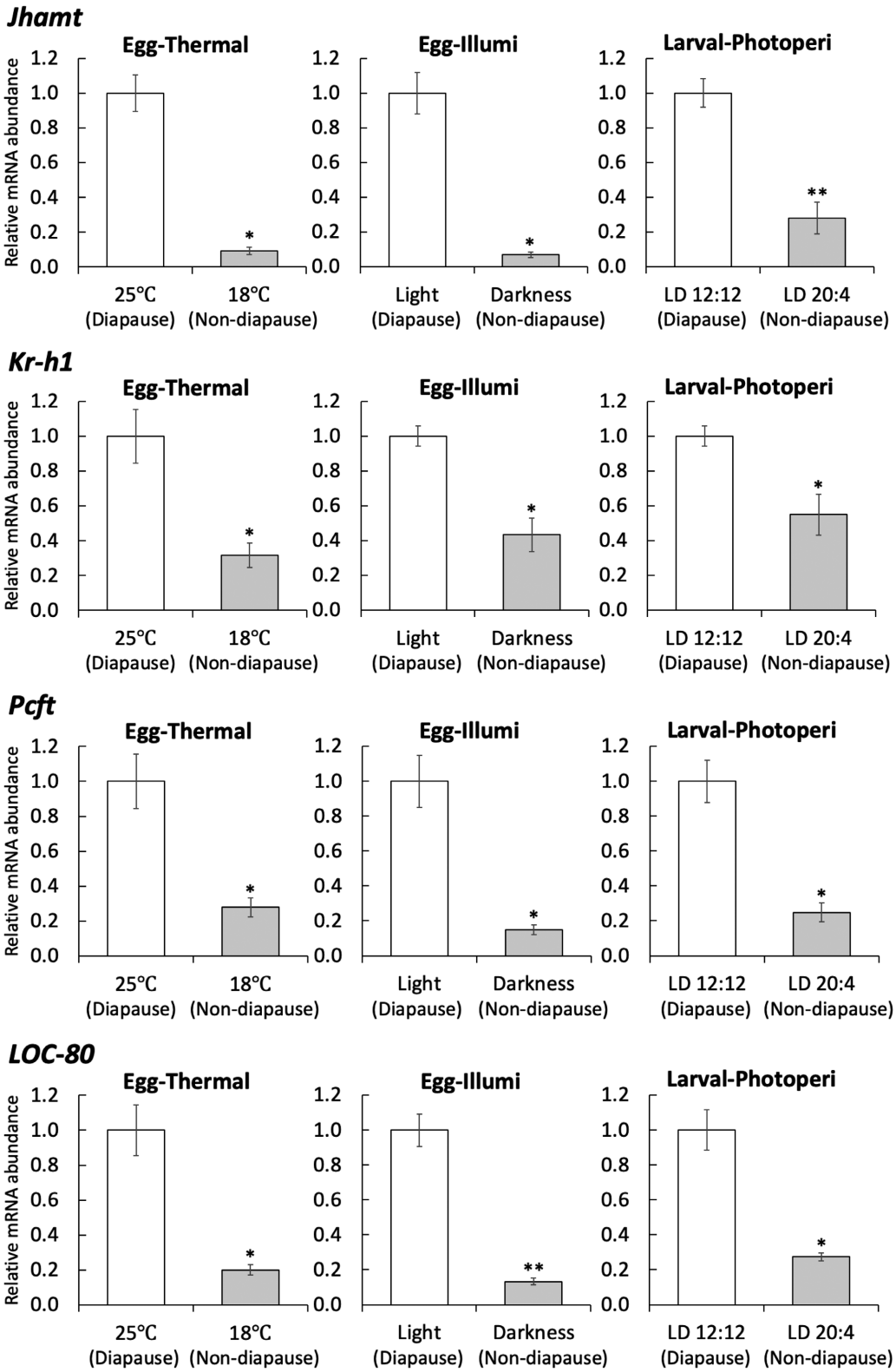


**Fig. 1.** Verification of CAGE data using RT-qPCR. Relative amounts of mRNA of *Jhamt*, *Kr-h1*, *Pcft* and *LOC-80* measured in brains of fifth instar larvae of producers of diapause eggs (white column) and non-diapause eggs (shaded column). All four genes were expressed more abundantly in producers of diapause eggs than non-diapause eggs under the three experimental conditions. In the egg thermal stimulation experiment (Egg-Thermal), diapause- and non-diapause-egg producers were induced by incubating eggs at 25°C and 18°C, respectively. In the egg illumination stimulation experiment (Egg-Illumi), diapause- and non-diapause-egg producers were induced by incubating eggs under continuous light and continuous darkness, respectively. In the larval photoperiod stimulation experiment (Larval-Photoperi), diapause- and non-diapause-egg producers were induced by rearing larvae under 12L : 12D and 20L : 4D, respectively. Data are shown as means ± S.E.M. ($n$ = 3–4: independent samples, each containing 10–20 brains). Value of diapause-egg producers in each transcript is expressed as 1.0 under each condition (Welch's $t$-test, $^{*}P < 0.05$, $^{**}P < 0.01$). CAGE – cap analysis of gene expression; *Jhamt* – juvenile hormone acid methyltransferase; *Kr-h1* – Krüppel homolog 1; LD – light/dark; *LOC-80* – uncharacterized LOC110385080; *Pcft* – proton-coupled folate transporter; RT-qPCR – real-time quantitative polymerase chain reaction.

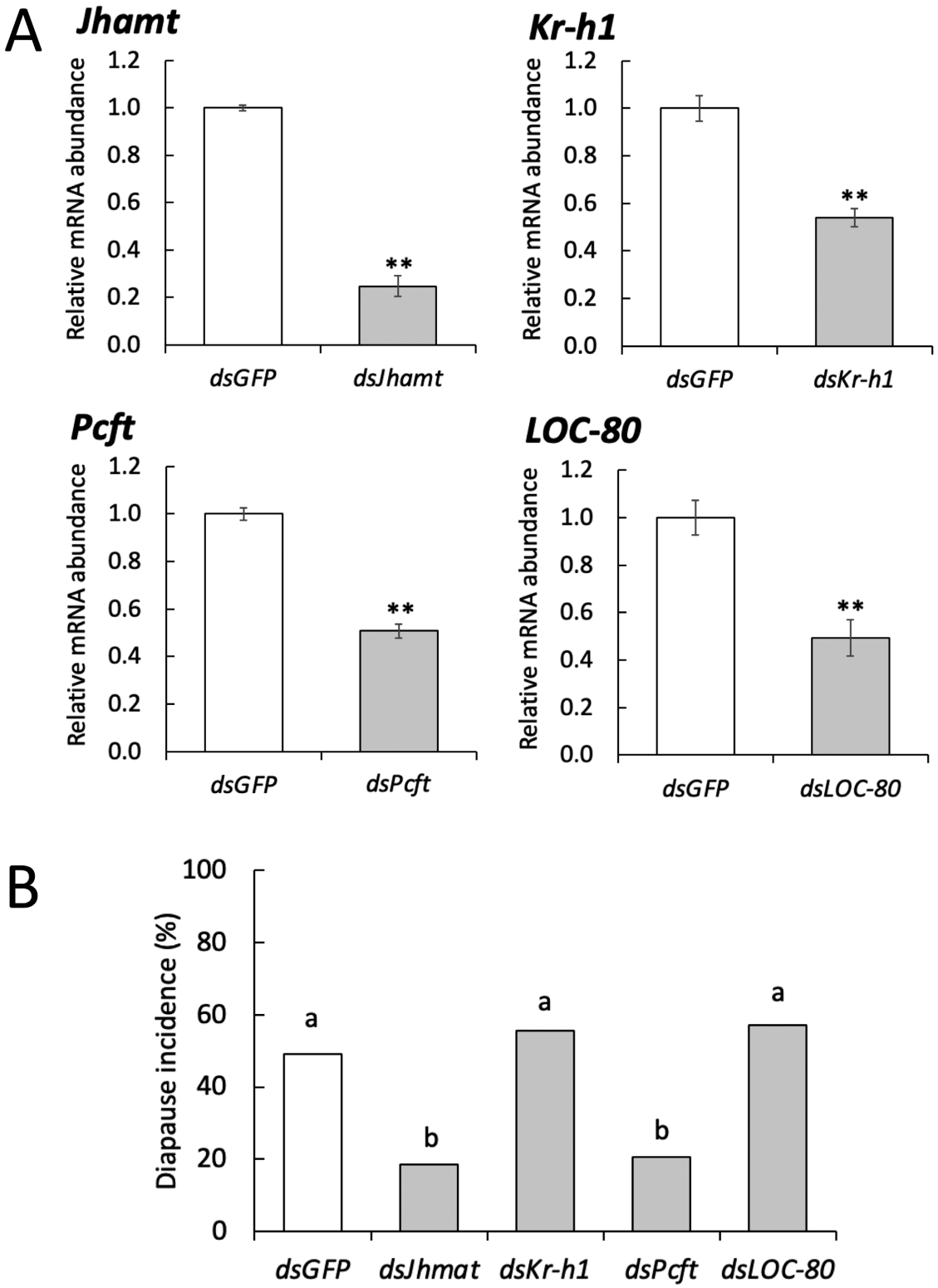


**Fig. 2.** Effects of RNAi against *Jhamt*, *Kr-h1*, *Pcft* and *LOC-80* on diapause in next generation. Larvae of diapause-egg producers were injected with gene-specific dsRNAs to knock down *Jhamt* (*dsJhamt*), *Kr-h1* (*dsKr-h1*), *Pcft* (*dsPcft*), *LOC-80* (*dsLOC-80*) or *GFP* (*dsGFP*, control). Eggs of diapause-egg producers were incubated at 25°C under continuous darkness, then the larvae were reared at 25°C under 12L : 12D. The larvae were injected with dsRNAs on day 0 of the fifth instar, then transferred from 12L : 12D to 20L : 4D, which induces producers of non-diapause eggs. (A) Relative amounts of mRNA of *Jhamt*, *Kr-h1*, *Pcft* and *LOC-80* in larval brains measured 3 days after injection to assess RNAi efficiency. Abundances of messenger RNA are expressed as means ± S.E.M. ($n$ = 5–6: independent samples, each containing 10–20 brains). Value of *dsGFP* control in each transcript is expressed as 1.0 (Welch's $t$-test, $^{**}P < 0.01$). (B) Comparison of diapause incidence among groups of silkworms injected with dsRNA. Incidence of diapause is defined as the percentage (%) of resultant female moths that produce diapause eggs in each group ($n$ = 27–65 individuals). Superscript letters indicate significant differences among groups (Fisher's exact test with Benjamini-Hochberg multiple-testing correction, $P < 0.05$). *Jhamt* – juvenile hormone acid methyltransferase; *Kr-h1* – Krüppel homolog 1; *LOC-80* – uncharacterized LOC110385080; *Pcft* – proton-coupled folate transporter.

### Effects of RNAi on candidate genes in diapause regulation

We investigated the functional involvement of the candidate genes in diapause regulation using dsRNA for RNAi. We assessed RNAi efficiency by measuring gene expression using RT-qPCR in silkworms injected with dsRNA. The gene expressions of the candidates were significantly decreased by the gene-specific dsRNA (Fig. 2A). However, the effects of RNAi on diapause regulation differed among genes (Fig. 2B). Knockdown of *Jhamt* and *Pcft* significantly decreased the incidence of diapause in next generation eggs, whereas *Kr-h1* or *LOC-80* knockdown did not have any significant effects on the incidence of diapause. These findings indicate that only *Jhamt* and *Pcft* are involved in the regulation of diapause in next generation silkworms.

## DISCUSSION

The present study revealed that *Jhamt* and *Pcft* were expressed more abundantly in the brains of fifth instar larvae of DEPs than NDEPs in three experiments in which only temperature, illumination or photoperiod was used as a diapause inducing factor during the egg or larval stage. Furthermore, RNAi knockdown of either *Jhamt* or *Pcft* decreased the incidence of diapause in eggs of the next generation. The silkworm larvae were injected with dsRNA and transferred to 20L : 4D, which induces producers of non-diapause eggs. We then examined the effects of RNAi without stimuli that induce diapause. It was unlikely that the decreased incidence of diapause was due to blockage of an input pathway that mediates stimuli to induce diapause. Our findings indicate that both *Jhamt* and *Pcft* were involved in diapause programming in the larval brain of silkworms. These genes could function by retaining information that leads to diapause in the next generation.

The present findings revealed a more abundant expression of *Jhamt* and *Kr-h1* in DEPs than NDEPs. Both genes are associated with juvenile hormone (JH) as the *Jhamt* gene encodes the rate-limiting enzyme for JH production (Shinoda & Itoyama, 2003) and *Kr-h1* is the early JH-inducible gene that mediates JH action (Minakuchi et al., 2008, 2009). These findings indicate that JH production is activated in larvae of diapause-egg producers. Juvenile hormone controls diapause and overwintering in various species of insects; for example, reproductive diapause in mosquitoes, larval diapause in moths and nymphal overwintering in crickets (Yagi & Fukaya, 1974; Nijhout, 1994; Denlinger et al., 2012; Denlinger & Armbruster, 2014). In fact, short days upregulate *Jhamt* expression, which results in increased JH production and delayed development of the nymphs of the cricket *Modicogryllus siamensis* (Miki et al., 2020). In addition, recently Mukai et al. (2022) report that JH is a causal factor in maternal induction of diapause in the jewel wasp *Nasonia vitripennis*. Maternal wasps downregulate the expression of *Jhamt* and decrease JH synthesis to produce diapause-destined offspring under short days or low temperatures. Although *Jhamt* expression is upregulated in diapause-egg producing silkworms, the JH signalling pathway probably also participates in programming the embryonic diapause in the next generation in silkworms.

However, *Kr-h1* knockdown by RNAi did not have any significant effects on the incidence of diapause in the next generation of silkworms. Insect *Kr-h1* encodes a transcriptional regulator mediating JH action that represses metamorphosis (Lozano & Belles, 2011; Kayukawa et al., 2012, 2014; Smykal et al., 2014). Thus, different pathways might regulate diapause and development in silkworms. In fact, the speed of larval development was not associated with the diapause status of eggs laid by resultant moths. The entire larval period was longer for DEPs than NDEPs in egg thermal and illumination stimulation experiments, whereas the entire larval period was shorter for DEPs than NDEPs in the larval photoperiod stimulation experiment (data not shown). It is well known that *Jhamt* is involved in the biosynthesis of JH in the corpora allata that secret JH to control metamorphosis (Shinoda & Itoyama, 2003; Daimon et al., 2012). However, the present study analysed gene expression in the larval brains from which the corpora allata were removed. It is likely that the JH signalling pathway that mediates diapause information only functions locally in the silkworm brain.

The *Pcft* gene encodes the principal folate transporter that absorbs dietary folate in the intestinal transport system of mammals (Zhao & Goldman, 2013; Hou et al., 2022). This transporter protein consists of 12 transmembrane domains and functions optimally in acidic conditions. Although its functions in insects remain somewhat obscure, *Pcft* might participate in folate uptake or transport in silkworms. Therefore, our finding that RNAi knockdown of *Pcft* significantly affected the incidence of diapause in the next generation implies that folate is involved in regulating diapause in silkworms. Previous findings on diapausing larvae of the sawfly *Acantholyda nemoralis* indicate that folate promotes mitotic activity and nucleic-acid biosynthesis in ovary cells and interrupts larval diapause (Zielińska & Grzelakowska, 1965a, b). Furthermore, a comparative analysis of differentially expressed proteins in non- and summer-diapausing pupae of the onion fly *Delia antiqua*, indicate that folate metabolism is associated with diapause regulation (You-Jin et al., 2014). It is plausible that folate could be a mediator of diapause induction in these species.

In conclusion, our findings indicate that *Jhamt* and *Pcft* are involved in mechanisms in larvae that program embryonic diapause in the next generation of *Bombyx mori*. The JH signalling pathway and folate might also participate in diapause regulation in silkworms. We believe that these findings are likely to help in elucidating the mechanisms that program diapause in insects.

**ACKNOWLEDGMENTS.** This study was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Number JP17K08151 (to K. Sakamoto).

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Received August 9, 2022; revised and accepted September 27, 2022
Published online November 7, 2022

Online supplementary file:
S1 (http://www.eje.cz/2022/042/S01.pdf). Tables S1–S4 and Fig. S1.