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Genome-wide microarray screening for *Bombyx mori* genes related to transmitting the determination outcome of whether to produce diapause or non-diapause eggs

Running tittle: Genes conveying information on diapause

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Abstract The bivoltine silkworm *Bombyx mori* (Lepidoptera: Bombycidae) exhibits a maternally controlled embryonic diapause. Maternal silkworms decide whether to lay diapause or non-diapause eggs depending on environmental factors such as the temperature and photoperiod during the egg and larval stages, and then induce diapause eggs during the pupal stage. However, little is known about the molecular mechanism that conveys the outcome of whether to produce diapause or non-diapause eggs from the egg or larval stages to the pupal stage. The present study used microarray analysis to investigate differentially-expressed genes in the larval brains of diapause- and non-diapause-egg producers, to which bivoltine silkworms were destined by thermal or photic stimulation during the egg stage. The *cytochrome P450 18a1* and *Krüppel homolog 1* genes were upregulated in producers of diapause eggs compared with those of non-diapause eggs under both experimental conditions. *Cytochrome P450 18a1* encodes a key enzyme for steroid hormone inactivation and *Krüppel homolog 1* is an early juvenile hormone-inducible gene that mediates the repression of metamorphosis. The upregulation of these genes during the larval stage might be involved in the signaling pathway that transmits information about the diapause program from the egg stage to the pupal stage in the silkworm.

Key words: *cytochrome P450 18a1*, *Krüppel homolog 1*, silkworm.

Introduction

Diapause is a hormonally regulated state of developmental arrest. Insects enter diapause to cope with predictable harsh seasons such as cold winters and hot summers (Tauber *et al*., 1986; Denlinger *et al*., 2012). Diapause is one of the most substantial adaptations among insects and it occurs at the embryonic, larval, pupal or adult stages in various species.

The silkworm *Bombyx mori* (Lepidoptera: Bombycidae) exhibits maternally controlled embryonic diapause. This species has served as a model system for studies of insect diapause, since it can be easily reared to generate large populations with a genetically uniform background and the large body size facilitates surgical procedures. Furthermore, egg color correctly indicates the diapause status (diapause or direct development) of embryos (Yamashita & Hasegawa, 1966).

Maternal bivoltine silkworms decide whether to lay diapause or non-diapause eggs depending on environmental factors such as the temperature and photoperiod experienced during the egg and larval stages (Watanabe, 1924; Kogure, 1933; Egi *et al*., 2014). After female silkworms are destined to produce diapause eggs, the brain then stimulates the sub-esophageal ganglion during the early pupal stage to secrete the hormone that induces diapause eggs (Fukuda, 1951, 1952). The findings of a transplantation study of cultured silkworm brains suggest that the brain memorizes information on the diapause status of eggs to be laid (Hasegawa & Shimizu, 1987). However, little is known about the molecular mechanism that conveys the determination

outcome of whether to produce diapause or non-diapause eggs from the egg or larval stages to the pupal stage.

The present study used microarray analysis to investigate differentially expressed genes in the larval brains of diapause- and non-diapause-egg producers in bivoltine silkworms. We generated producers of diapause and non-diapause eggs by adjusting the temperature or illumination during the egg stage under two different experimental conditions. In one experiment, producers of diapause eggs were incubated at high temperature (25°C) and those of non-diapause eggs at low temperature (18°C) both under continuous darkness: silkworms were destined by thermal stimulation. In the other experiment, producers of diapause eggs were incubated under continuous illumination and those of non-diapause eggs under continuous darkness both at low temperature (18°C): silkworms were destined by photic stimulation. We postulated that genes involved in the transmission of diapause program information might be differentially expressed at the larval stage in producers of diapause and non-diapause eggs regardless of the type of stimulation that was applied during the egg stage. We then rigorously detected candidate genes by dual screening using two kinds of experimental conditions. These differentially expressed genes might be reflected neither to high temperature nor continuous illumination, but to diapause-destined programming in the brain.

Materials and methods

Animals and tissue collection

The bivoltine silkworm strain p50 can be clearly destined to become producers of either diapause or non-diapause eggs by adjusting a single environmental parameter such as temperature or light during the egg stage (Egi *et al*., 2014). Strain p50 diapause eggs without acid treatment were 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). We incubated eggs at 25°C under continuous darkness, or at 18°C under continuous illumination to generate diapause-egg producers; and at 18°C under continuous darkness to generate non-diapause-egg producers. Hatched larvae were fed with the Silkmate PS artificial diet (Nihonnousan Kogyo Co. Ltd., Yokohama, Japan), and reared at 25°C under a daily 12 h light – 12 h dark cycle (lights on: 9:00 – 21:00 h). A white fluorescent lamp was the source of illumination (0.3 – 0.5 W/m^2 at the level of the insects).

We obtained tissues to isolate total RNA for microarray and real-time PCR analyses. Female larvae were killed at the midpoint (15:00 h) of the photophase on day 1 of the fifth (last) instar or on the day after the fourth larval molting, and then the brain was dissected, quickly frozen in liquid nitrogen and stored at -80°C. Four or five pools of tissues (n = 25 – 35 brains) were collected from each experimental group. We confirmed the diapause status of eggs laid by resultant female moths (n = 34 – 45 individuals) in each experimental group, as described (Egi *et al*., 2014).

The Animal Care and Use Committee of Kobe University approved all experiments and procedures.

Microarray analysis

Microarray experiments proceeded as described (Nakamura *et al*., 2011; Tabunoki *et al*., 2013), using newly designed silkworm 4x44 K custom oligo-microarray slides comprising 60-mer oligonucleotide probes on 43,864 spots corresponding to 25,324 gene sequences constructed from full-length silkworm cDNAs (Suetsugu *et al*., 2013), EST sequences and predicted genes (Agilent Technologies, Palo Alto, CA, USA; No. 2545996) according to the manufacturer's instructions. We then searched differentially-expressed genes in the larval brains of diapause- and non-diapause-egg producers. Four independent brain samples were obtained from each group of diapause- and non-diapause-egg producers in each microarray experiment; among the four samples of each group, two were labeled with Cyanine 3-CTP (Cy3) and the other two with Cyanine 5-CTP (Cy5). The Cy3-labeled samples from producers of diapause eggs were compared with the Cy5-labeled samples from those of non-diapause eggs, and the Cy5-labeled samples from producers of diapause eggs with the Cy3-labeled samples from those of non-diapause eggs. Consequently, each microarray experiment comprised four combinations of Cy3- and Cy5-labeled samples.

Total RNA was extracted from each pool of larval brains using RNeasy Plus Micro Kit (Qiagen, Tokyo, Japan) and quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies). Double-stranded cDNA was synthesized from 60 ng of total

RNA and cRNA was labeled using a Quick Amp Labeling Kit /Two-color (Agilent Technologies). The labeled cRNA was purified using the RNeasy Mini Kit (Qiagen). Hybridization proceeded using Gene Expression Hybridization Kits (Agilent Technologies). The Cy3- and Cy5-labeled cRNAs (825 ng each) were mixed, fragmented and hybridized to a microarray and rotary shaken at 10 rpm for 17 h at 65°C. The arrays were washed with Gene Expression Wash Buffer (Agilent Technologies). The intensity of hybridized probes was detected using a G3565BA Microarray Scanner (Agilent Technologies). Signals were extracted and quantified, and then signal spots that significantly differed from the background level were selected using G2565AA Feature Extraction Software v. 9.5 (Agilent Technologies).

Real-time quantitative PCR

Total RNA was extracted from each tissue pool using RNeasy Plus Micro Kits (Qiagen, Tokyo, Japan). First-strand cDNA was synthesized from 500 ng of each RNA sample using random primers and oligo-dT with the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). Real-time quantitative reverse transcription PCR proceeded using SYBR Green and THUNDERBIRD^{TM} qPCR Mix (Toyobo) and an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The primers were as follows: *Bombyx mori cytochrome P450 18a1* (GenBank accession number NM_001083609) forward, 5’-attgttcctttggcaaccac-3’ and reverse, 5’-agagattcggatccatgtgc-3’ (121 bp product); *Bombyx mori Krüppel homolog 1α* (GenBank accession number AB360766) forward, 5’-cacaacctacgccaacattagaaacg-3’

and reverse, 5’-actgatgaactcgctcctcgtcac-3’ (91 bp product; Kayukawa *et al*., 2012); *Bombyx mori Krüppel homolog 1β* (GenBank accession number, AB642242) forward, 5’-gaaacaatttcgttcttcaggtgacg-3’ and reverse, 5’-tcgtgcgtgtgctgtaagcg-3’ (100 bp product; Kayukawa *et al*. 2012); *Bombyx mori rp49* (GenBank accession number NM_001044157) forward, 5’-tcaatcggatcgctatgaca-3’ and reverse, 5’-atgacgggtcttcttgttgg-3’ (136 bp product). Cycling parameters were 95°C for 60 sec to activate DNA polymerase, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. The specificity of the PCR products was confirmed from melting curves using the software supplied with the ABI 7000 Sequence Detection System, and samples were resolved by electrophoresis on agarose gels. The amounts of amplified products were calculated from cDNA standard curves prepared for each PCR run. The expression level of each transcript was normalized by comparison with the amount of *rp49* mRNA.

The abundance of mRNA measured using real-time quantitative PCR is expressed as mean values with standard deviation (n = 4 – 5 independent samples). Mean values were statistically analyzed using Student’s *t*-test. $P < 0.05$ was considered to represent significance.

Results

Identification of differentially expressed genes in producers of diapause and non-diapause eggs by microarray analysis

We compared gene expression profiles in the brains of fifth-instar larvae between producers of diapause and non-diapause eggs in the following experiments.

Thermal stimulation experiment (Thermo-Exp)

We prepared experimental groups by adjusting the temperature during the egg stage. Producers of diapause eggs were incubated at 25°C and those of non-diapause eggs at 18°C both under continuous darkness. Microarray analysis showed that four genes (0.016% of the total examined) corresponding to nine probed spots were upregulated ≥ 1.5 fold in producers of diapause eggs compared with those of non-diapause eggs, and seven genes (0.028% of the total examined) corresponding to 11 probed spots were upregulated ≥ 1.5 fold in producers of non-diapause eggs compared with those of diapause eggs (Table S1, S2).

Photic stimulation experiment (Photo-Exp)

We prepared experimental groups by adjusting the illumination during the egg stage. Producers of diapause eggs were incubated under continuous illumination and those of non-diapause eggs under continuous darkness both at 18°C. Microarray analysis showed that 14 genes (0.055% of the total examined) corresponding to 25 probe spots were upregulated ≥ 1.5 fold in producers of diapause eggs compared with those of non-diapause eggs, and four genes (0.016% of the total examined) corresponding to seven probe spots were upregulated ≥ 1.5 fold in producers of non-diapause eggs compared with those of diapause eggs (Table S1, S2).

We found that two genes were differentially expressed in both experiments of Thermo-Exp and Photo-Exp. The genes *cytochrome P450 18a1* (*Cyp18a1*) and *Krüppel homolog 1* (*Kr-h1*) were upregulated in producers of diapause eggs compared with those of non-diapause eggs (Table 1). On the other hand, there were no genes upregulated in producers of non-diapause eggs compared with those of diapause eggs in both Thermo-Exp and Photo-Exp.

Verification of microarray data by real-time quantitative PCR

We examined mRNA levels of *Cyp18a1* and *Kr-h1* by real-time PCR under the same experimental conditions as in the microarray analysis (Thermo-Exp and Photo-Exp) to verify the microarray results. As the isoforms *Kr-h1α* and *Kr-h1β* have been identified in silkworm *Kr-h1* transcripts (Kayukawa *et al*., 2012), we separately measured the expression of these isoforms using isoform-specific primers designed by Kayukawa *et al*., (2012).

More *Cyp18a1* mRNA was expressed in producers of diapause eggs than those of non-diapause eggs in both experiments of Thermo-Exp and Photo-Exp (Fig. 1). Levels of *Kr-h1α* mRNA did not significantly differ between the two types of producers in Thermo-Exp, but were higher in producers of diapause eggs than those of non-diapause eggs in Photo-Exp (Fig. 1). Levels of *Kr-h1β* mRNA were higher in diapause-egg producers than in non-diapause-egg producers in both experiments (Fig. 1). Consequently, the microarray results were validated by the mRNA expression of

Cyp18a1 and *Kr-h1β*. Although the functional differences between the two *Kr-h1* isoforms in the silkworm are essentially unknown, *Kr-h1β* seems important for diapause regulation.

Discussion

The present study found that the genes *cytochrome P450 18a1* (*Cyp18a1*) and *Krüppel homolog 1* (specifically *Kr-h1β* transcripts) were expressed at higher levels in the brains of the fifth instar larvae of producers of diapause eggs than those of non-diapause eggs under the described rearing conditions (Thermo-Exp and Photo-Exp). Since the diapause mode of the experimental groups has already been determined by environmental stimuli during the egg stage, upregulation of the two genes during the larval stage might be involved in the signaling pathway that transmits information about diapause regulation from the egg stage to the pupal stage in the silkworm.

Cyp18a1 is a cytochrome P450 monooxygenase gene that is present in many insects and crustaceans (Guittard *et al*., 2011; Ai *et al*., 2011). This gene encodes a key enzyme of steroid hormone inactivation that is essential for metamorphosis in *Drosophila melanogaster* (Rewitz *et al*., 2010; Guittard *et al*., 2011). The CYP18A1 protein catalyzes the 26-hydroxylation and further oxidation of ecdysteroids to 26-carboxylic acids (Guittard *et al*., 2011). This enzymatic activity is probably conserved in the silkworm CYP18A1 (Li *et al*., 2014). Therefore, our findings suggest

that upregulated *Cyp18a1* lowered levels of the active ecdysteroid, 20-hydroxyecdysone, in the larval brain of diapause-egg producers.

The early juvenile hormone (JH)-inducible gene *Kr-h1* encodes a zinc-finger-type transcription factor that mediates the repression of metamorphosis by JH in insects including silkworms (Minakuchi *et al*., 2009; Lozano & Belles, 2011; Konopova *et al*., 2011; Kayukawa *et al*., 2014; Smykal *et al*., 2014). Juvenile hormone activates the *Kr-h1* gene through the Methoprene-tolerant (Met) JH receptor (Wilson & Fabian, 1986; Ashok *et al*., 1998; Miura *et al*., 2005; Charles *et al*., 2011) and binds to Met resulting in the formation of a complex with steroid receptor coactivator (SRC) (Li *et al*., 2011; Zhang *et al*., 2011; Kayukawa *et al*., 2012). Thereafter, the JH/Met/SRC complex interacts with a JH response element (*k*JHRE) in the *Kr-h1* gene (Kayukawa *et al*., 2012, 2014). Since the present study found that *Kr-h1* in diapause-egg producers was upregulated, JH signal transduction might play a role in the transmission system for diapause information in the silkworm. The notion that JH levels increase to activate the *Kr-h1* gene in diapause-egg producers is plausible.

Morohoshi (1976a, b) has suggested, based on extensive physiological studies using surgical techniques, that diapause-egg producers generate less molting hormone and more JH at the late larval stages than non-diapause-egg producers in bivoltine silkworm strains. He also proposed that the decrease in molting hormone and the increase in JH titers in hemolymph during the larval stages simultaneously induce late maturity and diapause. Actually, we found that the entire larval period was two days longer for producers of diapause eggs than those of non-diapause eggs under our

experimental conditions (data not shown).

The major hormones ecdysteroids and JH control development and metamorphosis in insects (Delanoue *et al*., 2010; Kozlova & Thummel, 2003; Riddiford *et al*., 2010) and, in fact, they are involved in diapause regulation (Nijhout, 1994; Denlinger *et al*., 2012). Ecdysteroids appear to promote the induction and maintenance of larval diapause of the gypsy moth, *Lymantria dispar* (Lee & Denlinger, 1997; Lee *et al*., 1997). In contrast, 20-hydroxyecdysone inhibits embryonic diapause in the eggs of the silkworm, *Bombyx mori* (Gharib *et al*., 1981b; Makka *et al*., 2002) as it does in the cochineal *Lepidosaphes ulmi* (Gharib *et al*., 1981a). On the other hand, a high JH titer is related to the induction and maintenance of larval diapause in the rice stem borer, *Chilo suppressalis* (Yage & Fukaya, 1974) and the beet webworm, *Loxostege sticticalis* (Jiang *et al*., 2011). Low levels of ecdysteroids and high levels of JH in fifth instar larvae might act as a signal to induce embryonic diapause in the next generation of silkworms.

However, data supporting this model for diapause regulation have not yet been obtained from hormone administration studies in the silkworm (Morohoshi *et al*., 1972, 1975; Sakamoto *et al*., 2015, unpublished data). In addition, our microarray analysis did not find any other genes that are possibly related to the hormone cascades. We can not deny the possibility that *Cyp18a1* and *Kr-h1* have an unknown function in the silkworm brain.

After all, further studies knocking down genes by RNA interference should clarify the function of *Cyp18a1* and *Kr-h1* and perhaps reveal a new pathway of insect diapause regulation. Since these two genes are likely to be essential for successful

completion of metamorphosis in insects as described above, we may have to establish a conditional gene knockdown system that works exclusively in the brain during a certain period of the larval stage in the silkworm.

Dual screening in the present study identified only two genes under our experimental conditions. Most screened genes differed between Thermo-Exp and Photo-Exp. This might be because some physiological processes responded differently to thermal and photic stimulation. Each stimulus might have unique significance within the ecological context of the silkworm. In fact, one gene encoding a putative forkhead box protein was upregulated in producers of diapause eggs compared with those of non-diapause eggs only in Thermo-Exp (Tabel S1, S2). The forkhead transcription factor promotes the diapause phenotype in the mosquito, *Culex pipinens* (Sim *et al*., 2015). Stimulus-dependent mechanisms probably also function in diapause regulation of the silkworm.

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Disclosure

We do not have any potential conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject of this manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publishere's web-site:

Table S1. Genes upregulated in diapause-egg producers.

Table S2. Genes upregulated in non-diapause-egg producers.

Figure legends

Figure 1 Real-time PCR verification of microarray data. Relative mRNA levels of *cytochrome P450 18a1* (*Cyp18a1*) and two *Krüppel homolog 1* isoforms (*Kr-h1α* and *Kr-h1β*) were measured in brains of fifth instar larvae of producers of diapause eggs (shaded column) and those of non-diapause eggs (white column) eggs. In experiment Thermo-Exp, producers of diapause eggs were incubated at 25°C and those of non-diapause eggs at 18°C both under continuous darkness during the egg stage. In experiment Photo-Exp, producers of diapause eggs were incubated under continuous illumination and those of non-diapause eggs under continuous darkness both at 18°C during the egg stage. Data are shown as means ± SD (n = 4 – 5: independent samples). The value of non-diapause-egg producers in each transcript is expressed as 1.0 under each experimental condition (Student's t-test, $^{*}P < 0.05$, $^{**}P < 0.01$).

Table 1. Genes upregulated in diapause-egg producers compared with non-diapause-egg producers in both experiments of Thermo-Exp and Photo-Exp

Gene name	Fold change		No. of probes
	Thermo-Exp	Photo-Exp	
Cytochrome P450 18a1 (*Cyp18a1*)	2.43	2.40	4
Krüppel homolog 1 (*Kr-h1*)	1.62	1.53	2

In experiment Thermo-Exp, producers of diapause eggs were incubated at 25°C and those of non-diapause eggs at 18°C both under continuous darkness during the egg stage. In experiment Photo-Exp, producers of diapause eggs were incubated under continuous illumination and those of non-diapause eggs under continuous darkness both at 18°C during the egg stage. Data represent average values of corresponding probes. Value for each probe is average of four arrays with independent samples (n = 4, see Table S1).

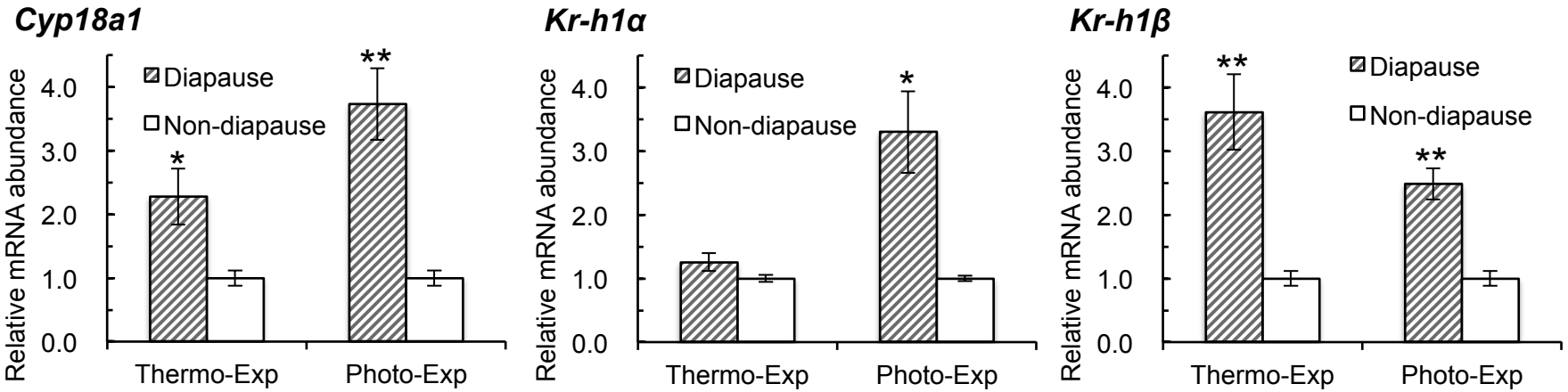

Fig. 1, Akitomo et al.