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Diapause specific gene expression in the eggs of multivoltine silkworm *Bombyx mori*, identified by suppressive subtractive hybridization

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ABSTRACT

Molecular mechanism controlling egg diapause remains obscure in silkworm, *Bombyx mori*. An attempt is made to decipher various molecular events occurring during embryonic diapause in multivoltine silkworm, *B. mori*. Using suppressive subtractive hybridization (SSH), 186 cDNA clones isolated from both diapause and nondiapause eggs were sequenced. Of the sequenced clones, 29 matched with silkbase entries and these identified putative genes were classified into six functional groups such as regulatory, food utilization, stress response, metabolic, ribosomal and transposable elements. Among these genes, twelve belonged to regulatory group while, one taste receptor type 2 member 117 gene was related to food utilization. One heat shock cognate 70 kDa protein and 3 of the ubiquitin family were identified under stress response category. Similarly, four genes were identified as metabolic genes, 3 belonging to chitin family and one propanediol utilization protein. Of the seven genes identified in ribosomal groups, most of them were 60s ribosomal protein subunits. However, one negative regulation of transcription gene identified was a transposable element. The qPCR analysis confirmed the expression of 21 of the above genes, wherein, 6 were upregulated during diapause, 12 during non-diapause, while, 3 remained unchanged.

1. Introduction

Among the many factors that contribute to the abundance of insects, a major element is the adaptation to survive under unfavourable environmental conditions through diapause at different developmental stages leading to suppressed metabolism. It has long been known that the major cues insects use to enter diapause are photoperiodic, sensing a reduction in day length and thermoperiodic, sensing a reduction in temperature. Suppression of metabolism enables the insect to stretch its food reserves to bridge the unfavourable period. Survival during diapause may also be enhanced by coupling of the suppression of metabolic activities with synthesis of polyols, other cryoprotective agents and heatshock proteins that reduce injury at low temperatures. Upon termination of diapause, the metabolic rate rapidly increases, which initiates development. Thus, the diapause and non-diapause phase of the insect's life cycle represent striking contrasts and these differences at the molecular level remain largely unknown.

The silkworm, *Bombyx mori*, is a holometabolous insect that has four distinct life stages including embryo, larva, pupa, and moth. It is a model organism for Lepidoptera in molecular genetics as well as functional genomics and has greatly contributed to the understanding of the mechanisms governing metamorphosis and diapause (Denlinger, 2002). It enters diapause at an early embryonic stage, prior to completion of dermal differentiation. Diapause hormone (*DH*), one of the neurohormones, has been identified as a major factor inducing diapause in the resulting embryos (Imai et al., 1991). The expression of *DH-Pheromone biosynthesis activating neuropeptide (DH-PBAN)* mRNA in the early pupal stage correlates to the incidence of diapause (Sato et al., 1993; Xu et al., 1995). Zhao and Shi (2009) suggested that hydrogen peroxide may also play a role in active release of *DH*. Although these findings clearly show that this hormone regulates the induction of embryonic diapause, whether the individual gene expression profile regulates the stage of initiation or termination of diapause is yet to be identified.

Several classes of diapause upregulated genes have been noted that include stress response genes, developmental arrest genes, and genes involved in regulating specific physiological activities that are unique to diapause. Although some genes are turned on at the onset of diapause and remain upregulated until diapause has been terminated/broken, others are uniquely expressed only in early or late diapause.

Earlier, Flanagan et al. (1998) identified genes differentially regulated during diapause in the flesh fly (*Sarcopahaga crassipalpis*) which included several significant observations like down regulation of proliferating cell nuclear antigen during diapause. Another interesting discovery was of the ribosomal protein P0, which was expressed in a cyclic pattern throughout diapause (Craig and Denlinger, 2000). However, the characterization of genes upregulated during the egg diapause of *B. mori* is still quite limited.

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Although the molecular mechanism controlling egg diapause remains obscure, it has recently been reported that Heat shock protein 70a, samui, Glycerol kinase and Sorbitol dehydrogenase1 genes play important roles in the mediation of egg diapause in silkworm, *B. mori* (Kihara et al., 2009, 2011; Rubio et al., 2011) but, the role of each gene in diapause regulation is unclear. Further, to decipher the molecular events, it is essential to identify additional genes upregulated during diapause. In the present study, efforts have been made to use SSH technique for identifying genes that are differentially expressed in the diapause induced multivoltine silkworm eggs of *B. mori*. Using this technique, several genes have been identified that are expressed during diapause and non-diapause. These genes offer insight into the molecular mechanisms contributing to egg diapause. In this context, the possible functional contributions and the underlying molecular pathways of each gene is discussed.

2. Materials and methods

2.1. Insect rearing

Generally, in tropical countries polyvoltine silkworms produce non-diapausing eggs, which are unsuitable for preservation and the silkworms have to be reared continuously five times per year. An earlier study indicated that rearing late instar polyvoltine silkworms under low temperature and less photoperiod tends to produce diapausing eggs. To induce egg diapause in polyvoltine silkworms, the methodology adopted by Saravanakumar and Ponnuvel (2007) was followed. The silkworm eggs of M13 race were incubated for hatching at 25 °C, 80% relative humidity, with photoperiod of 16 h L:18 h D. The first to third instar silkworms were reared under 26 °C and 80% relative humidity with 12 h L:12 h D. Subsequently, the fourth and fifth instar silkworms were reared at 18 °C to 20 °C with 6 h L:18 h D to obtain diapausing eggs. In parallel, to obtain non-diapausing eggs the larvae were reared under standard rearing conditions up to the final instar (Krishnaswami, 1978).

2.2. RNA isolation

After oviposition, the diapause and non-diapause egg samples were collected from 6 to 48 h at every 6 h time interval. Total RNA was extracted from the diapause and non-diapause eggs using TRIzol reagent (Invitrogen) and quantified by measuring the UV absorbance at 260 or 280 nm. The RNA sample was denatured in formaldehyde, formamide and electrophoresed in 2.0% agarose gels.

2.3. Construction of subtracted cDNA library through Suppressive Subtractive Hybridization

Suppressive Subtractive Hybridization (SSH) was performed using Clonetech PCR-Select™ cDNA Subtraction kit to select genes that are upregulated and down regulated during diapause and non-diapause. The forward selection of SSH consisted of cDNA from non-diapausing eggs as tester and diapause induced eggs as driver and the reverse selection had diapause induced eggs as tester and non-diapausing eggs as driver.

The forward and reverse subtracted libraries were cloned using InsTAclone™ PCR Cloning kit (Fermentas). Transformed plasmids were inserted into competent *Escherichia coli* cells and grown overnight on Luria-Bertani (LB) plates containing ampicillin. Over 100 colonies were isolated from each library and grown overnight in LB-ampicillin broth at 37 ^{0}C. Colonies were then purified with GeneJET™ Plasmid Miniprep kit (Fermentas), run on a 1% agarose gel to determine concentration and all subtracted clones were subjected to sequencing using M13 primer.

2.4. BLAST analysis

The sequences were edited and assembled using MEGA version 5 (Tamura et al., 2011). Putative sequence homologies were determined by BLASTn and BLASTx algorithms in Silkbase database (http://www.silkdb.org) and GenBank (http://www.ncbi.nlm.nih.gov/).

2.5. Primer design

Primers were designed specifically for their use in qPCR from the sequences obtained from SSH using primer3 software (http://frodo.wi.mit.edu/primer3/). These primers designed were confirmed for amplification in normal PCR followed by qPCR.

2.6. Real time PCR analysis

The total RNA isolated from diapause and non-diapause eggs from 6 to 48 h at each 6 h time interval was DNase treated and reverse transcribed in a 20 μl reaction using M-MLV Superscript III reverse transcriptase (Invitrogen). One μL of first strand cDNA was used in a 20 μL reaction mixture using the specific primers designed for Realtime PCR (qPCR). The reactions were conducted on a STRATAGENE Mx 3005P realtime PCR system. The relative expression levels of each gene at different time points were normalized using the Ct values obtained for the *β-actin* amplifications run in the same plate. All samples were tested in triplicate. The mean value ± SD were used for analysis of relative transcript levels for each time point using the ΔΔCt method. A non-template control (NTC) sample was also run to detect contamination if any.

3. Results

3.1. Diapause and non-diapause subtraction

Two SSH experiments were carried out under which, 186 cDNA clones each (94-Non-diapause, 92-diapause) were specifically identified from forward (non-diapause) and reverse (Diapause) subtraction. The plasmids were isolated from all 186 clones and run on a 0.8% agarose gel. Based on the size variations among the clones amplified by using M13 primers, 40 clones were selected from forward and reverse subtracted libraries and sequenced. The sequences obtained were subjected to BLAST analysis to know their homology and a total of 29 genes were identified from the diapause and non-diapause SSH libraries of which, 17 were non-diapause specific and 12 diapause specific (Tables 1a and 1b). The subtractive genes identified were classified into six functional groups (regulatory, food utilization, stress response, metabolic, ribosomal and transposable elements) (Table 2). Based on the above classification, specific primers were designed for all 29 genes identified through SSH (Table 3) and validated through qPCR.

3.2. Diapause upregulated genes identified through SSH

Out of 92 clones screened, the following 12 genes were upregulated during diapause namely, one regulatory gene (40S ribosomal protein S19), two stress responsive genes (Heat shock cognate 71 kDa protein and Ubiquitin c-terminal hydrolase), two metabolic genes (similar to chitin metabolic process and chitinase domain containing protein), six ribosomal protein genes (*Bm* ribosomal protein L41, 60S ribosomal protein L8,L18,L27a,L13, ribosomal protein L14), and one transposable element gene (negative regulation of transcription) (Fig. 1).