Insect Biochemistry and Molecular Biology 76 (2016) 95–108

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Histomorphometric and transcriptomic features characterize silk glands' development during the molt to intermolt transition process in silkworm

Wenbo Hu [a], Chun Liu [a, b], Tingcai Cheng [a], Wei Li [a], Niannian Wang [a], Qingyou Xia [a, b, *]

[a] State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing 400716, China
[b] Chongqing Engineering and Technology Research Center for Novel Silk Materials, Chongqing 400716, China

ARTICLE INFO

Article history:
Received 26 April 2016
Received in revised form
20 June 2016
Accepted 5 July 2016
Available online 6 July 2016

Keywords:
Silkworm
Molt to intermolt transition
Silk gland development
Cell renewal
Histomorphology
Transcriptome

ABSTRACT

The molt–intermolt cycle is an essential feature in holometabolous and hemimetabolous insects' development. In the silkworm, silk glands are under dramatic morphological and functional changes with fibroin genes' transcription being repeatedly turned off and on during the molt–intermolt cycles. However, the molecular mechanisms controlling it are still unknown. Here, silk gland's histomorphology and transcriptome analysis were used to characterize changes in its structure and gene expression patterns from molt to intermolt stages. By using section staining and transmission electron microscope, a renewable cell damage was detected in the silk gland at the molt stage, and an increased number of autophagosomes and lysosomes were found in silk gland cells' cytoplasm. Next, by using RNA sequencing, 54,578,413 reads were obtained, of which 85% were mapped to the silkworm reference genome. The expression level analysis of silk protein genes and silk gland transcription factors revealed that *fibroin heavy chain*, *fibroin light chain*, *P25/fhx*, *sericin1*, *sericin3* and *Dimm* had consistent alteration trends in temporal expression. In addition, differentially expressed genes (DEGs) were identified, and most of the DEGs associated with ecdysone signal transduction, mRNA degradation, protein proteolysis, and autophagy were significantly down-regulated in the transition from molt to intermolt, suggesting that these pathways were activated for the silk gland renewal. These findings provide insights into the molecular mechanisms of silk gland development and silk protein genes transcriptional regulation during the molt to intermolt transition process.

1. Introduction

Developmental timing, the temporal coordination of organismal development, is a fundamental aspect of biological systems (Ambros, 2000; Banerjee and Slack, 2002; Rougvie, 2005; Thummel, 2001). It may be involved in once in a lifetime events, such as mammalian puberty and insect metamorphosis, initiating a series of drastic morphological and physiological changes that allow organisms to be sexually mature, or involved in recurring events, such as insects' molt–intermolt cycle (Ebling, 2005; Niwa and Niwa, 2016), which is an essential feature of insects' life cycle. At the molt stage, insects stop eating, change cuticle, and many are inactive while at the intermolt stage they feed and grow. Thus, larval insect growth is punctuated by periodic molts to form larger cuticles able to accommodate their growing body. During the transition from molt to intermolt, insects undergo profound changes in behavior and in the patterns of gene expression, which are regulated by ecdysone (20E) and by the juvenile hormone (JH) (MacWilliam et al., 2015; Russel et al., 2011). The periodic variation in 20E and JH titers controls the molt–intermolt cycle (Gilbert et al., 2002; Jindra et al., 2013).

The silkworm *Bombyx mori*, a classic model for lepidopterans, has a typical four molt–intermolt cycle comprising major physiological events in which cuticular chitin structures are degraded and rebuilt (Daimon et al., 2005; Qu et al., 2014; Zhang et al., 2014; Zhuo

Abbreviations: IVM24thh, 24th hour of the fourth molt; V3rdD, 3rd day of the fifth instar; ASG, anterior silk gland; MSG, middle silk gland; PSG, posterior silk gland; MIT, molt to intermolt transition; RPKM, reads per kb per million reads; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; FibH, fibroin heavy chain; FibL, fibroin light chain; 20E, ecdysone; JH, juvenile hormone.

* Corresponding author. State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing 400716, China.
E-mail address: xiaqy@swu.edu.cn (Q. Xia).

http://dx.doi.org/10.1016/j.ibmb.2016.07.002
0965-1748/

et al., 2014). Still, only larvae at the intermolt stage are able to spin small amounts of silk to facilitate molting by anchoring the old cuticle to the substratum (Couble et al., 1981; Durand et al., 1992; Waga and Mizuno, 1993), meaning the silk gland is repeatedly activated and inactivated, which contrasts with the changes observed for the 20E titer in the hemolymph (Calvez et al., 1976; Lanzrein et al., 1985; Maekawa and Suzuki, 1980; Satake et al., 1998).

The fourth molt–intermolt cycle is the longest period spent in the larval stage, comprising about 30 h in the molt stage and seven days in the intermolt stage (Maekawa and Suzuki, 1980). Although fibroin messenger RNA (mRNA) synthesis rate is below 0.006 molecules/gene/min at the 24th hour of the fourth molt (IVM24thh), and over 98% of it has been degraded, at the 3rd day of the fifth instar (V3rdD), fibroin mRNA synthesis rate is about 19.2 molecules/gene/min, which is the highest level registered in the larval stage (Kimura et al., 1985; Maekawa and Suzuki, 1980). Studies focusing on silk gland structure showed that the fibroin and sericin deposited in the lumen of the silk gland during the fourth instar, were dissolved during the fourth molt stage and accompanied by remarkable cell structural changes (AKAI, 1971). During intermolt stages, the silk gland grows dramatically and synthesizes silk protein rapidly, and endomitotic DNA replication occurs in the middle silk gland (MSG) and posterior silk gland (PSG) resulting in a 200,000- to 400,000-fold increase in DNA content (Gage, 1974; Xia et al., 2014). However, the detailed molecular mechanism of silk gland development from molt to intermolt stages has remained unclear.

Over the past decade, transcriptomic and proteomic methods have been successfully applied to gene expression and protein component analysis in the silk gland. Expressed sequence tags (ESTs) and microarray techniques have been used to search for differentially expressed genes (DEGs) (Royer et al., 2011; Xia et al., 2007) and RNA sequencing technology (RNA-Seq) allowed detecting 282 up-regulated genes in the anterior silk gland (ASG), when compared to other parts of the silk gland (Chang et al., 2015). Transcriptomic sequencing of the wild silkworm *Bombyx mandarina* silk gland showed that 400 orthologous genes might have experienced or are experiencing positive selection (Cheng et al., 2015). Two-dimensional electrophoresis and computer-assisted analysis were used to characterize the differential protein distribution between the MSG and the PSG, and 98 proteins were identified and classified (Hou et al., 2007). Liquid chromatography-tandem mass spectrometry (LC-MS) analysis of silk proteins spun from different development stages allowed identifying 500 proteins from seven silks (Dong et al., 2013). However, the detailed mechanism of silk gland production in the transition from molt to intermolt is still poorly known, according to published reports.

In this study, we performed a comprehensive analysis of the silk gland histomorphology and transcriptome (MSG and PSG) at the IVM24thh of the molt stage and V3rdD of the intermolt stage. The silk gland and its subcellular structure were observed. Transcription factors involved in temporal and spatial expression of silk protein genes and the pathways correlated with silk gland renewal were identified and analyzed. The highly expressed cuticular genes were first identified in the silk gland at the molt stage. These results might provide insight into the molecular mechanism of silk gland renewal and silk protein genes transcriptional regulation during the molt to intermolt transition (MIT) process.

2. Material and methods

2.1. Silkworm and silk gland isolation

Bombyx mori, strain Dazao, were provided by the State Key Laboratory of Silkworm Genome Biology, Southwest University (Chongqing, China). Silkworms were reared at a stable temperature of 25 °C with fresh mulberry leaves on a 12:12 h light: dark cycle. Silk glands from IVM24thh and V3rdD larval stages were dissected in phosphate buffered saline (PBS, pH 7.4) and divided into MSG and PSG. Both parts were collected and stored in 1.5 mL microtubes at −80 °C until required.

2.2. HE staining

MSGs and PSGs were fixed in 4% (v/v) paraformaldehyde/PBS, dehydrated in a gradient ethanol series from 70% to 100%, embedded in paraffin, and cut into 5 μm sections. After deparaffinization, silk gland sections were stained with a hematoxylin and eosin (HE) staining kit (Beyotime, China) following the manufacturer's protocol, and examined by bright-field microscopy. Images of the sections were captured using the Axio Imager 2 microscope (Zeiss, Germany).

2.3. Transmission electron microscope observation

Fresh silk gland parts (MSG and PSG) from IVM24thh and V3rdD were dissected in PBS (pH 7.4) on ice, cut into 1 mm^3 sections, and fixed in ice-cold 2.5% glutaraldehyde solution (Sigma-Aldrich, USA) for 24 h. Sections were then dehydrated in an ethanol series from 50% to 90%, embedded in Spurr resin (Spi-Chem, USA), cured, and cut into 70 nm sections. Silk gland sections were stained with 3% uranyl acetate-lead citrate (Spi-Chem, USA). Images were captured using a JEM1230 transmission electron microscope (JEOL, Japan).

2.4. RNA preparation and transcriptome sequencing

Total RNA was obtained from three independent extractions, each using TRIzol™ reagent (Invitrogen, USA) and three to ten silk glands (MSG and PSG from IVM24thh and V3rdD). RNA quality and quantity were analyzed using an Agilent Bioanalyzer 2100 system (Agilent Technologies, USA) and the Qubit RNA assay kit (Life Technologies, USA). For each developmental stage of the two silk gland parts, RNA samples from the three individuals were pooled together in equal amounts to generate one mixed sample. These four mixed RNA samples were subsequently used in cDNA library construction and Illumina sequencing which was performed by Beijing Novogene Bioinformatics Technology Co., Ltd.

A total amount of 3 μg RNA per sample was used in sample preparation. Sequencing libraries were constructed using the NEBNext Ultra™ RNA library prep kit for Illumina™ (NEB, USA) following the manufacturer's instructions. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was performed using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer. First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H$^-$). Second strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 150–200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.