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Genomic transcriptional response to 20-hydroxyecdysone in the fat body of silkworm, *Bombyx mori*

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ABSTRACT

Insect growth and development are primarily controlled by two major hormones, juvenile hormone and molting hormone. 20-Hydroxyecdysone is the most active form of the molting hormone. Although intensive studies have been performed on its biological function and action mechanism, it is still unknown how many genes are directly or indirectly regulated by the molting hormone. Here, we analyzed the genomic transcriptional response to 20-hydroxyecdysone in the fat body of silkworm, by using high-throughput Illumina sequencing technology and bioinformatics tools. In total, 606 differentially expressed genes with 347 up-regulated and 259 down-regulated were detected. The 606 differentially expressed genes were significantly enriched in 118 GO terms, i.e. biological process (68), molecular function (37) and cellular component (13). The KEGG analysis revealed that the significantly enriched pathways were mainly focused on the metabolic processes. The differentially expressed genes were further aligned to the functionally verified sequences of *B. mori* in the NCBI database, and a total of 43 functional sequences were identified, of which 23 genes were down-regulated and 20 genes were up-regulated. The up-regulated genes mainly relate to metamorphosis, immune response and protein synthesis. RT-qPCR analysis further validated the correctness of the digital gene expression data. Our study gives an overall view of the regulating effect of 20-hydroxyecdysone on the whole-genome transcript expression in the silkworm, provides useful dataset, and will be helpful for the further studies.

1. Introduction

Insect growth and development are primarily controlled by two major hormones, juvenile hormone which is secreted from corpus allatum and molting hormone which is secreted form prothoracic gland. The titers of both hormones are precisely coordinated by biosynthesis and metabolism pathways and the levels of these hormones are tightly correlated with their regulation of physiological and developmental processes (Li et al., 2014). Silkworm, *Bombyx mori*, is an important economic insect and model organism of *Lepidoptera* in the fields of genetics, physiology and biochemistry. From the 1970 s, exogenous juvenile hormone and molting hormone have been widely used in sericulture to improve the economic efficiency. The exogenous hormones can significantly influence the growth and development of silkworm, metabolism of amino acid and synthesis of nucleic acid and protein within the silk gland (Cui and Zhang, 1993). 20-Hydroxyecdysone is the most active form of the molting hormone, which plays key roles in regulation of developmental processes (Mirth et al., 2009), remodeling of fat body (Bond et al., 2011), patterning of larval and pupal cuticle (Wang et al., 2010; Yamaguchi et al., 2013), apoptosis and autophagy during developmental transitions (Denton et al., 2013; Thummel, 2001) in different insect species. 20-Hydroxyecdysone exerts its function through a heterodimer composed of the ecdysone receptor (EcR) and ultraspiracle protein (USP) (Schubiger and Truman, 2000; Yao et al., 1992) and triggers downstream signal transduction in a strictly hierarchical manner (Beckstead et al., 2005). Proper development and homeostasis of eukaryotic organisms rely on the accurate spatiotemporal regulation of gene expression in response to diverse physiological and environmental signals. Although intensive studies have been performed on its biological function and action mechanism, it is still unknown how many genes are directly or indirectly regulated by the molting hormone, which is one of the important contents of functional genomics.

Gene expression in eukaryotes is regulated primarily at the level of transcription. Illumina Solexa sequencing is a technology used to obtain

Abbreviations: GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; NCBI, National Center of Biotechnology Information

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novel information for whole-genome transcript expression without prior sequence knowledge (Qian et al., 2016). It is a very convenient and accurate method to analyze various expressed genes between several samples (Ou et al., 2014; Das et al., 2016). In this study, we analyzed genomic transcriptional response to 20-hydroxyecdysone in the fat body of silkworm, by adopting the Illumina Solexa sequencing method, and validated some differentially expressed genes through real-time fluorescence quantitative PCR.

2. Materials and methods

2.1. Materials

The Dazhao breed of silkworm was raised with mulberry leaves at 26 ± 1 °C. 20-hydroxyecdysone was purchased from Baomanbio Ltd. (Shanghai, China). Fifth instar larvae of similar sizes were selected on day-1, day-3 and day-5 for the study and assigned to the control group and the hormone group. The biological functional assessment of exogenous hormone was done at dosages of 2 and 20 μg/larva. The control group was fed an equal volume of the dilution solution (2 μL). Larvae were dissected 24 h after exogenous hormone treatment, and fat body were collected for RNA extraction.

2.2. RNA isolation and RNA sequencing

To eliminate the individual error, total RNA of four larval fat body was extracted separately using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and mixed equally as a biological sample for mRNA isolation. Two biological repetitions were constructed for high throughput sequencing with each treatment (n = 2). A magnetic bead homogenizer was used to homogenize the tissue and TRIzol Reagent. The quality and concentration of RNA was detected by spectrophotometry and gel electrophoresis. Four cDNA libraries were constructed using the TruSeq Stranded Total RNA LT Sample Prep Kit (Illumina, Santiago, CA, USA). Solexa sequencing was used to detect the differentially expressed genes between the control and hormone groups. The library construction and Solexa sequencing were performed by Genergy biological technology Limited company (Shanghai, China).

2.3. Quality control and bioinformatics analysis for raw sequencing data

The raw sequencing data were in a FASTQ file, and the data with clean reads were obtained by trimming the adapter contaminants and filtering out low-quality reads. A quality control tool, HTSeq (https://pypi.org/project/HTSeq/), which can provide a quick impression of high throughput data, was utilized in this study.

Tophat (http://ccb.jhu.edu/software/tophat/tutorial.shtml), which uses Bowtie as an engine to align the sequencing reads to reference genome, was used to align each clean read to the silkworm genome database (http://silkworm.genomics.org.cn/). Subsequently, the transcriptome was assembled with the Cufflinks software (http://cole-trapnell-lab.github.io/cufflinks/), and these assemblies were merged using Cuffmerge in the presence of the reference genome. Cuffmerge provided a uniform basis to calculate the expression level of transcripts and genes at different conditions. Cuffdiff, a separate program contained in Cufflink, was used to calculate the expression level of each individual and test the statistical significance between the control and hormone groups. Cuffdiff used FPKM (fragments per kilobase of transcript per million mapped fragments) method to calculate the expression level.

2.4. Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG)

The gene ontology (GO) classification system (http://geneontology.org/) was employed to determine the possible functions of all differentially expressed genes. The *P* value was calculated by GO and Bonferroni corrected. *P* values less than −0.05 were selected as the threshold for the significance enrichment of the gene sets. Web gene ontology annotation plot (WEGO) software was used to visualize, compare, and plot the GO annotation results (Ye et al., 2006). Pathway-enrichment analysis can further identify significantly enriched metabolic pathways or signal transduction pathways using the KEGG database (http://www.kegg.jp/). Pathways with Q values < 0.05 were significantly enriched in differentially expressed genes.

2.5. Differential expression gene validation by real time RT-PCR

Quantitative real-time PCR was performed to validate the differentially expressed genes identified by the Solexa sequencing data. The housekeeping gene *Actin 3* (GENBANK accession number: AAC47446.1) was used as the internal reference gene. Fifteen differentially expressed genes were randomly selected for quantitative real-time PCR analysis. The Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, CA, USA) software was used to design the quantitative primers based on the cDNA sequence, and synthesized by Invitrogen Ltd. (Shanghai, China) (Table 1). RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for RNA reverse transcription following the manufacturer's instructions, and DNase I and RNase-free buffers (Thermo Fisher Scientific, Waltham, MA, USA) were used to treat the RNA sample to avoid genomic DNA contamination. The qPCR reaction was performed on a 7300 Sequence Detection System (ABI, USA) as follows: single cycle of denaturation at 95 °C for 10 min,

Table 1
Primers for real-time fluorescence quantitative PCR analysis.

Functional sequence	Upstream primer (5′ -3′)	Downstream primer (5′ -3′)
Acyl-coenzyme A dehydrogenase	ACTAGCGACCGAACAAATAAG	TATTCCGTGCAGAGTTTCGT
Arginase	TTAGATGTAAAAGACTTCGGG	GTCCATAGTAGGCAAATAAGG
Cytochrome P450 18a1	CGGCGTTGTTGTTGACTC	ATGCCAAGACCATCTAAAGTA
Chitinase-like protein	CTGAAGGCGGCTCCAAGT	CTCCCAGTCCAGATCCAAACC
Copper transporter	TTGGGTTCAGCAGTAGCA	GCCCGACAGCATAGAGTA
Cystathionine gamma-lyase	ACTAGAAACACCCTAGAAGA	TGATTTAGCAGAGCAACC
Estrogen sulfotransferase	AGTCACCTACCGTTGTCG	CATCGCTTCTTCTTTCATA
Glutathione S-transferase sigma 1	ACCCTGATATGCTGAAGAAG	ATGCTGGTGAACACGAAA
Glycerophosphoryl diester phosphodiesterase	GCACCTTCACGATTCCTA	TATTTGGCGACTTCCTTC
Immune-related protein	GATGGCAACTATGGGATAT	GATGGCAACTATGGGATAT
Juvenile hormone epoxide hydrolase	CGCCTTGCCGCTGTTAGT	ATGCTGGTATCCTGTTTCTGCT
Malate dehydrogenase	CACGAGTCAAGACGCAAGA	GCCCAGACCCAGAATACG
Peptidoglycan recognition protein S2	CATAGGGATCGGTTTCAT	TCCAGGACTTAGCGTGTT
Rab-related protein	TAATAAGATGGCAACTATGGGA	ATTTTGGCGGGACTATTGACTA
Serine protease inhibitor 9	ATTCTTGACTGTCGCCATTA	GACGCATTGTCGGTTTCT