Gene 527 (2013) 161–166

Contents lists available at SciVerse ScienceDirect

Gene

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

Differentially expressed genes in the ovary of the sixth day of pupal "Ming" lethal egg mutant of silkworm, *Bombyx mori*

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ARTICLE INFO

Article history:
Accepted 23 May 2013
Available online 11 June 2013

Keywords:
Bombyx mori
Lethal egg mutant
Digital gene expression
Vitelline membrane protein
sHSP
Ecdysone

ABSTRACT

The "Ming" lethal egg mutant ($l\text{-}e^m$) is a vitelline membrane mutant in silkworm, *Bombyx mori*. The eggs laid by the $l\text{-}e^m$ mutant lose water, ultimately causing death within an hour. Previous studies have shown that the deletion of BmEP80 is responsible for the $l\text{-}e^m$ mutation in silkworm, *B. mori*. In the current study, digital gene expression (DGE) was performed to investigate the difference of gene expression in ovaries between wild type and $l\text{-}e^m$ mutant on the sixth day of the pupal stage to obtain a global view of gene expression profiles using the ovaries of three $l\text{-}e^m$ mutants and three wild types. The results showed a total of 3,463,495 and 3,607,936 clean tags in the wild type and the $l\text{-}e^m$ mutant libraries, respectively. Compared with those of wild type, 239 differentially expressed genes were detected in the $l\text{-}e^m$ mutant, wherein 181 genes are up-regulated and 58 genes are down-regulated in the mutant strain. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis results showed that no pathway was significantly enriched and three pathways are tightly related to protein synthesis among the five leading pathways. Moreover, the expression profiles of eight important differentially expressed genes related to oogenesis changed. These results provide a comprehensive gene expression analysis of oogenesis and vitellogenesis in *B. mori* which facilitates understanding of both the specific molecular mechanism of the $1\text{-}e^m$ mutant and Lepidopteran oogenesis in general.

1. Introduction

Oogenesis can be divided into three broad developmental periods representing previtellogenesis, vitellogenesis (yolk uptake), and choriogenesis (eggshell formation) in *Bombyx mori*, respectively (Funaguma et al., 2007; Kendirgi et al., 2002). At two to three days after larval–pupal ecdysis, the terminal follicles in each ovariole will enter vitellogenesis (Swevers and Iatrou, 2003). At the end of yolk deposition, the vitelline membrane located in the space between the oocyte and follicle epithelial cells is secreted by follicle epithelial cells (Uchida et al., 2004). During choriogenesis, the chorion proteins are synthesized and secreted from the cells of the follicular epithelium to form chorion as a water barrier that protects the oocyte from dehydration (Kendirgi et al., 2002). At six to seven days after larval–pupal ecdysis, follicles at the different stages of development are simultaneously present and arranged in a linear array in each ovariole (Swevers and Iatrou, 2003).

In *B. mori*, the removal of the vitelline membrane or chorion can destroy the structural integrity of the follicle and result in egg mortality. For example, Gr^{col} is a chorion mutant, and its deficient secreted protein complement fails to form the highly ordered structure characteristic of a normal chorion (Nadel et al., 1980). The $l\text{-}e^m$ mutant is a vitelline membrane mutant in silkworm, *B. mori* (Chen et al., 2009). The eggs laid by the $l\text{-}e^m$ mutant lose water, start to become concave at around ten minutes, and completely lose water, which ultimately causes death within an hour (Chen et al., 2009). The results of positional cloning showed that the abnormal termination of BmEP80 expression is responsible for $l\text{-}e^m$ mutation (Chen et al., 2012; Xu et al., 2011).

Vitelline membrane proteins (VMPs) have an important role in maintaining the structural integrity of the eggs. Currently, only two VMPs have been found in *B. mori*. One vitelline membrane protein is BmEP80 (BmEP80 was also named BmVMP90 (Sdralia et al., 2012), which is involved in maintaining the structural integrity of the vitelline membrane (Chen et al., 2012; Xu et al., 2011). The other vitelline membrane protein is BmVMP30, which contributes to the structural integrity of the follicle, and is essential for the connection between the follicular epithelium and the oocyte (Kendirgi et al., 2002).

Abbreviations: $l\text{-}e^m$, The "Ming" lethal egg mutant; DGE, Digital gene expression; KEGG, The Kyoto Encyclopedia of Genes and Genomes; VMPs, Vitelline membrane proteins; qRT-PCR, The quantitative real-time PCR method; SilkDB, Silkworm genome database; TPM, Transcripts per million clean tags; FDR, False discovery rate; GO, The gene ontology; Hsp19.5, 19.5-kDa heat shock protein; sHsps, Some small Hsps; EO, Ecdysone oxidase; 20E, 20-hydroxy-ecdysone.

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http://dx.doi.org/10.1016/j.gene.2013.05.049

In this study, DGE was performed to screen the differentially expressed genes in ovaries between the wild type and the *l-e*^{m} mutant on the sixth day of the pupal stage to further understand the effect of *BmEP80* mutation on the expression of other genes. The materials for DGE analysis are based on the fact that the different developmental stages of follicles are present on the sixth day of the pupal stage (Swevers and Iatrou, 2003). In addition, we investigated the expression profiles of eight genes (*BmVMP30*, *Hsp19.5*, BGIBMGA014177-TA, BGIBMGA014208-TA, BGIBMGA005692-TA, BGIBMGA009715-TA, BGIBMGA009716-TA, BGIBMGA014163-TA) related to oogenesis in the ovary, from the first day of the pupal stage to the first day of the moth stage, using the quantitative real-time PCR method (qRT-PCR).

2. Materials and methods

2.1. Silkworm rearing and tissue isolation

The lethal egg mutant strain and the wild type were reared on mulberry leaves at 25 °C, and the head and ovary were dissected from the first day of the pupal stage to the first day of the moth stage, and subsequently stored at −80 °C.

2.2. Mutant identification and RNA extraction

DNA was extracted from the heads to identify the mutant, using a previously described method (Chen et al., 2012). And then, the total RNA of the ovaries were extracted from six wild types and six *l-e*^{m} mutants using RNAiso Plus (TaKaRa), according to the instructions of the manufacturer, and treated with DNase I (TaKaRa). Total RNA quality was determined at a 260/280 absorbance ratio via electrophoresis.

2.3. DGE tag profiling

We analyzed the gene expression in the ovaries between the wild type and the *l-e*^{m} mutant on the sixth day of the pupal stage using the DGE method. Considering the effects of individual differences, we dissected the ovaries of three wild types and three *l-e*^{m} mutants as a sample for DGE, respectively. The mRNA was purified from 6 μg of total RNA from each sample, and then synthesized the first- and second-strand cDNA. *Nla*III, a 4 base-recognition enzyme, was used to digest this cDNA, and Illumina adaptor 1 was ligated. *Mme*I was used to digest at a region 17 bp downstream of the CATG site; afterwards, an Illumina adaptor 2 was ligated at the 3′ end. After 15 cycles of linear PCR amplification, 105-bp fragments were purified by 6% TBE PAGE gel electrophoresis. After denaturation, the single-chain molecules were fixed onto an Illumina Sequencing Chip (flowcell). Each molecule was grown into a single-molecule cluster sequencing template through in situ amplification. Subsequently, four types of nucleotides, which were labeled by one of four colors, were added to the chip, and then sequencing was performed with the sequencing by synthesis method. Each tunnel could generate millions of raw reads, each with a sequencing length of 49 bp.

Adaptor sequences, low quality tags (tags with unknown nucleotides N), empty reads (reads with only 3′ adaptor sequences, but no tags), tags that were too short or too long, and tags with only one copy (probable sequencing error) were filtered to obtain clean tags. A preprocessed database of all possible CATG + 17-nucleotide tag sequences was created using the transcriptome reference database of SilkDB (silkworm genome database, http://www.silkdb.org/silkdb/). For annotation, all clean tags were mapped to the reference sequence, and a mismatch of only 1 bp was considered. Clean tags mapped to the reference sequences from multiple genes were filtered. The remaining clean tags were designated as unambiguous clean tags. For gene expression analysis, the number of unambiguous clean tags for each gene was calculated, and then normalized to the number of transcripts per million clean tags (TPM).

2.4. Analysis of differential gene expression

The differential expression detection of genes across samples was performed using a rigorous algorithm method (Benjamini and Yekutieli, 2001). False discovery rate (FDR) was used to determine the P value threshold in multiple tests and analyses. We obtained the significance of the gene expression difference through an FDR of ≤ 0.001 and the absolute value of $\log_2$ ratio ≥ 1.

2.5. GO and KEGG

The gene ontology (GO) classification system was used to determine the possible functions of all differentially expressed genes. P value was calculated by GO (http://www.geneontology.org/) and Bonferoni corrected. A corrected P value of ≤ 0.05 was selected as a threshold for significant enrichment of the gene sets.

Pathway enrichment analysis can further identify significantly enriched metabolic pathways or signal transduction pathways using the KEGG database. Pathways with a Q value of ≤ 0.05 are significantly enriched in DGEs.

2.6. qRT-PCR analysis

The genes selected according to the DGE-tag copy number were evaluated, and some of these genes were investigated by qRT-PCR. A total of 1 μg of total RNA from each sample was used to synthesize the first strand cDNA using the PrimeScript Reverse Transcriptase kit (TaKaRa) according to the protocol of the manufacturer. *BmActin A3* was used as a reference gene. qRT-PCR was carried out in an ABI PRISM® 7300 Sequence Detection System (Applied Biosystems) using SYBR Green Supermix (TaKaRa) according to the instructions of the manufacturer. The thermal cycle conditions were 94 °C for 10 min for denaturation, followed by 40 cycles of 94 °C for 15 s, and 57 °C for 40 s for annealing, and then an extension. The primers for the qRT-PCR analysis are listed in Table S1.

3. Results

3.1. Analysis of DGE libraries

The results of the two DGE libraries showed that 3,549,903 and 3,702,293 raw tags were generated, respectively. A total of 3,463,495 and 3,607,936 clean tags corresponding to 68,398 and 75,493 distinct clean tags for wild type and mutant libraries were filtered from the raw tags (Table 1), respectively. The distribution of the total and distinct tags over the different tag abundance categories showed highly similar patterns in each DGE library (Fig. 1). More than 75% of the total clean tags had a copy number higher than 100 counts, whereas less than 8% of distinct tags had a copy number higher than 100 counts.

The sequencing saturation was analyzed to estimate whether the sequencing depth was sufficient for transcriptome coverage. The results showed that the number of detected genes was almost saturated when the total tag number reached 2 million or higher (Fig. S1). Our sequencing depths reached approximately 3.5 million in each DGE library, which satisfied the requirement for the experiment. The aforementioned results revealed that the two DGE libraries were reliable.

3.2. Analysis of tag mapping

We mapped the tag sequences of the two DGE libraries to the reference database of silkworm, which contains 14,623 reference genes including 12,264 genes with the CATG site. In the wild type and the *l-e*^{m} mutant libraries, 24.92% and 24.47% of clean tags corresponding