Identifying potential maternal genes of *Bombyx mori* using digital gene expression profiling

Meirong Zhang¹,²*, Sheng Qin¹,²*, Pingzhen Xu¹,², Guozheng Zhang¹,²*

¹ School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang Jiangsu, China, ² Sericulture Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang Jiangsu, China

* These authors are co-first authors on this work.

OPEN ACCESS

Citation: Zhang M, Qin S, Xu P, Zhang G (2018) Identifying potential maternal genes of *Bombyx mori* using digital gene expression profiling. PLoS ONE 13(2): e0192745. https://doi.org/10.1371/journal.pone.0192745

Editor: Subba Reddy Palli, University of Kentucky, UNITED STATES

Received: September 27, 2017
Accepted: January 30, 2018
Published: February 20, 2018

Copyright: © 2018 Zhang et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: The raw data have been submitted to the GEO database with the accession number of GSE109815.

Funding: This research was financially supported by the National Natural Science Foundation of China (grant no. 31302035), Jiangsu Provincial Natural Science Foundation of China (grant no. BK2012273), National Natural Science Foundation of China (grant no. 31272499) and Natural science fund for colleges and universities in Jiangsu Province (grant no. 13KJD230001).

Abstract

Maternal genes present in mature oocytes play a crucial role in the early development of silkworm. Although maternal genes have been widely studied in many other species, there has been limited research in *Bombyx mori*. High-throughput next generation sequencing provides a practical method for gene discovery on a genome-wide level. Herein, a transcriptome study was used to identify maternal-related genes from silkworm eggs. Unfertilized eggs from five different stages of early development were used to detect the changing situation of gene expression. The expressed genes showed different patterns over time. Seventy-six maternal genes were annotated according to homology analysis with *Drosophila melanogaster*. More than half of the differentially expressed maternal genes fell into four expression patterns, while the expression patterns showed a downward trend over time. The functional annotation of these maternal genes was mainly related to transcription factor activity, growth factor activity, nucleic acid binding, RNA binding, ATP binding, and ion binding. Additionally, twenty-two gene clusters including maternal genes were identified from 18 scaffolds. Altogether, we plotted a profile for the maternal genes of *Bombyx mori* using a digital gene expression profiling method. This will provide the basis for maternal-specific signature research and improve the understanding of the early development of silkworm.

Introduction

Maternal genes whose RNA or protein products present in oocytes, fertilized egg or early stage of embryo play an important role in the proper developmental stages [1, 2]. In the early stages of development, the transition of material to the zygote is accomplished by two processes: the degradation of maternal products and the initiation of the transcription of the zygotic genome [3–5]. Maternal genes have been well studied in many species, such as humans, mouse, zebrafish and so on [3, 5–9]. In insects, maternal genes and their functions were well known across various *Drosophila* species [10–14].

*Bombyx mori* (*B. mori*) is an important economic insect in agriculture. The development of *B. mori* is significantly different from *Drosophila*. RFLP linkage analysis showed that maternal
genes might locate at all twenty-eight chromosomes of *B. mori* [15]. However, only a few maternal genes have been researched sufficiently. For example, *BmCad* was a homologue of *Drosophila Cad* which revealed a very similar but distinct genetic hierarchy compared to *Drosophila* [16]. *BmHNF-4* transcribed two isoforms as components of follicular cell differentiation [17]. The polypeptides encoded by the *BmHNF-4* gene were required during both early and more advanced stages of embryogenesis. *BmVLG*, whose mRNA or protein products were deposited in the oocyte cytoplasm, was expressed in both unfertilized eggs and embryonic stages of fertilized eggs [18]. Although these studies demonstrated the functions of maternal genes during the development of the zygote, it is still limited to explain the molecular mechanisms of early oogenesis and the embryogenesis of the silkworm.

In this study, we identified potential maternal genes in silkworm. Also, the expression profiles of maternal genes were determined 24 h after spawning. The whole workflow is as follows (Fig 1):

First, unfertilized eggs were collected from five points-in-time after spawning. Second, total RNA was isolated and cDNA libraries were constructed using the sample of each time. After that, analyses of digital gene expression profiling were performed on all cDNA libraries and 12,264 genes were annotated. The orthologous genes with the known maternal genes of *Drosophila melanogaster* were analyzed as potential maternal genes of *B. mori*. The expression patterns of these orthologous maternal genes were analyzed, and the molecular functions of differentially expressed genes was annotated. This study will provide a new perspective for understanding the early development of silkworm.

**Materials and methods**

**Sample preparation**

The *B. mori* strain Dazao was obtained from the Sericulture Research Institute of China Academy of Agricultural Sciences. The larvae were reared under standard conditions until spawning (25°C and 70% humidity). Unfertilized and fertilized embryos were collected at specific points-in-time (0 h, 6 h, 12 h, 18 h, 24 h). The fertilized embryos of five time points were only used to real-time quantitative PCR analysis. 0 h is defined as the 15th minutes after most female moths spawn. The conditions during embryo collection were 25°C and 70% humidity with 12-h light/12-h dark photoperiod. All samples were immediately stored in liquid nitrogen.

**RNA extraction**

Total RNA was extracted from embryos at various times using the total RNA isolation kit (Qiagen, USA) according to the manufacturer’s instructions. The mRNA was purified by Oligo (dT) magnetic beads (Invitrogen, USA), and then used as templates for cDNA synthesis.

**Digital gene expression profile**

The 5’ end of tags which recognized and cut off the CATG sites on cDNAs were generated by endonuclease Nla III. The fragments apart from the 3’ cDNA fragments connected to Oligo (dT) beads were washed away and the Illumina adaptor 1 was ligated to the sticky 5’ end of the digested bead-bound cDNA fragments. The junction of Illumina adaptor 1 and CATG site was the recognition site of MseI, which was a type of endonuclease with separated recognition sites and digestion sites. It cut 17bp downstream of the CATG site to produce tags with adaptor 1. After removing 3’ fragments with magnetic beads precipitation, Illumina adaptor 2 was ligated to the 3’ ends of tags to acquire tags with different adaptors of both ends to form a tag library. The raw tags were sequenced by the Illumina HiSeq™ 2000. Clean tags were generated
Collect unfertilized eggs of silkworm from five points in time

Isolate total RNA and prepare cDNA libraries

Sequencing and construct digital gene expression profiling

Collect known maternal genes of *Drosophila melanogaster*

Analyzing expression patterns of differentially expressed genes

Finding orthologous genes of known maternal genes in silkworm

Gene function annotation and enrichment analysis

Analyze gene cluster containing maternal genes on silkworm genome

Validation of maternal gene expression by qRT-PCR

---

**Fig 1. Workflow.** Experimental design and schematic diagram of the workflow to identify maternal genes of *Bombyx mori*. 

https://doi.org/10.1371/journal.pone.0192745.g001
by removing the 3' adaptor sequence, low quality tags, tags with a copy number of 1 and tags which were too long or too short, leaving only tags which were 21 bp long.

**Annotation of expressed genes**

A virtual library containing all of the possible CATG+17 bases sequences of the reference gene sequences were constructed based on the silkworm database (ftp://silkdb.org) for gene annotation. All clean tags were mapped to the reference sequences and only 1bp mismatches were considered. Clean tags mapped to reference sequences from multiple genes were filtered. The remaining clean tags were designated unambiguous clean tags. Unambiguous clean tags were used to identify genes and assess the expression level of genes. A gene mapped by at least one count tag is defined as an expressed gene.

**Identification of differentially expressed genes (DEGs)**

The DEGs in the samples were identified based on digital gene expression profiling following the method of Audic S et al. [19]. Multiple-test correction was performed to control the false discovery rate (FDR) [20]. Genes with FDR≤0.01 and |log2 Ratio|≥1 were defined as the DEGs. The DEGs were obtained following two strategies: (I) The sample from four time points, except 0 h, was compared with the sample from 0h. (II) Each sample was compared with the sample of the prior point in time.

**Orthologous analysis**

Maternal genes of *Drosophila melanogaster* were obtained from NCBI based on the retrieve conditions "(maternal) AND 7227[Taxonomy ID]". Orthologous genes in *B. mori* were collected from OrthoDB v9 [21].

**Genes expression patterns among different time points**

Based on strategy I, a gene with a log2 Ratio of TPM for all time points would be selected when its expression levels were significantly different in any comparison. The expression patterns were clustered using hierarchical cluster analysis. The Pearson’s correlation of log2 Ratio among the genes was used as the distance for clustering.

**GO annotation and enrichment analyses**

All 14,623 genes obtained from silkworm database were annotated for protein function using InterProScan 5.19.58 (http://www.ebi.ac.uk/interpro/) with the "-goterms" parameter [22]. The GO enrichment was evaluated using the Fisher’s exact test function implemented in Blast2GO (P≤0.01).

**Real-time quantitative PCR analysis**

In order to analyze the orthologous maternal differentially expressed genes (OMDEGs) identified by our transcriptome sequencing analysis between unfertilized and fertilized embryos, reverse transcription quantitative PCR (RT-qPCR) was adopted. Total RNA from each sample was used to synthesize the first strand cDNA using the PrimeScript Reverse Transcriptase kit (TaKaRa, China) according to the instructions of the manufacturer. RT-qPCR was carried out in an ABI PRISM® 7300 Sequence Detection System (Applied Biosystems) using SYBR Green Supermix (TaKaRa, China) according the instructions of the manufacturer. The thermal cycling conditions profile consisted of an initial denaturation at 94˚C for 10 min, followed
by 40 cycles of 94°C for 15 s, and 60°C for 31 s, and then an extension. The expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. *Bmactin3* was used as a reference gene.

**Results**

**Summary of digital gene expression (DGE) profiles**

Five time-series samples were prepared which were collected at 0 h, 6 h, 12 h, 18 h and 24 h after unfertilized embryo spawning. DGE tag libraries were constructed and sequenced using the Illumina HiSeq™ 2000. In total, 5.7–6.0 million raw tags were generated from each library, 96.97–98.07% of which were filtered as clean tags after quality control. Among the clean tags of each sample, a total of 0.08–0.11 million tags were distinct. The details of tag sequencing are listed in Table 1. In each sample, 7–9% of distinct clean tags had more than 100 copies, 35–38% of them had 6 to 100 copies and 54–57% of had 2 to 5 copies. This indicates that most of them were expressed at a low level and a small part of them were expressed at a very high level. The raw data have been submitted to the GEO database with the accession number of GSE109815.

**Analysis of tag mapping**

For tag mapping, a reference tag database containing 54,721 unambiguous reference tags and 12,264 genes with CATG sites was constructed based on the silkworm database (ftp://silkdb.org). In total, 17–20% of distinct tags in five DGE libraries were mapped to the genes and

| Summary                                    | 0h            | 6h            | 12h           | 18h           | 24h           |
|--------------------------------------------|---------------|---------------|---------------|---------------|---------------|
| **Raw Data**                               | Total         | 6033953       | 589636        | 5782715       | 586535        | 5785000       |
|                                            | Distinct Tag  | 282267        | 280867        | 277144        | 258012        | 191942        |
| **Clean Tag**                              | Total number  | 5854282       | 5717831       | 5607394       | 5704932       | 5673650       |
|                                            | Distinct Tag number | 105259 | 104536 | 104115 | 99597 | 82971 |
| **All Tag-Mapping to Gene**                | Total number  | 769935        | 791770        | 814159        | 1034126       | 1270324       |
|                                            | Distinct Tag number | 18456 | 19755 | 18374 | 18812 | 16892 |
| **Unambiguous Tag-Mapping to Gene**        | Total number  | 755083        | 778145        | 805111        | 1020601       | 1251238       |
|                                            | Distinct Tag number | 18217 | 19505 | 18139 | 18549 | 16626 |
| **All Tag-mapped Genes**                   | number        | 6374          | 6457          | 6344          | 6732          | 6133          |
|                                            | % of ref genes | 43.59% | 44.16% | 43.38% | 46.04% | 41.94% |
| **Unambiguous Tag-mapped Genes**           | number        | 6183          | 6286          | 6193          | 6516          | 5912          |
|                                            | % of ref genes | 42.28% | 42.99% | 42.35% | 44.56% | 40.43% |
| **Mapping to Genome**                      | Total number  | 4187834       | 4100044       | 3947447       | 3827770       | 3634083       |
|                                            | Distinct Tag number | 58257 | 58143 | 57482 | 58627 | 50707 |
| **Unknown Tag**                            | Total number  | 896513        | 826017        | 845788        | 843036        | 769243        |
|                                            | Distinct Tag number | 28546 | 26638 | 28259 | 22158 | 15372 |
|                                            | Distinct Tag % of clean tag | 27.12% | 25.48% | 27.14% | 22.25% | 18.53% |

Table 1. Summary of tags and mapped genes in the DEG libraries.

https://doi.org/10.1371/journal.pone.0192745.t001
40–45% of known silkworm genes were expressed in the sampled oocytes according to matched unambiguous tags (Table 1). In the remaining district tags, 55–61% were mapped to the silkworm genome; 19–27% of tags distinguished as unknown could not be mapped to the reference tag library.

**Differentially expressed genes among different times**

To identify genes with a significant change in expression during different developmental stages, the expression abundances of tag-mapped genes were normalized by counting the number of transcripts per million (TPM) clean tags. Tag abundances and TPM values of expressed genes are shown in S1 Table.

The DEGs among five samples were filtered with the threshold of FDR ≤ 0.01 and the |log₂ Ratio| ≥ 1. Gene expressions were compared based on two strategies, as described above.

Based on strategy I, the results showed that the number of DEGs was increased with time (Fig 2A). In total, 2,167 genes were identified as DEGs at each time point of 6 h, 12 h, 18 h and 24 h. These DEGs and their log2-fold changes of expression were used for clustering analysis in the next step (S2 Table). Eleven common genes were differentially expressed across all four time points (Fig 2B).

Based on strategy II, a similar trend was observed, where the number of DEGs increased with time (Fig 2C). A total of 372 genes showed significant up-regulation from 12 h to 18 h, but 751 genes showed significant down-regulation from 18 h to 24 h. There were 57 common DEGs between the 6 h and 12 h groups and the 12 h and 18 h groups. In total, 85.96% of them were down-regulated from 6 h to 12 h and 91.23% were up-regulated from 12 h to 18 h (Fig 2D). Conversely, there were 196 common DEGs between the 12 h vs. 18 h groups and the 18 h vs. 24 h groups. Overall, 79.08% were up-regulated from 12 h to 18 h, while 51.53% of them maintained high expression levels from 18 h to 24 h. Only two common genes were differentially expressed consistently over the entire period.

**Orthologous maternal genes (OMGs) between Drosophila melanogaster and Bombyx mori**

Ninety-nine maternal genes from *Drosophila melanogaster* were collected from the NCBI database. Fifty-one were found to be consistent with 76 OMGs in the *B. mori* genome (S3 Table). Sixty OMGs were detected as expressed genes, with twenty-three of them being DEGs according to strategy I. The expression levels of 56.52% (13/23) of orthologous maternal DEGs (OMDEGs) were up-regulated for more than 10 TPM for at least one point-in-time, but this proportion of orthologous maternal non-DEGs was only 16.22% (6/37). This indicates that most non-DEG maternal genes were poorly expressed even almost unexpressed.

**Analysis of time-series gene expression patterns**

The majority of expressed genes showed time-specific expression patterns and could be clustered into 27 distinct groups (S1 Fig). Twenty-three OMDEGs were distributed among 13 clusters (Table 2). Four clusters (Cluster 1, 3, 5, and 9) contained at least two OMDEGs and accounted for more than half of OMDEGs. The expression pattern of cluster 1 revealed a sustained downward trend after spawning, while the expression pattern of cluster 3 showed a flattening trend before 18h and a sharp decline after 18h (Fig 3). The expression trend of genes in cluster 5 continuously increased from 0 h to 24 h (Fig 3). The expression trend of genes in cluster 9 increased from 12 h to 18 h and then decreased after 18h (Fig 3). All clusters including DEGs are listed in S4 Table.
### Fig 2. Summary of differentially expressed genes among five points in time.

- **A** Compared with 0 h, up-regulated and down-regulated genes for the other four times.
- **B** Venn diagram of differentially expressed genes in Fig 2A.
- **C** Compared with prior time point, up-regulated and down-regulated genes for the current time point.
- **D** Venn diagram of differentially expressed genes in Fig 2C.

Blue bar indicates up-regulated gene and orange bar indicates down-regulated gene.

### Table 2. The distribution of OMDEGs among the clusters.

| Cluster | No. of OMDEGs | No. of total DEGs | Orthologous maternal DEGs |
|---------|---------------|-------------------|---------------------------|
| 1       | 4             | 294               | BGIBMGA010673, BGIBMGA006904, BGIBMGA005370, BGIBMGA001950 |
| 3       | 6             | 507               | BGIBMGA013421, BGIBMGA005594, BGIBMGA003747, BGIBMGA002655, BGIBMGA008249, BGIBMGA014089 |
| 5       | 2             | 276               | BGIBMGA004822, BGIBMGA010384 |
| 6       | 1             | 69                | BGIBMGA011857 |
| 7       | 1             | 44                | BGIBMGA012427 |
| 8       | 1             | 67                | BGIBMGA010471 |
| 9       | 2             | 64                | BGIBMGA012283, BGIBMGA013746 |
| 11      | 1             | 57                | BGIBMGA003731 |
| 12      | 1             | 49                | BGIBMGA010644 |
| 14      | 1             | 128               | BGIBMGA003207 |
| 15      | 1             | 41                | BGIBMGA006841 |
| 18      | 1             | 39                | BGIBMGA003186 |
| 23      | 1             | 24                | BGIBMGA005172 |

https://doi.org/10.1371/journal.pone.0192745.g002

https://doi.org/10.1371/journal.pone.0192745.t002

Potential material genes of silkworm

PLOS ONE | https://doi.org/10.1371/journal.pone.0192745 February 20, 2018 7 / 15
Gene clusters of OMDEGs in the *B. mori* genome

The neighboring genes in the linking chromosomal locations usually have similar expression patterns and functions [23]. To investigate the genes correlated with OMDEGs, linking chromosome locations of DEGs were analyzed. The genes which were less than 50 kb from the OMDEG were defined as a gene cluster. Twenty-two gene clusters including 123 genes were identified from 18 scaffolds (S5 Table). Sixteen DEGs were located on 14 gene clusters and neighbored OMDEGs. Among them, BGIBMGA008251 had a close relationship with an OMDEG (BGIBMGA008249) with a similar expression pattern. The expression levels of these genes were down-regulated from 0 h to 24 h.

GO annotation and enrichment analysis

A total of 14,623 unique genes matched proteins in the silkworm database and 8,156 (55.78%) were annotated with GO terms. The molecular functions of OMGs were filtered for further analysis. Fifty-eight OMGs were annotated into 108 GO terms about molecular functions, including 19 OMDEGs, 27 non-differential expressed OMGs and 12 unexpressed OMGs. The molecular functions of annotated OMGs are listed in S6 Table.

---

Fig 3. Four expression patterns of differentially expressed genes containing more than two maternal genes. Blue lines indicate the expression pattern of genes in current cluster. Red line indicates the average expression level of genes.

https://doi.org/10.1371/journal.pone.0192745.g003
GO enrichment analysis was performed for analyzing functions of DEG expression clusters using Fisher’s exact test in Blast2GO. GO terms with P-value ≤ 0.01 were considered to show significant enrichment between the DEGs (S7 Table).

In cluster 1, a total of 109 DEGs were enriched in 13 GO terms of molecular function (MF) and 35 DEGs were enriched in 11 GO terms of biological processes (BP). Two OMDEGs in cluster 1, including BGIBMGA010673 and BGIBMGA005370, were annotated in ATP binding, adenyl ribonucleotide binding and adenyl nucleotide binding functions. BGIBMGA005370 was involved in the protein modification process.

In cluster 3, a total of 148 DEGs were enriched in 15 GO terms of MF and 115 DEGs were enriched in 30 GO terms of BP. Three OMDEGs in cluster 3, including BGIBMGA005594, BGIBMGA008249 and BGIBMGA014089, were enriched in protein binding function and involved in the macromolecule metabolic process.

The enriched molecular functions were compared for four expression clusters (S2 Fig). The results showed that genes involved in different expression clusters had distinct functions. Only cluster 1 and 3 did have four common function annotations including protein binding, ATP binding, adenyl ribonucleotide binding and adenyl nucleotide binding. Besides, cluster 5 and 9 had a common function annotation for RNA finding.

**Partly OMDEGs expressional analysis by RT-qPCR**

The gene expression levels of a part of OMDEGs was analyzed by RT-qPCR. The primers of OMDEGs were listed in S8 Table. One gene was selected for each expression cluster analysis. The results were shown in Fig 4. The expression trends of the four selected DEGs in the transcriptome were consistent with RT-qPCR results (Fig 4A, 4B, 4C and 4D). The expression level of BGIBMGA005370 was gradually down-regulated from 6 h to 24 h in unfertilized embryos, different from this, in fertilized embryos, the expression level of BGIBMGA005370 was sharply down-regulated from 6 h to 12 h and gradually increased from 18 h to 24 h (Fig 4A and 4A1). Between unfertilized and fertilized embryos, the expression trend of BGIBMGA005594 was similar and showed significant down-regulation from 6 h to 24 h comparing to 0 h (Fig 4B and 4B1). The expression level of BGIBMGA004822 was gradually up-regulated from 6 h to 24 h in unfertilized embryos and down-regulated from 6 h to 18 h in fertilized embryos (Fig 4C and 4C1). The expression level of BGIBMGA013746 was obviously down-regulated from 12 h to 24 h in fertilized embryos (Fig 4D1).

**Discussion**

The functions of OMDEGs, non-differentially expressed OMGs and unexpressed OMGs

All 58 OMGs with GO annotation were divided into three groups for further analysis, including 19 OMDEGs, 27 non-differentially expressed OMGs and 12 unexpressed OMGs. The molecular function annotation showed that many OMDEGs were related to the transcriptional regulation functions, such as nucleic acid binding (BGIBMGA001950, BGIBMGA003186, BGIBMGA004822, BGIBMGA010644, BGIBMGA010673, and BGIBMGA012283), transcription factor activity (BGIBMGA013421), growth factor activity (BGIBMGA010384), methyltransferase activity (BGIBMGA011857) and so on. However, the non-differentially expressed OMGs were focused on metal ion bindings (BGIBMGA001083, BGIBMGA001789, BGIBMGA002518, BGIBMGA002519, BGIBMGA004416, and BGIBMGA003866) or some receptors (BGIBMGA000601, BGIBMGA000602, and BGIBMGA007355). In silkworm, the recombinant BmPAH (BGIBMGA003866) protein had high phenylalanine hydroxylase (PAH) activity, decreasing BmPAH mRNA levels in
Potential material genes of silkworm

Silkworm eggs reduced neonatal larval tyrosine and caused insect coloration to fail [24]. Unexpressed OMGs were similar to non-differentially expressed genes.

**Differential expressed patterns of OMGs**

In this study, the expression levels of most OMGs decreased along points-in-time, such as Clusters 1 and 3. BGIBMGA003747, one of the OMDEGs, is the homologous gene of Dmcyclin

https://doi.org/10.1371/journal.pone.0192745.g004
B (the maternal gene) in *Drosophila melanogaster* and has been previously cloned from silkworm eggs [25]. The accumulation and degradation of cyclin B played a critical role in mitosis and karyokinesis [26–29]. The BmCyclin B was necessary to complete the cell cycle in BmN cells and had revealed mitotic cell divisions function in silk gland development of embryogenesis in *B. mori* [30, 31]. The BmCyclin B mRNA, abundant in matured eggs, began to degrade sharply just after oviposition, which suggested that the penetration of sperm triggered the degradation of maternal BmCyclin B mRNA in the first meiosis [25]. The expression profiling of BmCyclin B was noticed in the middle silk glands (MSGs) during stage 24 and completely stopped after embryonic stage 25 by *in situ* hybridization and immunohistochemistry [30]. We found that the BmCyclin B mRNA amount distinctly declined, which suggested the cyclin B was degraded by the ubiquitin pathway [32].

PIWI-interacting RNAs (piRNAs) play a crucial role in transposon silencing and other targets to maintain genome integrity [33, 34]. BGIBMGA011857 is homologous to TUDOR in *Drosophila*, and is known as BmTUDOR. The depletion of BmTUDOR by RNAi increased the levels of the piRNAs in *B. mori* [35]. The result was also in agreement with previous reports on *Drosophila* ovaries, which implicated TUDOR in piRNA biogenesis, and the TUDOR mutant contained increasing piRNA levels [36]. Siwi (BGIBMGA010644) had been identified as a PIWI subfamily gene in *B. mori*; the expression profiles of Siwi were abundantly expressed in the testis and ovary on day 3 of the fifth instar larvae and reached a relatively high level at 8–12 h after diapause-destined embryos oviposition [37]. In silkworm, there were two PIWI proteins, Siwi and Ago3, and two typical Heterochromatin Protein 1 (HP1) proteins, HP1a and HP1b, and silkworm PIWI proteins interact with HP1s in the nucleus acting as a chromatin regulator [38].

DNA methyltransferases (DNMTs) are some of the most important proteins associated with DNA methylation and play a major role in the silencing of gene expression, among other important functions [39]. BmDNMT-1(BGIBMGA005382) bound to unmethylated and hemimethylated DNA equally, but preferentially methylated the hemimethylated DNA, acting as a maintenance DNMT in *B. mori* [40]. BmDNMT-1 was highly transcribed during early embryogenesis [41]. Interestingly, knockdown of the BmDNMT-1 gene resulted in lower hatchability [42]. In this study, BmDNMT-1 was significantly down-regulated in the unfertilized eggs. These results indicate that BmDNMT-1 had an important biological function during silkworm embryogenesis.

Thus, most maternal genes showed a downward trend at the beginning of development. Besides, some OMGs with an increasing trend in the first 24 h were also observed, such as OMGs in cluster 5, which indicated that these OMGs might function downstream of biological processes. When the upstream regulators finished translation, the expression levels of OMGs would be reduced over time.

Decapentaplegic (Dpp) is a subgroup of bone morphogenetic protein (BMP)-type ligands which are members of the transforming growth factor-β (TGF-β) superfamily [43]. In *Drosophila*, CHES-1-like activated TGF-β signaling by directly binding to the Dpp promoter and up-regulating Dpp expression [44]; Dpp played a significant role in immunity and several distinct stages of Drosophila development [45, 46]. BGIBMGA010384 was identified as BmDpp, which functioned in response to the BmNPV infection in *B. mori* [47]. Our data show that BmDpp was clearly up-regulated in unfertilized egg oviposition from 18 h to 24 h. Based on the analysis of protein domain using blastp, we found that BGIBMGA008311 was homologous to CHES-1-like in Drosophila. Interestingly, the mRNA expression level CHES-1-like was increased at 6 h of unfertilized egg oviposition in contrast to 0 h (data not shown). Therefore, CHES-1-like might act genetically upstream of Dpp and play an important role in activating TGF-β signaling in *B. mori*.
Conclusion

In the current work, we detected the gene expression profile of unfertilized eggs of *B. mori* from five points-in-time. A total of 76 OMGs in silkworm were identified by comparison with known maternal genes of other species. More than half of the OMGs were differentially expressed and showed 13 different kinds of expression patterns. Four major expression patterns contained more than half of the OMDEGs. We also identified 22 gene clusters containing OMGs in the silkworm genome. Our findings expanded the collection of silkworm maternal genes and provided a perspective for the early embryo development of *B. mori*.

Supporting information

S1 Table. Tag abundances and TPM values of expressed genes.
(XLSX)

S2 Table. Summary of differentially expressed genes.
(XLSX)

S3 Table. Maternal genes of *Drosophila melanogaster* and their orthologous genes in *Bombyx mori*.
(XLSX)

S4 Table. Clusters of DEG expression.
(XLSX)

S5 Table. The gene cluster of OMDEGs.
(XLSX)

S6 Table. Molecular functions of orthologous maternal genes.
(XLSX)

S7 Table. GO enrichment results of four DEG expression clusters.
(XLSX)

S8 Table. Primers of genes for RT-qPCR.
(XLSX)

S1 Fig. All expression patterns of DEGs. The majority of expressed genes showed time-specific expression patterns and could be clustered into 27 distinct groups.
(TIFF)

S2 Fig. Comparison of molecular function annotation for four expression patterns. The enriched molecular functions were compared for expression cluster 1, 3, 5 and 9. P-value was ranged from 0~1.
(TIFF)

Acknowledgments

This research was financially supported by the National Natural Science Foundation of China (grant no. 31302035), Jiangsu Provincial Natural Science Foundation of China (grant no. BK2012273), National Natural Science Foundation of China (grant no. 31272499) and Natural science fund for colleges and universities in Jiangsu Province (grant no. 13KJD230001).

Author Contributions

Conceptualization: Guozheng Zhang.
Data curation: Sheng Qin.
Investigation: Meirong Zhang.
Methodology: Pingzhen Xu.
Writing – original draft: Guozheng Zhang.

References

1. Mager J, Schultz RM, Brunk BP, Bartolomei MS. Identification of candidate maternal-effect genes through comparison of multiple microarray data sets. Mammal Genome. 2006; 17(9):941–9. https://doi.org/10.1007/s00335-006-0034-6 ISI:000240797300004. PMID:16964442
2. Stitzel ML, Seydoux G. Regulation of the oocyte-to-zygote transition. Science. 2007; 316(5823):407–8. https://doi.org/10.1126/science.1138236 ISI:000245813400044. PMID:17446393
3. Desvignes T, Nguyen T, Chesnel F, Bouleau A, Fauvel C, Bobe J. X-linked retinitis pigmentosa 2 is a novel maternal-effect gene required for left-right asymmetry in Zebrafish. Biol Reprod. 2015; 93(2). doi:ARTN42 10.1095/biolreprod.115.130575. ISI:000368003300004.
4. Tadros W, Lipshitz HD. The maternal-to-zygotic transition: a play in two acts. Development. 2009; 136(18):3033–42. https://doi.org/10.1242/dev.033183 ISI:000245813400044 . PMID:19700615
5. Walser CB, Lipshitz HD. Transcript clearance during the maternal-to-zygotic transition. Curr Opin Genet Dev. 2011; 21(4):431–43. https://doi.org/10.1016/j.gde.2011.03.003 ISI:000295351700001 . PMID:21497081
6. Assou S, Boumela I, Haouzi D, Anahory T, Dechaud H, De Vos J, et al. Dynamic changes in gene expression during human early embryo development: from fundamental aspects to clinical applications. Hum Reprod Update. 2011; 17(2):272–90. https://doi.org/10.1093/humupd/dmq36 ISI:000287491400011 . PMID:20716614
7. Messerschmidt DM, de Vries W, Ito M, Solter D, Ferguson-Smith A, Knowles BB. Trim28 is required for epigenetic stability during mouse oocyte to embryo transition. Science. 2012; 335(6075):1499–502. https://doi.org/10.1126/science.1216154 ISI:000301837000050 . PMID:22442485
8. Feng H, Chang BH, Lu CL, Su JM, Wu YY, Lv P, et al. Nlrp2, a maternal effect gene required for early embryonic development in the mouse. PLoS One. 2012; 7(1). doi:ARTN e30344 10.1371/journal.pone.0030344. ISI:000301640600027.
9. Rauwerda H, Wackers P, Pagano JFB, de Jong M, Ensink W, Dekker R, et al. Mother-specific signature in the maternal transcriptome composition of mature, unfertilized Zebrafish eggs. PLoS One. 2016; 11(1). doi:ARTN e0147151 10.1371/journal.pone.0147151. ISI:000368655300004.
10. Bushtai N, Stark A, Brenneckje J, Cohen SM. Temporal reciprocity of miRNAs and their targets during the maternal-to-zygotic transition in Drosophila. Curr Biol. 2008; 18(7):501–6. https://doi.org/10.1016/j.cub.2008.02.081 ISI:000254860600026 . PMID:18394895
11. Lehmarn R, Nüsslein-Volhard C. The maternal gene nanos has a central role in posterior pattern formation of the Drosophila embryo. Development. 1991; 112(3):679 . PMID:1935684
12. Rouget C, Papin C, Bourex A, Meunier AC, Franco B, Robine N, et al. Maternal mRNA deadenylation and decay by the piRNA pathway in the early Drosophila embryo. Nature. 2010; 467(7319):1128–14. https://doi.org/10.1038/nature09465 ISI:0002835486000052 . PMID:20953170
13. Tomancak P, Berman BP, Beaton A, Weiszmann R, Kwan E, Hartenstein V, et al. Global analysis of patterns of gene expression during Drosophila embryogenesis. Genome Biol. 2007; 8(7). doi:Artn R145 10.1186/Gb-2007-8-7-R145. ISI:000249416000025.
14. Yan LY, Yang MY, Guo HS, Yang L, Wu J, Li R, et al. Single-cell RNA-Seq profiling of human pre-implantation embryos and embryonic stem cells. Nat Struct Mol Biol. 2013; 20(9):1131+. https://doi.org/10.1038/nsmb.2660 ISI:000324160900018 . PMID:23934149
15. Kadono-Okuda K, Kosegawa E, Mase K, Hara W. Linkage analysis of maternal EST cDNA clones covering all twenty-eight chromosomes in the silkworm, Bombyx mori. Insect molecular biology. 2002; 11(5):43–51. Epub 2002/09/17. PMID:12230543.
16. Xu X, Xu PX, Suzuki Y. A maternal homeobox gene, Bombyx caudal, forms both mRNA and protein concentration gradients spanning anteroposterior axis during gastrulation. Development. 1994; 120(2):277. PMID:7906628
17. Swevers L, Iatrou K. The orphan receptor BmHNF-4 of the silkmoth Bombyx mori: ovarian and zygotic expression of two mRNAs encoding polypeptides with different activating domains. Mech Develop. 1998; 72(1–2):9–13. Epub 1998/05/12. PMID:9533948.
18. Nakao H. Isolation and characterization of a *Bombyx* vasa-like gene. Develop Genes Evol. 1999; 209 (5):312–6. Epub 2001/03/17. PMID: 11252184.

19. Audic S, Claverie JM. The significance of digital gene expression profiles. Genome Res. 1997; 7 (10):896–95. Epub 1997/10/23. PMID: 9331369.

20. Benjamin Y, Yekutieli D. The control of the false discovery rate in multiple testing under dependency. Ann Stat. 2001; 29(4):1165–88.

21. Kriventseva EV, Tegenfeldt F, Petty TJ, Waterhouse RM, Simao FA, Pozdniyakov IA, et al. OrthoDB v8: update of the hierarchical catalog of orthologs and the underlying free software. Nucleic Acids Res. 2015; 43(D1):D250–D6. https://doi.org/10.1093/nar/gku1220 ISI:000350210400039. PMID: 25428351.

22. Jones P, Binns D, Chang HY, Fraser M, Li WZ, McAnulla C, et al. InterProScan 5: genome-scale protein function classification. Bioinformatics. 2014; 30(9):1236–40. https://doi.org/10.1093/bioinformatics/btu031 ISI:000336095100007. PMID: 24451626.

23. Michalak P. Co-expression, co-regulation, and co-functionality of neighboring genes in eukaryotic genomes. Genomics. 2008; 91(3):243–8. https://doi.org/10.1016/j.ygeno.2007.11.002 PMID: 18082363.

24. Chen P, Li L, Wang JY, Li HY, Li Y, Lv Y, et al. BmPAH catalyzes the initial melanin biosynthetic step in *Bombyx mori*. PLoS One. 2013; 8(8). doi: ARTN e71984 10.1371/journal.pone.0071984. ISI:000324228800032.

25. Takahashi M, Niimi T, Ichimura H, Sasaki T, Yamashita O, Yaginuma T. Cloning of a B-type cyclin homolog from *Bombyx mori* and the profiles of its mRNA level in non-diapause and diapause eggs. Develop Genes Evol. 1996; 206(4):288–91. Epub 1996/11/01. https://doi.org/10.1007/s004270050054 PMID: 24173568.

26. Minshull Jeremy, Julian J, Hunt Tim. Translation of cyclin mRNA is necessary for extracts of activated *Xenopus* eggs to enter mitosis. Cell. 1989; 56(6):947–56. PMID: 2564315.

27. Glotzer M, Murray AW, Kirschner MW. Cyclin is degraded by the ubiquitin pathway. Nature. 1991; 349(6305):132. PMID: 1846030.

28. Senti KA, Brenneck A. The piRNA pathway: a fly’s perspective on the guardian of the genome. Trends Genet. 2010; 26(12):499–509. https://doi.org/10.1016/j.tig.2010.08.007 ISI:000285237400002. PMID: 20934772.

29. Siomi MC, Sato K, Pezic D, Aravin AA. PIWI-interacting small RNAs: the vanguard of genome defense. Nat Rev Mol Cell Bio. 2011; 12(4):246–58. https://doi.org/10.1038/nrm3089 ISI:000288696700012. PMID: 21427766.

30. Honda S, Kirino Y, Maragkakis M, Alexiou P, Ohtaki A, Muraki R, et al. Mitochondrial protein BmPAPI modulates the length of mature piRNAs. RNA. 2013, 19(10):1405–18. https://doi.org/10.1261/rna.040428.113 ISI:000324524300010. PMID: 23970546.

31. Nishida KM, Okada TN, Kawamura T, Mituyama T, Kawamura Y, Inagaki S, et al. Functional involvement of Tudor and dPRMT5s in the piRNA processing pathway in *Drosophila* germelines. EMBO J. 2009; 28(24):3820–31. https://doi.org/10.1038/emboj.2009.365 ISI:000272833700005. PMID: 19959991.

32. Kawaoka S, Katsumoto S, Mita K, Shimada T. Developmentally synchronized expression of two *Bombyx mori* Piwi subfamily genes, SIWI and BmAGO3 in germ-line cells. Biochem Bioph Res Co. 2008; 367(4):765–60. https://doi.org/10.1016/j.bbrc.2008.01.013 ISI:000253248600008. PMID: 18191035.

33. Jurkowski TP, Jeltsch A. On the evolutionary origin of eukaryotic DNA methyltransferases and Dnmt2. PLoS One. 2011; 6(11). doi: ARTN e28104 10.1371/journal.pone.0028104. ISI:000298168100036.
40. Mitsudome T, Mon H, Xu J, Li ZQ, Lee JM, Patil AA, et al. Biochemical characterization of maintenance DNA methyltransferase DNMT-1 from silkworm, *Bombyx mori*. Insect Biochem Molec. 2015; 58:55–65. https://doi.org/10.1016/j.ibmb.2015.01.008 ISI:000350711000006. PMID: 25623240

41. Xiang H, Zhu JD, Chen QA, Dai FY, Li X, Li MW, et al. Single base-resolution methylome of the silkworm reveals a sparse epigenomic map (vol 28, pg 516, 2010). Nat Biotechnol. 2010; 28(7):756-. https://doi.org/10.1038/nbt0710-756d ISI:000279723900038.

42. Xiang H, Li X, Dai FY, Xu X, Tan AJ, Chen L, et al. Comparative methylomics between domesticated and wild silkworms implies possible epigenetic influences on silkworm domestication. BMC Genom. 2013; 14. doi: Artn 646 10.1186/1471-2164-14-646. ISI:000326171800003.

43. Massagué J. The transforming growth factor-beta family. Ann Rev Cell Biol. 1990; 6(1):597.

44. Yu J, Liu Y, Xiang L, Hao W, Yang W, Zhou Z, et al. CHES-1-like, the ortholog of a non-obstructive azoospermia-associated gene, blocks germline stem cell differentiation by up-regulating Dpp expression in *Drosophila* testis. Oncotarget. 2016; 7(27):42303–13. https://doi.org/10.18632/oncotarget.9789 PMID: 27281616

45. Hadar N, Yaron S, Oren Z, Elly O, Itamar W, Johnathan G, et al. A screen identifying genes responsive to Dpp and Wg signaling in the *Drosophila* developing wing. Gene. 2012; 494(1):65–72. https://doi.org/10.1016/j.gene.2011.11.047 PMID: 22192913

46. Li Z, Zhang Y, Han L, Shi L, Lin X. Trachea-derived Dpp controls adult midgut homeostasis in *Drosophila*. Develop Cell. 2013; 24(2):133–43.

47. Hu X, Jiang Y, Gong Y, Zhu M, Zhu L, Chen F, et al. Important roles played by TGF-β member of Bmdpp and Bmdaw in BmNPV infection. Mol Immunol. 2016; 73:122. https://doi.org/10.1016/j.molimm.2016.04.004 PMID: 27077706