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Comparative proteomic analysis of the silkworm middle silk gland reveals the importance of ribosome biogenesis in silk protein production

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ABSTRACT

The silkworm middle silk gland (MSG) is the sericin synthesis and secretion unique sub-organ. The molecular mechanisms of regulating MSG protein synthesis are largely unknown. Here, we performed shotgun proteomic analysis on the three MSG subsections: the anterior (MSG-A), middle (MSG-M), and posterior (MSG-P) regions. The results showed that more strongly expressed proteins in the MSG-A were involved in multiple processes, such as silk gland development and silk protein protection. The proteins that were highly expressed in the MSG-M were enriched in the ribosome pathway. MSG-P proteins with stronger expression were mainly involved in the oxidative phosphorylation and citrate cycle pathways. These results suggest that the MSG-M is the most active region in the sericin synthesis. Furthermore, comparing the proteome of the MSG with the posterior silk gland (PSG) revealed that the specific and highly expressed proteins in the MSG were primarily involved in the ribosome and aminoacyl-tRNA biosynthesis pathways. These results indicate that silk protein synthesis is much more active as a result of the enhancement of translation-related pathways in the MSG. These results also suggest that enhancing ribosome biogenesis is important to the efficient synthesis of silk proteins.
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1. Introduction

The silk gland of the silkworm is worthy of study, as its prominent function of synthesizing silk protein in both basic and applied researches [1–4]. The silk gland is anatomically and physiologically divided into three subparts: the anterior silk gland (ASG), middle silk gland (MSG), and posterior silk gland (PSG). Each subpart has a unique function in cocoon formation. Silk fibers are mainly composed of the core protein fibroin and the coat proteins sericins, which are synthesized by the PSG and MSG, respectively. As a result, the MSG is more suitable for expressing exogenous proteins, as it is more practical to extract proteins from the sericin layer than the fibroin layer [2,4]. Considering the economic significance of silk production, understanding the molecular basis of MSG protein synthesis is highly important.

The MSG, with approximately 230 cells, develops as a one-cell layered glandular epithelium and is the unique sub-organ responsible for synthesis of sericin [5]. Sericins are a group of hydrophilic glue proteins mainly composed of sericins 1, 2 and 3 that surround the fibroin core and make up 20–30% of silk protein [5,6]. These sericin proteins are synthesized in different MSG subsections, including the anterior (MSG-A), middle (MSG-M), and posterior (MSG-P) regions [5]. The sericin genes, encoding glue proteins, are expressed specifically in the MSG with sub-organ localized specificity. The expression of *Ser1* is restricted to only the MSG-P in early larval instars, and expands to the MSG-M in the last instar [5,7,8]. Meanwhile, the *Ser2* and *Ser3* genes are expressed mainly in the MSG-A [5,7,9–11]. This spatio-temporal regulation of sericin gene expression in the three MSG regions suggests unique control of sericin synthesis in the MSG [10,12]. The expression of sericin genes is regulated by various factors. For example, *Ser1* has two binding sites, SA (around −90) and SC (around −200), in its promoter that stimulate its transcription in vitro [13,14]. Although *Bombyx* fork head (Fkh) protein and POU-homeodomain protein POU-M1 can bind to the SA and SC, respectively [15,16], POU-M1 can negatively regulate the expression of *Ser1* [8,17,18]. Besides, the Hox protein Antp, a component of the MSG–intermolt-specific complex (MIC), binds to the essential promoter element of *Ser1* and activates its expression [19].

In addition to the studies on the transcriptional regulation factors of sericin genes, large-scale expression profiling analyses of the silk gland have been carried out. Comparative analysis of MSG and PSG transcriptomes has shown that MSG cells have a wide spectrum of functions in addition to their major role in sericin synthesis and secretion [20,21]. Differential expression of proteins from different sections of the silk gland has been analyzed [22,23]. The PSG expression profiles at transcriptional, translational, and post-translational modification levels during the fifth instar have been characterized [24]. However, the proteome of the MSG is not well understood, and proteomic

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differences among the three subsections of the MSG have not been deeply analyzed. The molecular basis of the functional differences between the MSG and PSG is still not clear. In the present study, we analyzed the proteome of the three subparts of the MSG using shotgun approaches with label-free quantification. We also compared the protein expression between the three MSG regions, as well as between the MSG and PSG, with the aim of revealing the molecular basis of the biological functions of these regions.

2. Materials and methods

2.1. Silkworm tissue collection

Silkworm strain P50 was reared on fresh mulberry leaves under the standard conditions (25 °C, and 80% R.H.). The MSG was dissected in cold physiological saline at the third day of the fifth instar (V3). The whole MSG was divided into three subsections: MSG-A, —M, and -P. The silkworms were from a homozygous strain with high genetic similarities. We randomly selected 15 silkworm larvae and divided them into three groups as biological repeats for proteomic analysis. To avoid contamination by secreted sericins into the gland lumen, the MSG was immersed in pre-chilled 60% ethanol for 1 min to denature the sericin proteins, which were then drawn out from the MSG lumen with nippers. For gene expression analysis, RNase free tips and solutions were used for all steps of experiment.

2.2. Protein sample preparation and SDS-PAGE

Protein extraction from the MSG-A, —M, and -P was performed as described previously [24,25]. The extracted protein samples were quantified using the 2-D Quant Kit (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. Totally 200 μg of proteins for each sample were separated by SDS-PAGE using a 12.5% resolving gel followed by Coomassie Brilliant Blue (CBB) staining.

2.3. Nano-LC–MS/MS analysis

The MSG proteins were separated by SDS-PAGE, and the gels were sliced into 12 sections followed by in-gel digestion and mass spectrometry (MS) analysis, according to our previously described methods [24,25]. Briefly, the digested peptide sample was re-suspended and subjected to an Ettan MDLC nanoflow/capillary LC system (GE Healthcare, Pittsburgh, PA) coupled to a linear ion trap Orbitrap mass spectrometer (LTQ-Orbitrap XL™, Thermo Fisher Scientific, Bremen, Germany). The LTQ-Orbitrap machine was operated with XCalibur software (version 2.0, Thermo Electron, San Jose, CA, USA). Collision-induced dissociation (CID) was controlled with normalized collision energy of 35%, and activation q of 0.25 for MS/MS acquisition. The five most intense ions were isolated for CID fragmentation and measured in the linear ion trap with the dynamic exclusion settings: repeat count 2, repeat duration 30 s, exclusion duration 180 s. Triplicate replicates were performed for each sample.

2.4. Protein identification

The retrieved MS/MS data was searched against the same database which was previously used, containing 1739 entries of silkworm protein sequences from NCBI Refseq and 14,623 entries of the predicted silkworm genome coding sequences [24]. The MS/MS data were automatically submitted to the in-house Mascot server for database search using Mascot Daemon software (version 2.2, Matrix Science, London, U.K.). The parameters for database searching were the same as in our previous study with minor changes [24]. Briefly, the parent and fragment ion mass tolerances were set at 50 ppm and 0.6 Da, respectively. Two missing cleavage sites were allowed for tryptic digestion. A fixed (carbamidomethyl) modification on cysteine and variable modifications on oxidation (M) were specified. To control for the false discovery rate (FDR), the resultant database search files were subjected to further processing by the Trans-Proteomic Pipeline (TPP, version 4.6) using PeptideProphet and ProteinProphet algorithms with the probability thresholds at 0.7 and 0.9, respectively [24,26]. The proteins identified with at least two assigned peptides were acceptable. To reduce the redundancy of the identifications, the proteins assigned in one group with common peptides were manually screened according to the previous method [24].

2.5. Label-free quantification

The relative expression levels of the proteins identified in the MSG-A, —M, and -P were evaluated by Absolute Protein Expression (APEX) scores [27]. All parameters were consistent with our previous report [24]. To compare differential protein expression between the MSG and PSG at V3 of the silkworm larvae, we analyzed the TPP processed data from the MSG (this study) and the PSG (previous research) [24] using the APEX analysis software [27]. The relative abundance of a protein could be compared based on its APEX value.

2.6. Real time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was separately extracted from the MSG-A, -M, and -P using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China) was used for the reverse transcription experiment. RT-qPCR was carried out with a LightCycler® 480 (Roche Diagnostics, Switzerland) in a 20-μL reaction volume containing 50 ng of cDNA, 10 μL of 2× SYBR Premix Ex Taq™ (TaKaRa, Dalian, China), and 4 μM each of the forward and reverse primers (Supplementary Table 1). The thermocycler program began at 95 °C for 30 s for DNA denaturation, followed by 40 cycles of amplification with 95 °C for 5 s, 60 °C for 20 s, and 72 °C 15 s. The relative gene expression level was calculated based on the delta Ct value using the $2^{-\Delta CT}$ method [28]. GAPDH (accession no. NM_001043921) was used as a reference gene. The statistical analysis of gene expression was performed by using SPSS software (Version 18). Multiple comparison for MSG-A, -M, and -P was analyzed with Duncan's test. Comparison between MSG and PSG was done with Student's t test. P values < 0.05 were taken to be statistically significant.

2.7. Bioinformatic analysis

Gene Ontology (GO) terms for the identified proteins were retrieved by searching against the latest InterPro member databases using InterProScan software. GO annotations of the proteomes were plotted by subjecting the retrieved GO terms in native format to the Web Gene Ontology Annotation Plot (WEGO) website (http://wego.genomics.org.cn/cgi-bin/wego/index.pl). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed with the Molecule Annotation System (MAS 3.0, http://bioinfo.capitalbio.com/mas3/).

3. Results

3.1. Proteome profiling of the different MSG sections

For better understanding the molecular details of MSG function, the whole proteomes of MSG subsections were analyzed by shotgun LC–MS/MS (Fig. 1A). There were no significant differences in the expression patterns among the three subsections samples separated by SDS-PAGE (Fig. 1B). Stringent filtering and manual checking were done after protein identification. We totally identified 8078, 7125, and 9600 peptides with a minimum probability of 0.7 from the MSG-A, -M, and -P, respectively (Supplementary Tables 2–4). Assembled with

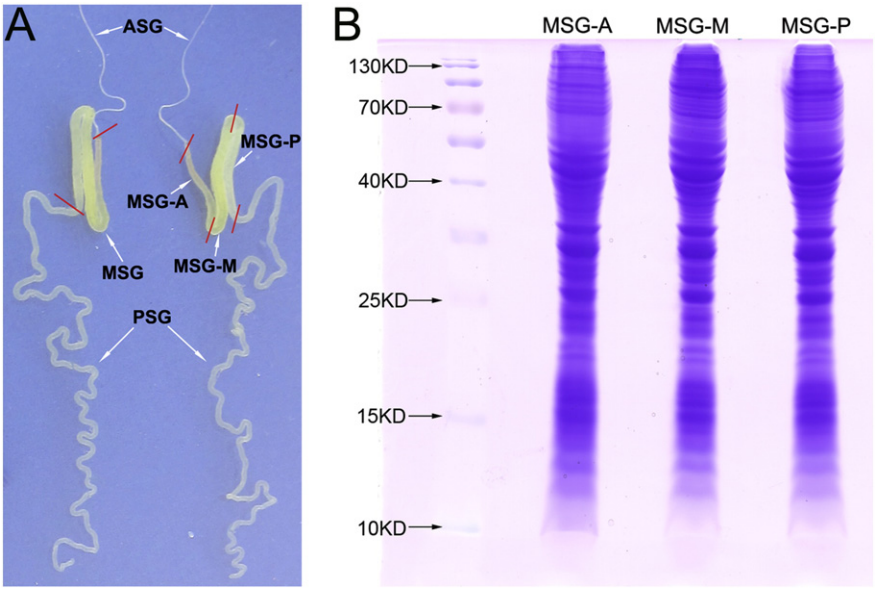


Fig. 1. Silk gland of *Bombyx mori* at V3. The silk gland is divided into anterior (ASG), middle (MSG), and posterior (PSG) regions (A). The MSG has three subsections including anterior (MSG-A), middle (MSG-M), and posterior (MSG-P) regions. The proteins from the three subsections of MSG were separated by SDS-PAGE with a 12.5% separating gel and stained with CBB (B).

these peptides, totally, 643, 594, and 823 proteins were identified from the MSG-A, -M, and -P, respectively, with a FDR of less than 0.5% (Supplementary Table 5). Among these identified proteins, 486 proteins overlapped in the three MSG regions (Fig. 2). There were 55, 14, and 173 identified proteins that were specific to the MSG-A, -M, and -P, respectively (Supplementary Table 5).

3.2. Proteins specifically expressed in the MSG-A

It is reported that sericin 2 is the major sericin protein expressed in the MSG from the third instar to the middle period of the fifth instar [11]. We specifically identified sericin 2 isoform 1 precursor (BGIBMGA011901-PA) in the MSG-A with a high level (APEX score 263.66). The strong expression level of sericin 2 in MSG-A was consistent with previous reports [11,12,29]. For the other sericin proteins, sericin 1 was identified at an extremely low expression level (Supplementary Table 5), and no sericin 3 was detected in the MSG-A.

The expression of silk protein genes is controlled by both tissue-specific and general transcriptional factors in regulation processes involving hormones [30]. In this study, several proteins related to primary insect hormones, including juvenile hormone (JH) and ecdysteroids, were specifically identified in the MSG-A. Ecdysone oxidase (BGIBMGA000158-PA) is a key enzyme involved in the transformation of ecdysteroid into inactivated 3-dehydroecdysteroid [31]. This protein was detected in the silk gland by expressed sequence tag (EST) analysis [32]. On the other hand, ecdysone oxidase (EO) was not found in the PSG proteome of fifth instar larva in our previous study [24]. These results showed that EO was specifically sublocalized in the MSG-A. Another hormone-related protein was juvenile hormone epoxide hydrolase-like protein 2 precursor (BGIBMGA009211-PA). Juvenile hormone epoxide hydrolase (JHEH) plays a pivotal role in regulating insect JH titer. Genome-wide screening using DNA microarrays showed that JHEH (probe ID: sw06428) was specifically expressed in the ASG and MSG at V3 [33].

3.3. Proteins uniquely expressed in the MSG-M

Several important proteins involved in development and translation initiation were uniquely expressed in the MSG-M, including Ras protein, exportin-7, eIF4E and eIF3-S4. Ras protein is a small GTPase that plays important roles in development, especially in cell proliferation and differentiation. Exportin-7 (Exp7), which is involved in a general export

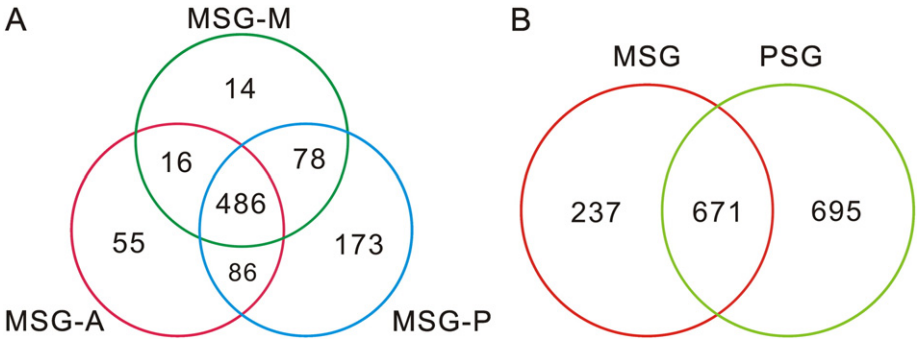
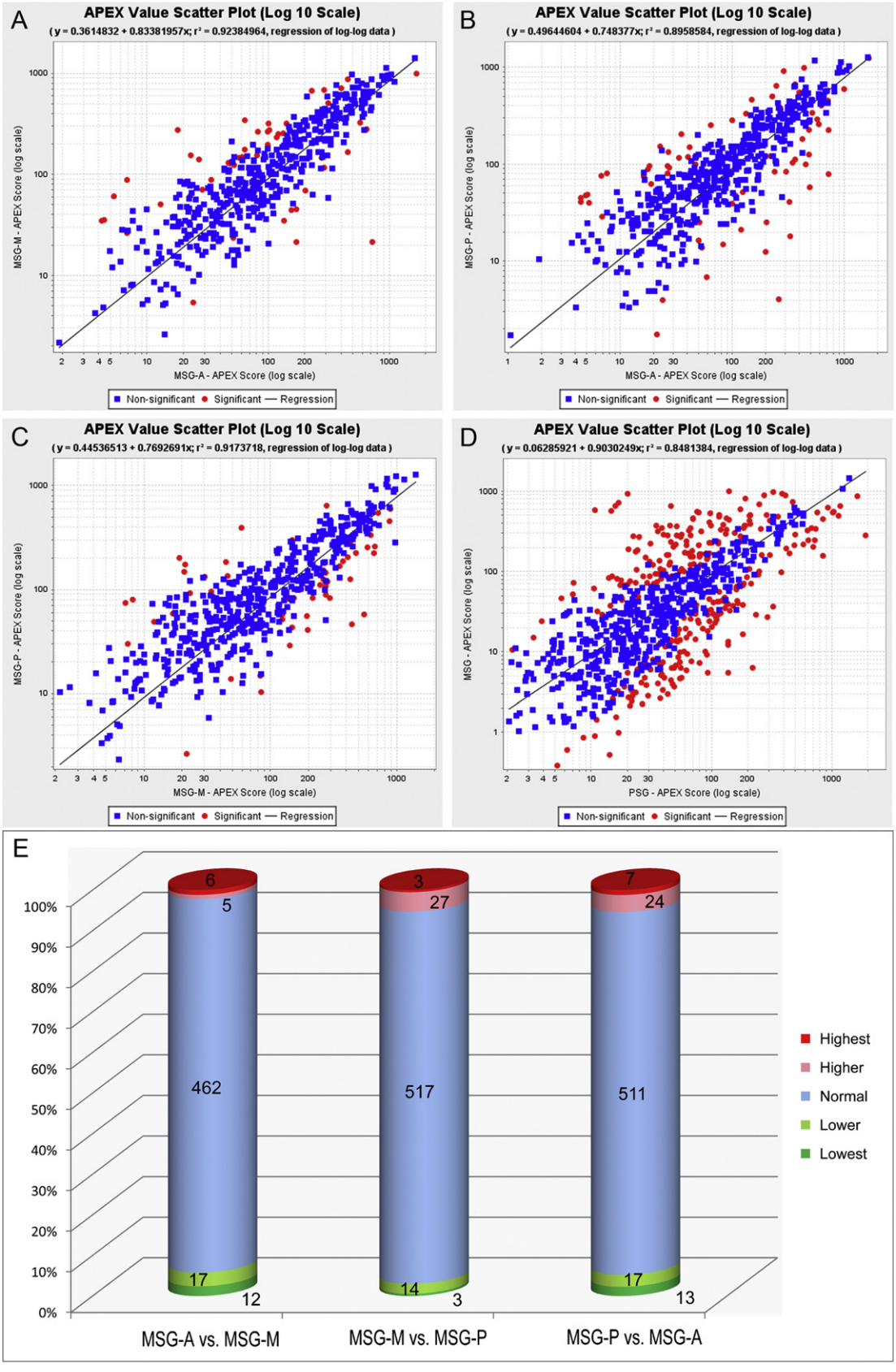


Fig. 2. Venn diagram of the pairwise comparison of the proteome identifications. A, the pairwise comparison of identifications from the three subparts of MSG; B, the proteome comparison of MSG and PSG at V3 of silkworm larvae.

pathway, plays a broad role in maintaining the identities of the nuclear cytoplasmic compartments [34]. Exp7 can be recognized and bound by eIFs, including eIF4AI in the cytoplasm of HeLa cells [34]. In addition to eIF4E and eIF3-S4, we identified 11 of the 28 known and predicted translation initiation factors with higher expression in the MSG-M than in the MSG-A or MSG-P (Supplementary Table 5).



3.4. MSG-P-specific proteins

The MSG-P-specific proteins, such as cameo2, imply that MSG-P has a function that is different with the other two regions (Supplementary Table 5). Cameo2 cooperates with carotenoid-binding protein (CBP) to facilitate selective transport from the midgut into the silk gland and cellular uptake of lutein. Cameo2 is also associated with the yellow-red color formation of cocoons [35]. In this study, only cameo2 was found to have a low abundance in the MSG-P, and CBP was not detected in any of the three MSG regions. This result is consistent with the mRNA expression of the corresponding genes [35]. The cocoon color of P50 (Dazao) strain which we used in this experiment is green; therefore the absence of CBP in the MSG could explain the failure to form a yellow cocoon.

3.5. Pairwise comparison of the MSG-A, -M, and -P proteomes

To identify differentially expressed proteins (≥2-fold, $P < 0.05$), a pairwise comparison of the MSG-A, -M, and -P proteomes was performed based on the APEX score for relative abundance of the proteins as evaluated by a label-free quantification method [27] (Fig. 3). The proteins with differential expression were clustered into a heat map according to previous studies [36,37] (Fig. 4), which showed that most of the differences were between the MSG-A and the other two regions. Compared with the MSG-M, the expression of 11 proteins increased and that of 29 proteins decreased in the MSG-A (Fig. 3A, E; Supplementary Table 6). Of the 11 proteins with enhanced expression in the MSG-A, 6 were also expressed at higher levels than observed in the MSG-P (Fig. 4E).

Among the 564 identified proteins common to both the MSG-M and MSG-P, 30 were more highly expressed in the former and 17 were more highly expressed in the latter (Fig. 3B, E; Supplementary Table 7). Among the 30 proteins with higher expression in MSG-M than in the MSG-P, 9 were related to protein synthesis. Moreover, sericin 1, the primary sericin protein in the cocoon, was expressed much more highly in the MSG-M than in the other two regions (Supplementary Table 5).

Compared with the MSG-A, 31 proteins were more highly expressed in the MSG-P (Figs. 3 and 4, Supplementary Table 8). Seven proteins, including silk fibroin light chain precursor, were expressed at significantly higher levels in the MSG-P than in both the MSG-A and MSG-M (Fig. 3E; Supplementary Table 9). The silk fibroin light chain, a component of fibroin protein, is specifically expressed in the PSG. We detected this protein in the MSG most likely because of contamination from fibroin proteins secreted from the PSG moving into the MSG. It is also possible that no clear border exists between MSG and PSG. RT-qPCR analysis revealed that the silk fibroin light chain gene (Fib-l) was expressed in the MSG-P, not in the MSG-A and MSG-M (Fig. 5D).

3.6. Proteome comparison between the MSG and the PSG

We compared the proteome of the MSG with PSG at V3, to better understand the functional differences between the silk gland regions at the molecular level [24]. We found 671 common proteins and 237 proteins specifically expressed in the MSG (Fig. 2B). In addition to the well-known silk glue proteins and some tissue-specific proteins mentioned above, many MSG-specific proteins were involved in protein biosynthesis, including six translation initiation factors, two elongation factors, and 14 ribosomal proteins. Although the protein expression patterns of the MSG and PSG have been compared using two-dimensional electrophoresis-based proteomics, only a few proteins were identified as being differentially expressed [23]. These differentially expressed proteins were all identified and they were consistent with our work on the MSG and PSG [23] (Supplementary Table 10). These results provided strong evidence for the credibility of our MS identification and label-free quantification. Moreover, the increased coverage of the MSG and PSG proteomes will greatly contribute to the understanding of the molecular mechanisms that govern their functional differences.

Of the 671 proteins common to both the MSG and PSG, 98 proteins showed significantly higher expression in the former than in the latter (fold changes ≥2, $P < 0.05$, Supplementary Table 10). Among these more highly expressed proteins in the MSG, there were 20 ribosomal proteins and six key factors involved in protein translocation, including translocon-associated protein gamma, transport protein Sec61 alpha subunit, TRAPγ, signal peptidase 18 kDa subunit, signal peptidase complex subunits 2 and 3, and signal recognition particle 54 kDa protein. In addition, there were seven predicted enzymes involved in forming aminoacyl-tRNA that exhibited higher expression levels in the MSG than in the PSG (Supplementary Table 10). However, elongation factor Tu, which not only plays a central role in prokaryotic protein synthesis by delivering amino-acylated tRNAs to the ribosome but is also thought to be a molecular switch in protein biosynthesis [38,39], was expressed more highly in the PSG than in the MSG.

3.7. Comparison at the translational and transcriptional levels

To reveal the expression levels at the transcriptional level, the genes of some of the differentially expressed proteins were selected for RT-qPCR analysis. We found that MSG-A specific proteins, including EO, JHEH, Ser2, and Suc1, were also specifically or highly expressed at the transcriptional level (Fig. 5A, B). Proteins in the MSG-A with significantly higher expression than in both the MSG-M and MSG-P, including HSD and serpin-16, also had higher expression at the transcriptional level (Fig. 5A, B). For the proteins specifically and highly expressed in the MSG-M and MSG-P, their transcriptional expression level was inconsistent with their protein expression (Fig. 5C, D). These inconsistencies may be the result of differential regulation at the transcriptional and translational levels [40]. In addition, the transcriptional level expression of the proteins found to be differentially expressed between the MSG and PSG was also compared (Fig. 5E, F). The transcriptional level expression was mostly consistent with the translational level expression (Fig. 5E, F).

3.8. Bioinformatic analysis

To provide some insight into the functional differences among the tissues as a result of these differences in their proteomes, the specifically expressed proteins and the differentially expressed proteins with higher expression levels were combined and subjected to Gene Ontology analysis (Fig. 6). By comparing the proteome of MSG-A and MSG-M, we found 152 proteins with specific and higher expression in the MSG-A and 121 proteins with specific and higher expression in the MSG-M (Figs. 2A, 3E and 6A). A significantly higher percentage of these proteins in the MSG-M than in the MSG-A (accounting for 14% and 6.6% of the total provided gene number, respectively) were involved in the biosynthetic process (GO: 0009058). KEGG pathway analysis revealed that the 121 proteins in the MSG-M were enriched in aminoacyl-tRNA biosynthesis, whereas the 152 proteins in the MSG-A were involved in

Fig. 3. Differential protein expression analysis with label-free quantification. The comparison of protein expression levels was based on the APEX scores. A, MSG-A vs. MSG-M; B, MSG-M vs. MSG-P; C, MSG-A vs. MSG-P; D, MSG vs. PSG. The red spots represent the proteins with significantly differential expression ($P < 0.05$), while the blue ones represent the proteins without significantly differential expression ($P < 0.05$). E, pairwise comparison of the differential expression of proteins from the three subsections of the MSG. Blue (normal) represents the proteins without significantly differential expression, and the others represent the proteins with significantly differential expression ($P < 0.05$, fold-changes $>= 2$); Red (highest) and green (lowest) represent the number of proteins with higher and lower expressions than in the other two MSG sections, respectively. While reddish (higher) and light green (lower) represent the number of proteins with higher and lower expressions than in only one MSG section, respectively.

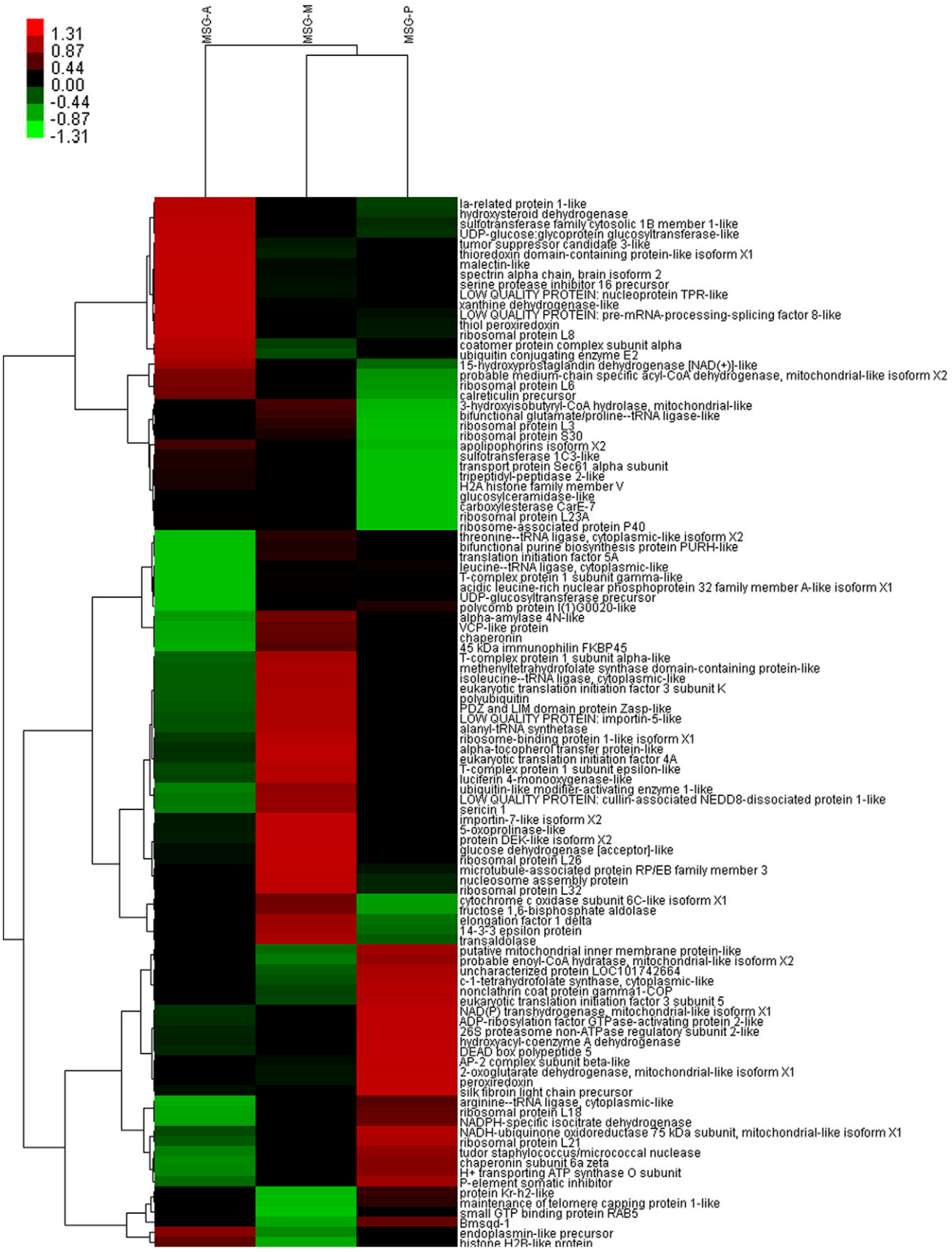


Fig. 4. Clustering of the differentially expressed proteins among the MSG-A, -M, and -P ($P < 0.05$). Pairwise comparison of the proteomes among the three regions of MSG was performed based on the relative abundance. The abundance values of the proteins didn't identify in one tissue were designated as zero. The columns represent the different samples, and the rows represent the individual protein. The proteins with higher and lower expressions are indicated in red and green, respectively. The intensity of the color increases with increasing expression differences as noted on the key bar on the top left side.

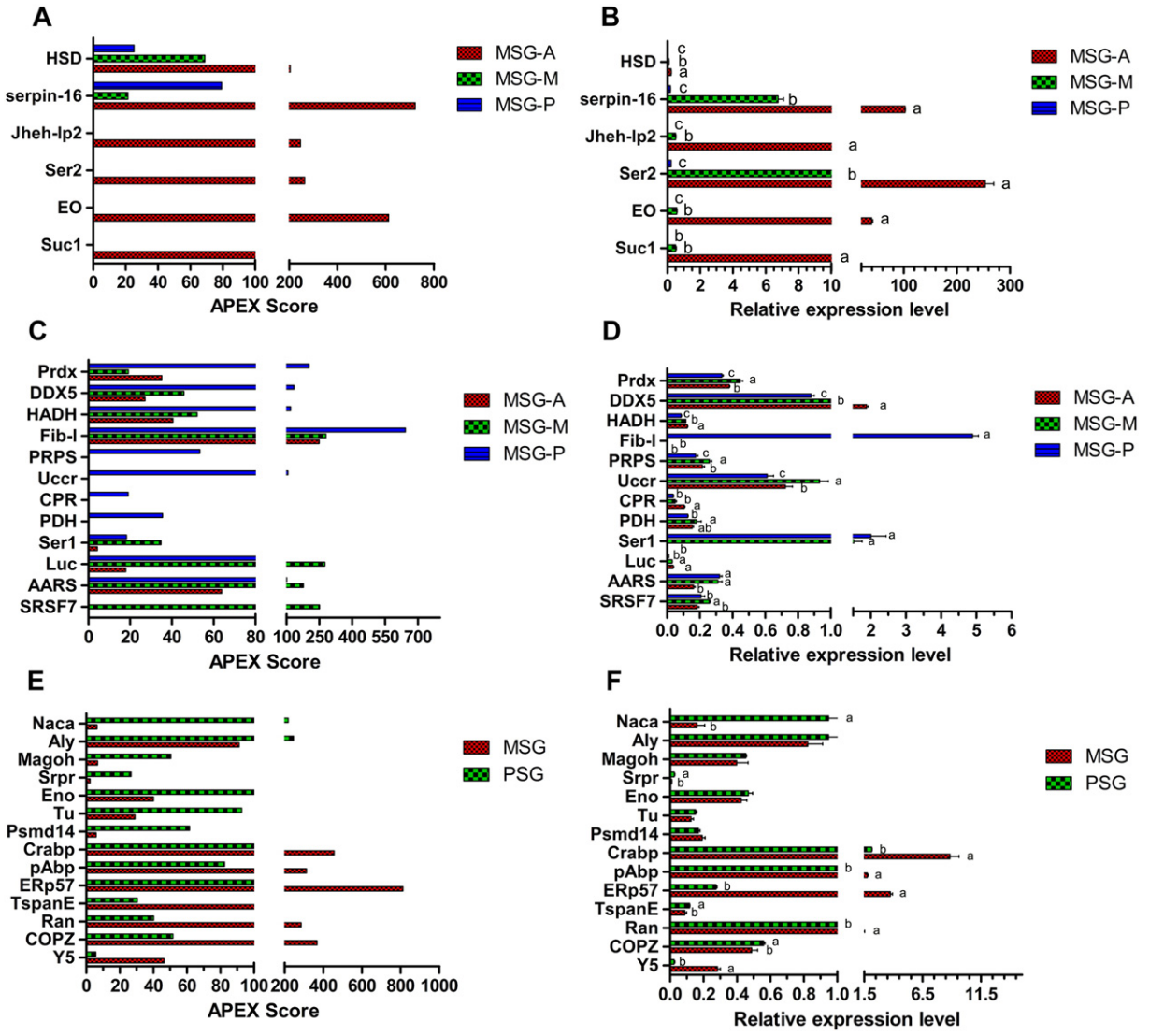


Fig. 5. Comparison of translational and transcriptional levels of the identified proteins. Translational level comparison was based on the APEX scores of the identified proteins. The transcriptional level comparison of the identified proteins was performed by RT-qPCR with relative expression. The mRNA expression was normalized with the reference gene (GAPDH). A, C, and E as the translational level comparisons; B, D, and F, as the transcriptional level comparisons; and E and F, as the translational and transcriptional level comparisons of proteins in the MSG and PSG, respectively. The significance of the difference between each comparison pair in qPCR results was analyzed that p values < 0.05 were taken to be statistically significant. (a) is significantly higher than (b) and (c), and (b) is significantly higher than (c).

energy metabolism and development related pathways (Supplementary Table 11).

Compared with the MSG-M, the MSG-P contained 259 specific proteins and 17 additional highly expressed proteins (Figs. 2A and 3E). In contrast, only 60 proteins were identified to have specific expression and higher expression in the MSG-M. However, there was a significant difference in the biosynthetic processes (GO: 0009058) between the MSG-M and MSG-P (accounting for 16.7% and 8.3% of the total provided gene number, respectively).

There were 101 and 282 proteins that were specifically and highly expressed in the MSG-A and MSG-P, respectively (Figs. 2A, 3E and 6C). Significantly higher percentages of proteins with catalytic (GO: 0003824) and binding (GO: 0005488) activities were expressed in the MSG-A than in the MSG-P ($P < 0.05$). By comparison to the MSG-P, the proteins that were specific to and more highly expressed in the MSG-A and MSG-M were enriched in the ribosome pathway. In contrast, the proteins that were specific to and more highly expressed in the MSG-P were enriched in the citrate cycle (TCA cycle) and oxidative phosphorylation pathways. In addition, in each comparison pair, the specifically expressed proteins and differentially expressed proteins involved in the pathways were further classified according to the KEGG definition (Fig. 7A). Most of these proteins were involved in metabolism pathways (C1). The proteins with higher expression in the MSG-P than in the MSG-A and MSG-M were involved in 7 and 8 carbohydrate metabolism related pathways, respectively.

The specifically expressed proteins and the differentially expressed proteins in the MSG and PSG were also classified by GO analysis (Fig. 6D). When categorized by molecular function, there was a significant difference in the proteins with structural molecular activity (GO: 0005198), primarily the structural constituents of ribosomes (GO: 0003735), which account for 10.7% and 1.9% of the total provided gene number in the MSG and PSG, respectively ($P < 0.05$). Compared with the PSG, the proteins with specific and higher expression in the MSG were enriched in pathways such as the ribosome pathway, aminoacyl-tRNA biosynthesis, and lysine degradation, whereas those in the PSG were enriched in energy metabolism related pathways, such as oxidative phosphorylation and glycolysis/gluconeogenesis (Table 1). In fact, among the 28 proteins with the highest differences (fold changes ≥ 5) between the MSG and PSG, 11 were ribosomal proteins, including ribosomal proteins S9, L18, S24, L10, and L9

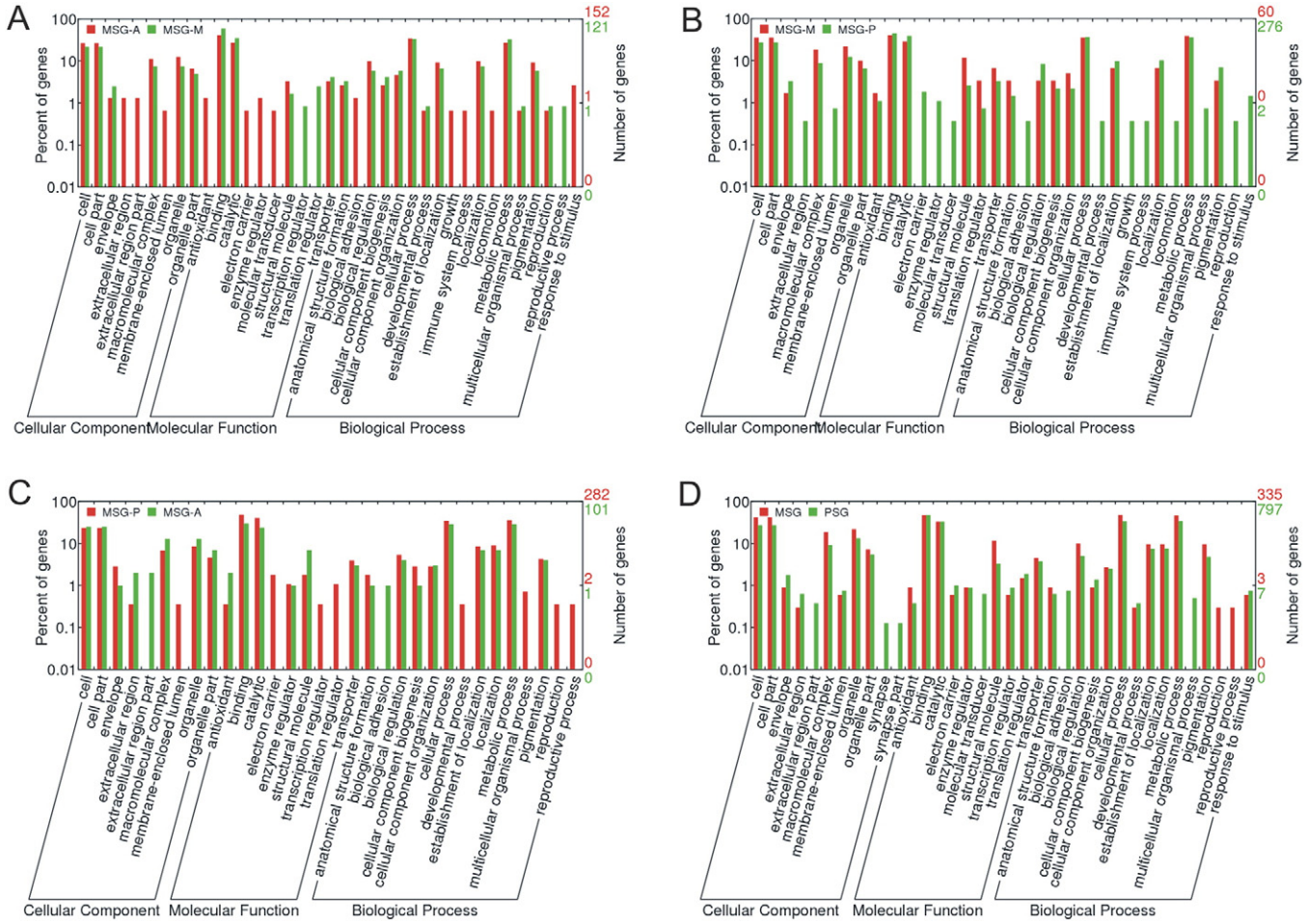


Fig. 6. GO categories of the differentially expressed proteins. A, the GO categories of proteins with specific and significantly higher expression in the MSG-A and MSG-M; B, the GO categories of proteins with specific and significantly higher expression in the MSG-M and MSG-P; C, the GO categories of proteins with specific and significantly higher expression in the MSG-A and MSG-P; D, the GO categories of proteins with specific and significantly higher expression in the MSG and PSG.

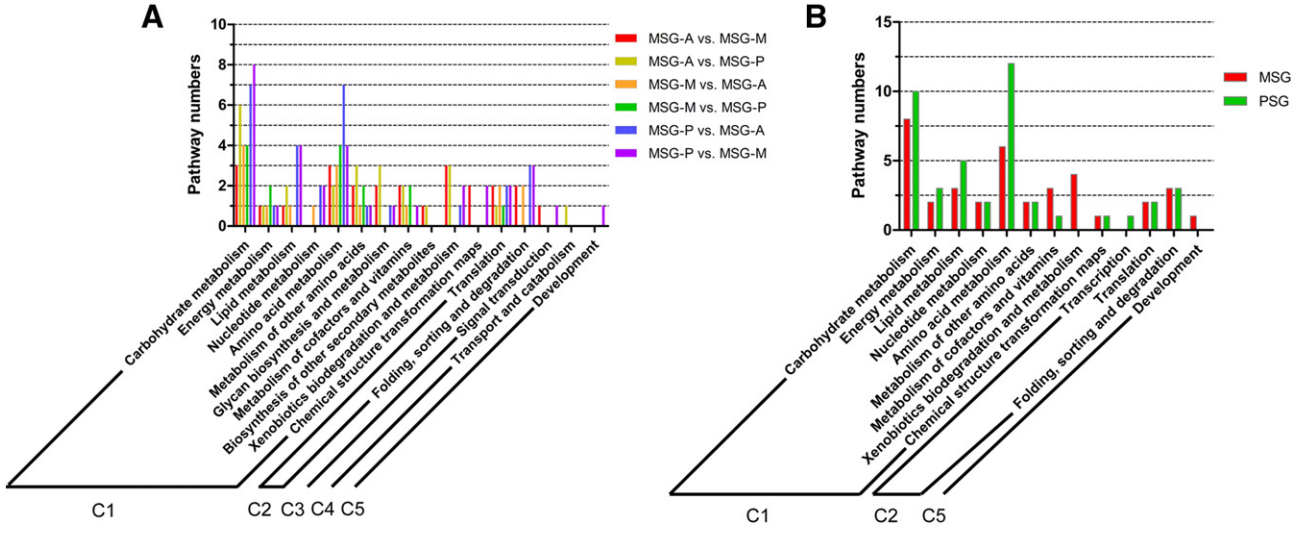


Fig. 7. KEGG pathway classification of the differentially expressed proteins. The differentially expressed proteins involved pathways were further classified based on KEGG definitions into metabolism (C1), genetic information processing (C2), environmental information processing (C3), cellular processes (C4), and organismal systems (C5). A, pairwise comparisons of pathways of the differentially expressed proteins involved among MSG-A, MSG-M, and MSG-P. In each comparison pair, the former represents the proteins with specific and higher expression compared with the latter involved pathways. B, the specific and more highly expressed proteins involved pathways in the MSG and PSG.

Table 1
KEGG pathway enrichment analysis of the differentially expressed proteins between the MSG and PSG.

Pathway	Count	p-Value	q-Value
Proteins with specific and higher expression in the MSG than in the PSG			
Ribosome	30	1.50E−39	8.19E−38
Aminoacyl-tRNA biosynthesis	7	1.43E−08	8.65E−08
Lysine degradation	6	2.25E−07	1.17E−06
Proteasome	6	2.08E−06	9.45E−06
Pyruvate metabolism	5	1.06E−05	3.84E−05
Valine, leucine and isoleucine degradation	5	1.06E−05	3.84E−05
Citrate cycle (TCA cycle)	5	1.24E−05	4.16E−05
Biotin metabolism	3	2.93E−05	8.76E−05
Fatty acid elongation in mitochondria	3	2.93E−05	8.76E−05
Glutathione metabolism	4	7.43E−05	1.95E−04
Glyoxylate and dicarboxylate metabolism	3	9.84E−05	2.50E−04
Oxidative phosphorylation	7	1.08E−04	2.62E−04
Fatty acid metabolism	4	2.09E−04	4.38E−04
Pentose phosphate pathway	3	5.31E−04	9.64E−04
Glycine, serine and threonine metabolism	3	7.46E−04	1.32E−03
SNARE interactions in vesicular transport	3	8.71E−04	1.51E−03
Dorso-ventral axis formation	3	1.16E−03	1.98E−03
Caprolactam degradation	2	1.85E−03	3.01E−03
Metabolism of xenobiotics by cytochrome P450	3	2.13E−03	3.36E−03
Butanoate metabolism	3	2.62E−03	3.91E−03
Protein export	2	3.89E−03	5.55E−03
Pentose and glucuronate interconversions	2	5.64E−03	7.59E−03
Reductive carboxylate cycle (CO2 fixation)	2	6.62E−03	8.66E−03
Bile acid biosynthesis	2	7.68E−03	9.79E−03
Proteins with specific and higher expression in the PSG than in the MSG			
Oxidative phosphorylation	21	8.15E−15	1.23E−13
Glycolysis/Gluconeogenesis	12	8.32E−12	5.60E−11
Pyrimidine metabolism	11	1.74E−08	7.03E−08
Butanoate metabolism	8	7.06E−08	2.44E−07
Starch and sucrose metabolism	8	1.04E−06	3.24E−06
Purine metabolism	11	1.80E−06	5.33E−06
Pyruvate metabolism	7	2.10E−06	6.05E−06
Ribosome	10	7.50E−06	1.83E−05
Carbon fixation	5	2.87E−05	5.99E−05
Fatty acid metabolism	6	2.96E−05	6.02E−05
Valine, leucine and isoleucine degradation	6	2.96E−05	6.02E−05
Citrate cycle (TCA cycle)	6	3.58E−05	7.10E−05
RNA polymerase	5	3.76E−05	7.23E−05
Fructose and mannose metabolism	5	1.19E−04	2.14E−04
Bile acid biosynthesis	4	1.34E−04	2.37E−04
Lysine degradation	5	2.09E−04	3.56E−04
Tryptophan metabolism	5	3.44E−04	5.21E−04
Methionine metabolism	3	4.12E−04	6.15E−04
SNARE interactions in vesicular transport	4	5.91E−04	8.41E−04
Phenylalanine, tyrosine and tryptophan biosynthesis	3	8.56E−04	1.17E−03
Protein export	3	8.56E−04	1.17E−03
Glutamate metabolism	4	1.03E−03	1.28E−03
Proteasome	5	1.15E−03	1.42E−03
Sphingolipid metabolism	3	1.95E−03	2.20E−03
Arginine and proline metabolism	4	2.20E−03	2.40E−03
Aminoacyl-tRNA biosynthesis	4	3.23E−03	3.44E−03
Pentose phosphate pathway	3	4.37E−03	4.50E−03
Galactose metabolism	3	6.05E−03	5.95E−03
Glycine, serine and threonine metabolism	3	6.05E−03	5.95E−03
Fatty acid elongation in mitochondria	2	7.83E−03	7.43E−03

(Supplementary Table 10). The highly expressed proteins in the PSG were involved in 10 carbohydrate metabolism related pathways and 12 amino acid metabolism related pathways (Fig. 7B).

We previously revealed that a large number of ribosomal proteins were up-regulated in the PSG from V3 to V5 when the silk proteins are efficiently synthesized [24]. To uncover the relationship between ribosomal protein expression and silk proteins synthesis, we compared the proteins in the MSG with specific and higher expression than the PSG with the up-regulated proteins in the PSG from V3 to V5. We found 33 overlapped proteins that were involved in some pathways, including the ribosome pathway (14), oxidative phosphorylation (2), protein export (1), glycine, serine and threonine metabolism (1), glutathione metabolism (1), and aminoacyl-tRNA biosynthesis (1) (Table 2). Clearly, nearly half (14/33) of the proteins with increased expression are involved in protein synthesis.

4. Discussion

The silkworm MSG is an important tissue for sericin synthesis and secretion. However, the molecular nature of how it accomplishes this function is not well understood. Furthermore, the three subsections of the MSG share the same proteome and biological function still remains elusive. This study performed an in-depth comparison of the MSG-A, -M, and -P proteomes, as well as of the proteomes of the MSG and PSG. Differential expression proteins and related pathways which were identified in our experiment contribute to better understanding of the molecular mechanisms underlying the different functions of the different parts of silk gland.

The MSG-A exhibited more differentially expressed proteins, as it was not clustered with MSG-M and MSG-P in the heat map (Fig. 4), suggesting that MSG-A has a unique function. The specifically expressed protein sericin 2 is thought to be related to cocoon reelability and to the high adhesiveness of silk fibers produced before each molt and just before cocoon spinning [29,41]. Thus, MSG-A is thought to be critical for cocoon spinning and for regulation of the cocoon characteristics. In addition, the highly expressed proteins in the MSG-A include serine protease inhibitor 16 precursor. Serine protease inhibitors (SPIs) are an important protease inhibitor family and may be involved in synthesizing and protecting silk protein [42]. DNA microarray analysis showed that SPIs 16, 18 and 22, which are group F members of the serpin family, were exclusively expressed in the silk gland during the fifth instar [42]. Proteomic analysis revealed that these proteins were expressed highly or exclusively in the MSG [23]. In our study, we further confirmed the high expression of SPI 16 (serpin-16) in the MSG-A and not in the MSG-M and MSG-P at both the transcriptional and translational levels (Fig. 5A, B). This result suggests that the MSG-A is responsible for silk protein protection via the expression of group F members of the serpin family. In addition, the MSG-A specific proteins EO, JHEH, and MSG-A highly expressed protein hydroxysteroid dehydrogenase suggest that the MSG-A is involved in insect hormone metabolism and may play important roles in silk gland growth and development as well as in control of silk protein gene expression [31,43,44]. Pathway analysis revealed that the proteins with specific expression and higher expression in the MSG-A than in the MSG-M were mainly involved in the energy metabolism and development related pathways. For example, the Wnt signaling pathway is involved in animal development. In *Drosophila*, it has been shown that Wnt signaling involves cross-talk with JH signaling by suppressing the transcription of genes that encode putative JH receptors [45]. This result further supports the role of MSG-A in silk gland development.

The MSG-M is the thickest part of the MSG and provides storage for the synthesized silk, which suggests that it is the primary location for sericin synthesis and secretion. Of the few specific proteins, the Ras protein may closely correlate with the function of the MSG-M. Of the three *Bombyx Ras* genes with different expression patterns [46], the activated Ras1 in the PSG of silkworms can increase cell and nuclei sizes, enrich subcellular organelles related to protein synthesis, and stimulate ribosome biogenesis, which together leads to improved silk yields [47]. In addition, highly expressed sericin 1, eIFs and ribosomal proteins were identified, confirming that the MSG-M is the most important region for sericin synthesis. Pathway analysis reveals that the proteins with higher expression levels in the MSG-M were involved in the aminoacyl-tRNA biosynthesis and ribosome pathways. These pathways are related to protein translation, implying highly active proteins synthesis in the MSG-M.

Among the highly expressed proteins in the MSG-P, nonclathrin coat protein gamma1-COP is involved in the early secretory pathway by binding to the cytoplasmic dilysine motif of membrane proteins [48].

Table 2
Comparison of proteins with significantly higher expression in the MSG and PSG (V5) than in the PSG (V3).

Protein Accession No.	Protein Annotation	APEX (PSG(V3))	APEX(MSG)	APEX (PSG(V5))	APEX_FOLD (MSG/PSG(V3))	APEX_FOLD (PSG(V5/V3))
BGIBMGA002504-PA	PREDICTED: GTP-binding protein SAR1b-like isoform X1	22.31	272.54	413.39	12.22	18.53
BGIBMGA003335-PA	PREDICTED: proteasome subunit beta type-6-like	14.90	86.08	60.39	5.78	4.05
BGIBMGA003977-PA	PREDICTED: serine--tRNA ligase, cytoplasmic-like	17.68	48.06	39.59	2.72	2.24
BGIBMGA005270-PA	PREDICTED: translocation protein SEC62-like isoform X2	17.57	67.85	58.07	3.86	3.31
BGIBMGA008066-PA	aminopeptidase N-like precursor	11.24	32.01	35.40	2.85	3.15
BGIBMGA008894-PA	PREDICTED: ADP-ribosylation factor 2-like	68.77	178.86	468.56	2.60	6.81
BGIBMGA011467-PA	ribosomal protein L18A	70.29	456.99	410.98	6.50	5.85
BGIBMGA012030-PA	PREDICTED: hypothetical protein	23.76	105.29	132.17	4.43	5.56
BGIBMGA013327-PA	tudor staphylococcus/micrococcal nuclease	35.84	316.54	137.55	8.83	3.84
gi|112982661	ribosomal protein S6	115.38	476.78	263.52	4.13	2.28
gi|112982844	ribosomal protein L7	116.52	504.71	278.61	4.33	2.39
gi|112983314	ras-related GTP-binding protein Rab11	40.16	103.53	116.28	2.58	2.90
gi|112983495	ribosomal protein L9	76.14	553.57	500.33	7.27	6.57
gi|112983527	ribosomal protein L27A	56.76	288.84	181.29	5.09	3.19
gi|112983546	ribosomal protein L12	51.75	280.87	484.79	5.43	9.37
gi|112984022	ribosomal protein S9	20.15	920.30	481.35	45.68	23.89
gi|112984164	ribosomal protein L35	70.02	451.95	238.87	6.45	3.41
gi|112984318	ribosomal protein L18	46.17	649.91	722.45	14.08	15.65
gi|112984334	ribosomal protein L11	62.09	235.88	200.93	3.80	3.24
gi|112984404	ribosomal protein L15	140.54	1001.14	516.16	7.12	3.67
gi|112984422	ribosomal protein L17	78.75	543.15	653.87	6.90	8.30
gi|114050851	tetraspanin E	30.42	132.98	116.71	4.37	3.84
gi|114051097	H + transporting ATP synthase subunit g	43.46	280.11	286.85	6.45	6.60
gi|114051996	nonclathrin coat protein zeta 1-COP	51.61	367.70	205.30	7.13	3.98
gi|114052751	GTP-binding nuclear protein Ran	40.16	286.13	98.35	7.12	2.45
gi|114052797	signal peptidase 18 kDa subunit	101.30	226.79	474.49	2.24	4.68
gi|114053277	H + transporting ATP synthase O subunit	45.07	375.67	200.15	8.34	4.44
gi|148298648	ribosomal protein S13	96.61	409.64	395.95	4.24	4.10
gi|148298726	ADP-ribosylation factor	55.33	270.30	683.32	4.88	12.35
gi|148298732	ribosomal protein S20	78.94	214.31	403.52	2.71	5.11
gi|151301000	ribosomal protein S24	34.99	368.69	679.30	10.54	19.41
gi|154146197	translocon-associated protein gamma	207.32	492.28	1114.53	2.37	5.38
gi|160333861	ribosomal protein L10	38.55	348.92	224.47	9.05	5.82

The biogenesis of most secretory proteins begins with a signal sequence-directed translocation of the polypeptide into the endoplasmic reticulum (ER) where they undergo processing and folding [49]. Newly synthesized secretory proteins arrive at the cis-Golgi after membrane fusion and progress through the Golgi complex. They may traffic within the Golgi and undergo retrograde Golgi–ER transport through COPI-mediated vesicular transport [49]. COPI vesicles are coated by seven coatomer subunits: α-, β-, β′-, γ-, δ-, ε-, and ζ-COP. In this study, we identified all these secretory pathway related COPs, and α- and γ- COP with significantly higher expression in the MSG-A and MSG-P, respectively. Given that the sericins are secretory proteins that are mainly synthesized in the MSG-M, it is elusive to determine the function of the COPI in the secretion of sericin. However, protein secretion is not only essential for cellular function but also provides the driving force for cell growth via the delivery of newly synthesized proteins, which permits cell expansion [49]. COPI has a proven essential role in the tube expansion of the silkworm PSG [50]. Thus, these results suggest that the MSG-A and MSG-P are still at an active tube expansion stage. The proteins with specific expression and higher expression in the MSG-P were mainly involved in the TCA cycle and oxidative phosphorylation pathways, which occur in the matrix of the mitochondrion to provide ATP. The vigorous energy metabolism may provide the driving force for silk gland growth and tube expansion. However, the active oxidative phosphorylation can produce large amount of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, which lead to damage of cells. ROSs may be the reason that the peroxiredoxin was expressed in the MSG-P at about ten-fold higher levels than in the MSG-M.

Pairwise comparison of proteomes among the MSG-A, -M, and -P revealed their diverse functional assignments; furthermore, the MSG as a whole was found to perform biological functions that were molecularly distinct from PSG. With the exception of ribosomal proteins, several important proteins expressed at higher levels in the MSG compared with the PSG were involved in active protein translocation. Ribosomal L10-like protein and the translocon protein gamma subunit-like protein interact with constitutive transport elements involved in the cellular translational and translocation machinery [51]. Secretory and membrane proteins are translocated across or integrated into ER membranes, forming a protein-conducting channel. The largest subunit of a heterotrimer, Sec61α, is a multi-spanning membrane protein that forms the central pore of the channel [52,53]. Sec61 interacts with heterotetrameric translocon associated-protein (TRAP) and plays a role in protein translocation with the help of transmembrane proteins [54,55]. Co-translational translocation begins with a targeting process in which the 54 kDa subunit of a signal recognition particle (SRP) binds to the signal sequence in a ribosome-bound nascent polypeptide chain [52]. Then, the nascent polypeptide-associated complex (Naca) serves as an inhibitor of the SRP-independent interaction of the ribosome with the ER membrane. These translocation related proteins were all highly expressed in the MSG, whereas the Naca that acts as an inhibitor was expressed much more highly in the PSG. These results suggest a much more active protein processing in the MSG than in the PSG. In addition, protein disulfide-isomerase-like protein ERp57 was also abundantly expressed in the MSG at both the transcriptional and translational level (Fig. 5E, F). ERp57 is a luminal ER protein involved in the glycoprotein folding by cooperation with calnexin and calreticulin [56]. In fact, the calreticulin precursor was also highly expressed in the MSG. Furthermore, ERp57 is also presumably involved in the synthesis of functional silk protein, as it was down regulated in the PSG of silkworms with lower silk yield [57]. These results imply that the MSG is more active in protein translation, processing, and secretion than the PSG.

To reveal the underlying relationship between protein expression and silk protein synthesis efficiency, a comparison of the whole proteomes of the MSG and PSG was performed, and dozens of differentially expressed proteins were identified that were mainly involved in ribosome pathway. Protein synthesis is the basis of cell growth. The enhanced expression of ribosome proteins reflects the fast growing of the silk gland. On the other hand, the expanded silk gland provides more units for silk proteins synthesis and secretion. Quantitative proteomic and transcriptomic analyses of the PSG from normal and low-yield silkworm strains revealed numerous down-regulated transcripts that were involved in the ribosome pathway, whereas the transcripts involved in energy metabolism related pathways, including oxidative phosphorylation, glycolysis/gluconeogenesis, and the TCA cycle, were enhanced [58]. Likewise, the stronger translation activity in the MSG implies a higher efficiency for silk protein synthesis at the early stage of the fifth instar, although the sericin protein weight in the cocoon is much lighter than the fibroin proteins secreted from the PSG. Thus, it is reasonable that at beginning of cocooning the silkworm larva must firstly spin sericin to fix the cocoon by synthesizing sericin prior to fibroin.

By contrast, the identification of numerous proteins involved in ribosome pathway also implies highly active ribosome biogenesis. Ribosome biogenesis involves a series of processes, i.e., synthesis and processing of both rRNA and the ribosomal proteins, as well as assembly of the components. All of these events must be tightly regulated and coordinated to prevent energy losses and imbalances in cell physiology [59]. The ribosomal protein S9 is required in the early steps of ribosome biogenesis. It has three motifs that mediate its nucleolar localization and interaction with NPM1/nucleophosmin, which is a multifunctional nucleolar protein playing an important role in genomic stability, ribosome biogenesis, and anti-apoptotic signaling [60]. Depletion of S9 by RNA interference in human cancer cell lines results in decreased global protein synthesis in association with p53 target genes and is followed by cell cycle arrest or apoptosis [61]. It is clear that the much higher expression of S9 in the MSG indicates active ribosome biogenesis. In addition, ribosomal proteins S6, L7, and S24 that were highly expressed in the MSG are also involved in ribosome biogenesis [62]. Ribosome biogenesis underlies the cell's capacity to grow [63]. The active ribosome biogenesis in MSG also indicates its status of rapid growing. In turn, the enlarged cell size provides capacity to synthesize more proteins. It is reasonable that expanded silk gland is apt to produce more silk proteins. Fox example, activation of Ras1 in the silkworm PSG can increase cell and nuclei sizes and stimulate ribosome biogenesis leading to improvement of silk yields [47]. On the other hand, enhancing the expression of ribosome protein genes such as S9 may provide an alternative approach to activate ribosome biogenesis. Numerous researches have been carried out to improve the silk protein yields by modifying the cis-acting elements and regulation factors of the silk genes [8,13–19]. However, instead of improving silk yield through improved transcriptional regulation, a novel alternative approach may be to increase the amount of translational "factories" – i.e., ribosomes.

Supplementary Tables 1–11 are available free of charge via the Internet. Supplementary data associated with this article can be found, in the online version, at doi: http://dx.doi.org/10.1016/j.jprot.2015.06.001.

Conflicts of interest

The authors declare no competing financial interest.

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