Comparative Proteomic Analysis Reveals Immune Competence in Hemolymph of *Bombyx mori* Pupa Parasitized by Silkworm Maggot *Exorista sorbillans*

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**Abstract:** The silkworm maggot, *Exorista sorbillans*, is a well-known larval endoparasitoid of the silkworm *Bombyx mori* that causes considerable damage to the silkworm cocoon crop. To gain insights into the response mechanism of the silkworm at the protein level, we applied a comparative proteomic approach to investigate proteomic differences in the hemolymph of the female silkworm pupae parasitized by *E. sorbillans*. In total, 50 differentially expressed proteins (DEPs) were successfully identified, of which 36 proteins were upregulated and 14 proteins were downregulated in response to parasitoid infection. These proteins are mainly involved in disease, energy metabolism, signaling pathways, and amino acid metabolism. Eight innate immune proteins were distinctly upregulated to resist maggot parasitism. Apoptosis-related proteins of cathepsin B and 14-3-3 zeta were significantly downregulated in *E. sorbillans*-parasitized silkworm pupae; their downregulation induces apoptosis. Quantitative PCR was used to further verify gene transcription of five DEPs, and the results are consistent at the transcriptional and proteomic levels. This was the first report on identification of possible proteins from the *E. bombycis*-parasitized silkworms at the late stage of parasitism, which contributes to furthering our understanding of the response mechanism of silkworms to parasitism and dipteran parasitoid biology.

**Keywords:** *Bombyx mori*; *Exorista sorbillans*; parasitoid; proteomics; two-dimensional gel electrophoresis

1. Introduction

To combat microbial infection and eukaryotic parasite infestation, insects have efficient and potent innate immune systems [1–6]. Infection and infestation activate defense mechanisms, including cellular and humoral immune responses. Exogenous invaders are first recognized by recognition factors to trigger cellular immune reactions and humoral immune reactions [2,4]. After the recognition procedure, modulating and signaling factors are next activated [2]. Signaling transduction is stimulated in major immune tissues, such as the fat body and hemocytes, and the gene encoding effectors are activated through signaling cascades [2,4]. The effector molecules are produced in specific tissues and secreted into the hemolymph [2].

The domesticated silkworm originated in China, has been distributed to different parts of the world [7], and is an economically important animal. The silkworm maggot *Exorista sorbillans*, a well-
known larval endoparasitoid of the silkworm, is found in all silk producing areas of Asia, severely
damaging the silkworm cocoon crop [8,9]. The mated gravid female *E. sorbillans* oviposit on the
epidermis of a silkworm larva. After about 48 hours, the parasitic maggots hatch and invade the
*Bombyx mori* larvae. In silkworms, immune reactions are triggered after infestation by *Exorista bombycis* [2,8,9]. The expression of immune proteins and the encoding genes are enhanced in the epithelium of *E. bombycis*-infested silkworm larvae. In hemocytes of the host *B. mori* larva after infestation by the parasitoid larva of *E. bombycis*, the level of reactive oxygen species (ROS) as measured by H$_2$O$_2$ production increases from six hours and continues to increase, significantly
reaching maximum at 48 h; the H$_2$O$_2$ production causes cytotoxicity, lipid peroxidation, and
membrane porosity that suppress both the humoral- and cell-mediated immune responses of
hemocytes in *B. mori* [10]. In the early stage of parasitization, the antioxidative enzyme levels are
maintained at a high level in silkworm hemocytes, revealing the continuous need for antioxidative
enzymes to prevent immune suppression by enduring parasitism in the host [11]. Under parasitic
influence, the expressions of cell apoptosis-associated genes, including autophagy 5-like (*Atg5*),
apoptosis-inducing factor (*AIF*), and nedd2-like caspase, are enhanced in the larval integument of *B. mori* [12], which indicates parasitism-induced activation of apoptosis in the host [8,9].

At a stable temperature of 25 °C, the eggs of silkworm maggot, *E. sorbillans* are incubated for
about two days before hatching, after which the newly hatched first instar parasitoid larvae invade
the host *B. mori* cuticle. Maggots invade the silkworm body and parasitize between the body wall and
muscle, which is identified by the presence of black markings on the epidermis at the point of
infection [9]. The parasitoid maggot completes larval stages inside the silkworm fifth instar larvae for
about five days [9]. Some molecular mechanisms of the early stage of parasitization have been
reported previously, but the mechanism during the late stage is not yet clear. Otherwise, during
silkworm larval–pupal metamorphosis, degradation of tissues that are no longer needed is an
essential process [13]. *B. mori* undergo distinct morphological changes; the 9th and 12th ventral
segments of larva are healed in pupa, which may impact maggot parasitism.

In this study, we used two-dimensional gel electrophoresis (2-DE) combined with mass
spectrometry (MS) to explore the differences in hemolymph protein expression in the day-1 female
silkworm pupae parasitized by *E. sorbillans*. This is the late stage and the third (final) instar larva of
the endoparasitic maggot. Then, the third instar maggot was elicited from the silkworm body and
pupated outside. We successfully identified 50 differentially expressed proteins (DEPs), obtained
protein information, and annotated the molecular function. Our study provides an overview of the
proteomic profile in the hemolymph of *B. mori* response to *E. sorbillans* parasitic infection and lays a
foundation for clarifying the mechanism of silkworm resistance to *E. sorbillans*.

2. Materials and Methods

2.1. Experimental Animals and Sample Preparation

Larvae of the *B. mori* strain Baiyu were reared on mulberry leaves at a stable temperature of 25
°C. The day-2 fifth instar larvae were exposed to mated gravid females of *E. sorbillans* for oviposition
for 3 hours. Only one egg was allowed on the larval surface of each host through physical removal of
other eggs if any were present. Control larvae were maintained without infestation. The infected and
control silkworm larvae were fed until the silkworm matured to avoid the effect of starvation. The
larvae, larvae–pupae, and pupae were maintained under a 12-h light/dark photoperiod at 25 °C and
70% humidity. Hemolymph, hemocytes, and the fat body were collected from the infected and
control female silkworm pupae on the first day of pupation. This time is the late stage and the third
(final) instar larva of the endoparasitic maggot. Then, the third instar maggot was obtained from the
silkworm body and pupated outside. The hemolymph samples were centrifuged for 10 minutes at
12,000 rpm at 4 °C and stored in a lysis buffer of 9 M urea, 4% the zwitterionic 3-[3-cholamidopropyl]
dimethylamino]-1-propanesulfonate (CHAPS), 1% dithiothreitol, 1% immobilized pH gradient (IPG)
buffer, and a 1% protease inhibitor cocktail. The total protein content was quantified using a Bradford
assay kit (Bio-Rad, Hercules, CA, USA). The hemolymph is an open circulatory system. The major
proteins in the hemolymph are produced in other specific tissues and secreted into the hemolymph, and the fat body is one of the specific tissues [14]. Thus, the hemocytes and fat body samples were used to verify gene expression analysis.

2.2. 2-DE and Protein Digestion

The proteins were separated with 2-DE. In short, 200 µg of each sample protein was added to a 24-cm broad range IPG strip (nonlinear, pH 3 to 10) for isoelectric focusing (IEF), and 2-DE was performed in 12.5% polyacrylamide gel. The gel was stained with silver nitrate following the 2-DE procedure [15]. Spots were scanned at 300 dpi using a high-resolution image scanner and analyzed using PDQuest 8.0 software (Bio-Rad, Hercules, CA, USA). For statistical analysis of the data, we used a Student's t-test and the fold ratio was calculated. Three replicates were performed, and a threshold of \( p \leq 0.05 \) and fold changes of \( \geq 2.5 \) or \( \leq 0.4 \) were used to identify differently expressed protein spots. The marked protein spots were identified. Differentially expressed protein spots were cut from the gel with a scalpel and washed twice with ultrapure water. The samples were destained for 5 min, the destaining solution was removed, and the samples were washed twice and incubated in 50% acetonitrile for 5 min, removing the acetonitrile with the addition of 100% acetonitrile for 5 min. Each sample was rehydrated in 4.0 µL of trypsin solution (Promega, Madison, WI, USA) for 30 min, and we added 16 µL of cover solution. After digestion at 37 °C for 16 h, the supernatant was transferred into a new tube and extracted once with 50 µL extraction buffer (67% acetonitrile and 5% trifluoroacetic acid). The combined extraction solution was completely dried. The dried peptides were dissolved in 5 µL 0.1% trifluoroacetic acid (TFA) and then mixed in a 1:1 ratio with a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% TFA.

2.3. MALDI-TOF/TOF-MS/MS Analysis and Protein Identification

The mass spectrometry (MS) spectra of digested peptides were performed on 5800 MALDI-TOF/TOF Plus mass spectrometer (Applied Biosystems, Foster City, CA, USA). The data were obtained in a positive MS reflector using a CalMix5 standard to adjust by an I 5800 TOF-TOF Proteomics Analyser (Applied Biosystems, Framingham, MA, USA). The databases used in this analysis were obtained from NCBI (http://www.ncbi.nlm.nih.gov; 6391 sequences) and SilkDB (http://www.silkdb.org/silkdb; 20361 sequences). The analysis of both the MS and MS/MS spectra data and the protein identification processes were implemented in accordance with a previous study [16]. The peptides of the quantified proteins were provided in Table S1. Gene Ontology (GO) assignments were completed using Blast2GO (https://www.blast2go.com/) and obtained corresponding GO identification numbers (IDs) of the identified proteins using InterproScan 5.0 sequence search [17,18]. GO annotation results of the DEPs were provided in Table S2. The Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to perform pathway enrichment analysis of the identified proteins [19]. Descriptions of the KEGG pathways were provided in Table S3.

2.4. Quantitative PCR

The genes selected according to the DEPs were investigated by quantitative PCR (qPCR) at the transcriptional level. Total RNA from the hemocytes and fat body of the infected and control samples was used to synthesize the first strand cDNA using a PrimeScript Reverse Transcriptase kit (TaKaRa, Dalian, China) according to the manufacturer’s instructions. Specific primers of genes for qPCR are listed in Table S4. qPCR was performed as previously described [20]. The gene expression levels were calculated using the 2\(^{-ΔΔCt}\) method. There were three biological sample replicates, and each biological sample replicate included three independent experiments. The reference gene was B. mori ribosomal protein gene BmRPL3. The statistical analysis was conducted using ANOVA, followed by an LSD a posteriori test via SPSS statistical software (version 16.0; SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Identification of DEPs
In the interest of understanding the molecular mechanism of the late stage of *E. sorbillans*-parasitized silkworms at the protein level, we collected the hemolymph of female silkworm pupae on the first day of pupation and conducted 2-DE combined with MALDI-TOF/TOF-MS/MS analysis to identify the DEPs. The protein molecular mass varied within a normal range of approximately 10 to 116 kDa, and isoelectric point (pl) values ranged from 3 to 10 (Figure 1, Table S1), suggesting that protein extraction was correctly performed and that most proteins from the hemolymph were obtained. A differentially expressed spot was defined as a spot with a 2.5-fold or greater change in intensity and frequency higher than 40%. According to the criteria, 36 upregulated and 14 downregulated spots were selected and subjected to MS/MS identification (Table 1). The DEPs ranged from 10 to 82 kDa in molecular mass and from pH 4 to 10 in pl. Detailed information, including the spot numbers, accession numbers, predicted molecular weights (MWs), pIs, sequence coverage, peptide count, fold change, and signal peptide, is provided in Table 1. The following eight innate immune proteins were upregulated in the infected group: PPO2 (spot 1), paralytic peptide binding protein (spot 4), antitrypsin (spot 6), CTL10 (spot 10), apolipophorin III (spot 22), translationally controlled tumor protein (spot 23), peptidoglycan recognition protein (spot 27), and ubiquitin-like protein SMT3 (spot 36) (Figure 1 and Table 1). Apoptosis-related proteins of cathepsin B (spot 43) and 14-3-3 zeta (spot 45) were significantly downregulated in the infected group (Figure 1 and Table 1). In this study, we analyzed the tissue expression patterns of the genes encoding the DEPs based on the microarray data. The raw data of 10 silkworm tissues on day 3 of the fifth instar were obtained from SilkMDB. We found many highly expressed genes in the epidermis amongst the 10 tissues (Figure S1). The expressed genes are defined as previously described [21]. This finding may be closely related to the epidermal invasion of maggots in the silkworm. Our data contribute to the understanding of the infection pathway of maggots.

Figure 1. Two-dimensional gel electrophoresis (2-DE) maps of the control and infected hemolymph collected from day-1 female silkworm pupae: The differentially expressed proteins (DEPs) are indicated by circles and numeric labels, which correspond to the numbers presented in Table 1. All samples were processed in parallel.
Table 1. Data of the 50 DEPs identified by MALDI-TOF/TOF-MS/MS.

| Spot No. | Protein Name | SilkDB Accession No. | NCBI Accession No. | Theoretical (kDa/pI) | Sequence Coverage (%) | Peptides Identified | Fold Change | Signal Peptide | Function |
|----------|--------------|----------------------|--------------------|----------------------|-----------------------|--------------------|-------------|---------------|----------|
| 1        | phenoloxidase subunit 2 precursor | BGIBMGA013115-PA | gi|112983448 | 80.12/5.62 | 18 | 11 | 3.66 | - | Serine protease involved in melanization |
| 2        | lipoprotein receptor isoform 1 precursor | BGIBMGA006214-PA | gi|160333138 | 61.54/5.20 | 4 | 3 | 4.53 | - | Chitin metabolic |
| 3        | protein, mitochondrial-like paralytic peptide binding protein uncharacterized | BGIBMGA007349-PA | gi|512896628 | 61.06/5.51 | 22 | 8 | 2.61 | - | Protein refolding |
| 4        | very low-density lipoprotein receptor isoform 1 precursor | BGIBMGA010168-PA | gi|11298982 | 50.01/6.33 | 30 | 11 | 3.17 | - | Extracellular region |
| 5        | 60 kDa heat shock protein, mitochondrial-like | BGIBMGA002604-PA | gi|512931715 | 49.40/5.42 | 15 | 6 | 2.94 | - | Farnesoic acid o-methyltransferase Peptidase inhibitor activity |
| 6        | antitrypsin | BGIBMGA009953-PA | gi|1253809709 | 43.43/5.41 | 26 | 8 | 9.88 | - | |
| 7        | failed axon connections isoform X | BGIBMGA000352-PA | gi|51292131 | 42.89/5.31 | 11 | 3 | 3.64 | - | N/A |
| 8        | proliferation-associated protein 2G4 DNA supercoiling factor | BGIBMGA008269-PA | gi|148298818 | 36.56/5.33 | 6 | 2 | 3.59 | - | Carbohydrate binding |
| 9        | 32 kDa apolipoprotein precursor | BGIBMGA002763-PA | gi|126501956 | 32.06/4.79 | 7 | 3 | 2.91 | 1 | Pigment binding |
| 10       | spermidine synthase small glutamine-rich tetratricopeptide repeat-containing protein alpha-like uncharacterized | BGIBMGA005897-PA | gi|1252899761 | 32.43/5.54 | 26 | 8 | 3.15 | - | Catalytic activity |
| 11       | low molecular mass 30 kDa lipoprotein 21G1 isoform X1 | BGIBMGA004395-PA | gi|1827538310 | 30.24/6.84 | 33 | 9 | 5.78 | 1 | Extracellular region |
| Gene ID/Name                                                                 | Description                                                                 | Accession/ID                      | S1/2 | S2/3 | p-value 1 | p-value 2 | p-value 3 | Pathway                                  |
|---------------------------------------------------------------------------|----------------------------------------------------------------------------|----------------------------------|------|------|-----------|-----------|-----------|------------------------------------------|
| low molecular 30 kDa lipoprotein PBMHPC-19-like precursor charged multivesicular body protein 5 | BGIBMGA0043498-PA gi|525343846                          | 28.50/5.72 | 13  | 5.77      | 1         | Extracellular region                      |
| Rab7                                                                      | BGIBMGA007712-PA gi|1140/51368                          | 23.42/5.16 | 4   | 8.85      | -         | Small GTPase mediated signal transduction |
| uncharacterized protein LOC101746349 apolipopophin III translationally controlled tumor protein | BGIBMGA006731-PA gi|51292615                            | 25.26/4.71 | 16  | 4.34      | -         | N/A                                      |
| translation initiation factor 5A                                          | BGIBMGA0007466-PA gi|112982832                           | 17.52/5.16 | 14  | 2.93      | -         | N/A                                      |
| abnormal wing disc-like protein                                          | BGIBMGA0007701-PA gi|153791847                           | 17.31/6.74 | 26  | 11.04     | -         | N/A                                      |
| cyclophilin-like protein                                                  | BGIBMGA002429-PA gi|60592747                            | 17.96/7.74 | 17  | 3.81      | -         | N/A                                      |
| peptidoglycan recognition protein                                        | BGIBMGA008038-PA gi|1129829046                          | 21.63/6.70 | 33  | 3.32      | 1         | N/A                                      |
| probable pterin-4-alpha-carbinolamine dehydratase                        | BGIBMGA008038-PA gi|1129829046                          | 17.93/9.94 | 30  | 18.51     | -         | N/A                                      |
| odorant-binding protein 6 isoform X1                                      | BGIBMGA008354-PA gi|1827551076                           | 15.96/4.94 | 18  | 3.41      | 1         | Odorant binding                          |
| E3 ubiquitin-protein ligase ZNF2                                           | BGIBMGA001980-PA gi|1512987556                           | 21.91/5.42 | 7   | 4.56      | -         | Zinc ion binding                         |
| ribosomal protein S12                                                     | BGIBMGA004374-PA gi|112982671                           | 15.03/5.79 | 24  | 4.62      | -         | Structural constituent of ribosome       |
| chemosensory protein 4 precursor                                         | BGIBMGA004048-PA gi|112983094                           | 14.55/5.17 | 8   | 2.51      | 1         | Transports of pheromone/odor molecules   |
| chemosensory protein 7 precursor                                         | BGIBMGA004048-PA gi|112983052                           | 13.52/4.97 | 18  | 4.77      | -         | Isomerase activity                       |
| FK506-binding protein uncharacterized protein LOC101736984 isoform X3 ubiquitin-like protein SMT3 | BGIBMGA004331-PA gi|1140/51243                           | 11.82/7.85 | 43  | 10.66     | -         | N/A                                      |
| heat shock protein R3                                                     | BGIBMGA004612-PA gi|112983556                           | 82.42/4.98 | 3   | 0.11      | -         | Unfolded protein binding                  |
|   | Gene Description                                      | Accession | Score 1 | Score 2 | Coverage 1 | Coverage 2 | Activity     | Notes                                      |
|---|-------------------------------------------------------|-----------|---------|---------|------------|------------|--------------|-------------------------------------------|
|38 | ATP synthase serine proteinase-like protein isoform X1 ornithine | BGIBMGA001853-PA gi|114052278 | 59.66/9.21 | 4          | 2          | 0.21         | ATP binding                              |
|39 | aminotransferase, mitochondrial aldose 1-epimerase aldo-ketoreductase AKR2E4-like isoform X1 | BGIBMGA009551-PA gi|1827563139 | 44.88/5.73 | 15         | 6          | 0.01         | 1 Serine-type endopeptidase activity     |
|40 | ornithine aminotransferase, mitochondrial aldose 1-epimerase aldo-ketoreductase AKR2E4-like isoform X1 | BGIBMGA003564-PA gi|512922127 | 44.70/6.36 | 5          | 2          | 0.21         | Pyridoxal phosphate binding              |
|41 | ornithine aminotransferase, mitochondrial aldose 1-epimerase aldo-ketoreductase AKR2E4-like isoform X1 | BGIBMGA009232-PA gi|512891308 | 39.71/5.88 | 27         | 7          | 0.28         | Transaminase activity                    |
|42 | ornithine aminotransferase, mitochondrial aldose 1-epimerase aldo-ketoreductase AKR2E4-like isoform X1 | BGIBMGA003134-PA gi|512908850 | 38.96/5.82 | 10         | 5          | 0.26         | Oxidoreductase activity                  |
|43 | ornithine aminotransferase, mitochondrial aldose 1-epimerase aldo-ketoreductase AKR2E4-like isoform X1 | BGIBMGA007061-PA gi|112983908 | 37.56/5.95 | 18         | 5          | 0.17         | Regulation of catalytic activity         |
|44 | ornithine aminotransferase, mitochondrial aldose 1-epimerase aldo-ketoreductase AKR2E4-like isoform X1 | BGIBMGA002644-PA gi|512901366 | 35.84/6.10 | 20         | 6          | 0.39         | Oxidoreductase activity                  |
|45 | ornithine aminotransferase, mitochondrial aldose 1-epimerase aldo-ketoreductase AKR2E4-like isoform X1 | BGIBMGA0012152-PA gi|114059001 | 28.17/4.90 | 6          | 2          | 0.21         | Protein domain specific binding           |
|46 | ornithine aminotransferase, mitochondrial aldose 1-epimerase aldo-ketoreductase AKR2E4-like isoform X1 | BGIBMGA002644-PA gi|512901366 | 35.84/6.10 | 20         | 6          | 0.39         | Oxidoreductase activity                  |
|47 | ornithine aminotransferase, mitochondrial aldose 1-epimerase aldo-ketoreductase AKR2E4-like isoform X1 | BGIBMGA0012152-PA gi|114059001 | 28.17/4.90 | 6          | 2          | 0.21         | Protein domain specific binding           |
|48 | ornithine aminotransferase, mitochondrial aldose 1-epimerase aldo-ketoreductase AKR2E4-like isoform X1 | BGIBMGA002644-PA gi|512901366 | 35.84/6.10 | 20         | 6          | 0.39         | Oxidoreductase activity                  |
|49 | ornithine aminotransferase, mitochondrial aldose 1-epimerase aldo-ketoreductase AKR2E4-like isoform X1 | BGIBMGA002644-PA gi|512901366 | 35.84/6.10 | 20         | 6          | 0.39         | Oxidoreductase activity                  |
|50 | ornithine aminotransferase, mitochondrial aldose 1-epimerase aldo-ketoreductase AKR2E4-like isoform X1 | BGIBMGA002644-PA gi|512901366 | 35.84/6.10 | 20         | 6          | 0.39         | Oxidoreductase activity                  |

Note: - no signal peptide was predicted and no SilkDB accession number was found; N/A not applicable.
3.2. GO Annotation and KEGG Pathway Enrichment Analysis of the DEPs

According to the GO annotation, 49 of the 50 identified DEPs were discovered with at least one GO match (Table S2). Based on the GO database, the identified DEPs were classified into three categories: cellular component, molecular function, and biological process (Figure 2). In the cellular component category, the DEPs are involved in different cellular processes and the extracellular region of the cell and macromolecular complex were the main members. The DEPs are mainly related to binding and catalysis in the molecular function category. The biological process category showed DEPs that are mainly involved in cellular, metabolic, and single-organism processes. KEGG pathway enrichment analysis of the identified DEPs showed that the main pathways are involved in disease, energy metabolism, signaling pathways, and amino acid metabolism (Table 2 and S3).

Figure 2. Distribution of the Gene Ontology (GO) terms for all proteins identified from the control and infected hemolymph: The DEPs were classified into cellular component, molecular function, and biological process by GO.
Table 2. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEPs.

| Number | Pathway                                      | Pathway ID | Accession No. | Description                  | Fold Change |
|--------|----------------------------------------------|------------|---------------|------------------------------|-------------|
| 1      | Galactose metabolism                         | ko00052    | gi|512891308   | aldose 1-epimerase            | 0.28        |
|        |                                              |            | gi|512908850   | aldehyde reductase            | 0.26        |
|        |                                              |            | gi|512901366   | aldehyde reductase            | 0.39        |
| 2      | Huntington’s disease                         | ko05016    | gi|160592747   | peptidyl-prolyl isomerase     | 3.81        |
|        |                                              |            | gi|114052278   | ATPase                        | 0.21        |
|        |                                              |            | gi|68144076   | superoxide dismutase          | 0.15        |
| 3      | Glycerolipid metabolism                      | ko00561    | gi|512908850   | aldehyde reductase            | 0.26        |
|        |                                              |            | gi|512901366   | aldehyde reductase            | 0.39        |
| 4      | Longevity regulating pathway—worm            | ko04212    | gi|512896628   | chaperonin                    | 2.61        |
| 5      | Lysosome                                     | ko04142    | gi|114050901   | protein binding               | 0.21        |
|        |                                              |            | gi|112983908   | cathepsin B                   | 0.17        |
|        |                                              |            | gi|151301100   | Niemann–Pick C2 protein       | 0.01        |
| 6      | Pentose and glucuronate interconversions     | ko00040    | gi|512908850   | aldehyde reductase            | 0.26        |
|        |                                              |            | gi|512901366   | aldehyde reductase            | 0.39        |
| 7      | Fructose and mannose metabolism              | ko00051    | gi|512908850   | aldehyde reductase            | 0.26        |
|        |                                              |            | gi|512901366   | aldehyde reductase            | 0.39        |
| 8      | Parkinson’s disease                          | ko05012    | gi|60592747   | peptidyl-prolyl isomerase     | 3.81        |
|        |                                              |            | gi|114052278   | ATPase                        | 0.21        |
| 9      | Amoebiasis                                   | ko05146    | gi|1253809709  | serpin B                      | 9.88        |
|        |                                              |            | gi|114051368   | Ras-related protein           | 8.85        |
| 10     | Oxidative phosphorylation                    | ko00190    | gi|114052278   | ATPase                        | 0.21        |
|        |                                              |            | gi|114052088   | ATPase                        | 0.19        |
| 11     | Tuberculosis                                 | ko05152    | gi|512896628   | chaperonin                    | 2.61        |
|        |                                              |            | gi|114051368   | Ras-related protein           | 8.85        |
| 12     | Antigen processing and presentation          | ko04612    | gi|112983556   | molecular chaperone           | 0.11        |
| 13     | Phagosome                                    | ko04145    | gi|112983908   | cathepsin B                   | 0.17        |
|        |                                              |            | gi|114051368   | Ras-related protein           | 8.85        |
|        |                                              |            | gi|114052088   | ATPase                        | 0.19        |
| 14     | Endocytosis                                  | ko04144    | gi|512926615   | charged multivesicular body   | 4.34        |
|        |                                              |            | gi|114051368   | protein                       |             |
| 15     | PI3K-Akt signaling pathway                   | ko04151    | gi|112983556   | molecular chaperone           | 0.11        |
|        |                                              |            | gi|114050901   | protein binding               | 0.21        |
| 16     | Arginine and proline metabolism              | ko00330    | gi|512899761   | spermidine synthase           | 3.15        |
|        |                                              |            | gi|512922127   | ornithine–oxo-acid transaminase | 0.21        |
| 17     | NOD-like receptor signaling pathway          | ko04621    | gi|112983556   | molecular chaperone           | 0.11        |
| 18     | Legionellosis                                | ko05134    | gi|512896628   | chaperonin                    | 2.61        |
| 19     | Epstein-Barr virus infection                 | ko05169    | gi|114050901   | protein binding               | 0.21        |
| 20     | Glycolysis/Gluconeogenesis                   | ko00010    | gi|512891308   | aldose 1-epimerase            | 0.28        |

Note: phosphatidylinositol-3-kinase (PI3K); phosphorylation of protein kinase B (Akt); nucleotide-binding and oligomerization domain (NOD).
3.3. Verification of Gene Expressions by Quantitative PCR

To validate the 2-DE result, we also performed qPCR on some selected targets in the control and *E. sorbilans*-infected hemocytes and fat bodies. The insect hemolymph is an open circulatory system. During larval–pupal metamorphosis, the fat body maintains intracellular homeostasis and meets the requirements of metamorphosis. Thus, the fat body was also used to verify gene transcription of the DEPs in the hemolymph. The fat body and hemocytes were collected from day-1 female silkworm pupae at the same time point. The information on the five selected differentially expressed genes and *B. mori* ribosomal protein gene *BmRPL3* primers are presented in Table S4. In the hemocytes and fat body, the transcriptional expression levels of phenoloxidase subunit 2 precursor (PPO2), CTL10, and peptidoglycan recognition protein (PGRP-S1) were increased with infection, whereas the gene expression levels of ecdysteroid-regulated 16 kDa protein (ESR16) and 14-3-3 protein zeta (14-3-3z) decreased with infection (Figure 3A,B). The induced fold-change of every gene was different, whereas the tendencies to expression changes were consistent between the hemocytes and fat body tissues. In summary, the changes in the gene transcription were consistent with their corresponding proteins in the 2-DE data.

![Figure 3](image-url)

**Figure 3.** Expression profiles of five genes in the control and infected (A) hemocytes and (B) fat bodies collected from day-1 female silkworm pupae: For each gene, the transcriptional level of the control was set to 1. The data are the means ± SD of three independent experiments. Statistical analysis was performed using SPSS software. *p < 0.05, **p < 0.01.

4. Discussion

In this study, we successfully identified 50 host-responsive DEPs via MALDI-TOF/TOF-MS in the hemolymph of female silkworm pupae after *E. sorbilans* infection. Parasitoids induce host responses such as enhancing innate immunity proteins expression and cell apoptosis.

4.1. Innate Immune System Enhanced Resistance to *E. sorbilans* Infestation

Parasitoids induce host responses. In particular, the following eight innate immune proteins were successfully identified and upregulated in the infected group: PPO2 (spot 1), paralytic peptide binding protein (spot 4) [22,23], antitrypsin (spot 6) [24], CTL10 (spot 10), apolipophorin III (spot 22) [25], translationally controlled tumor protein (spot 23) [26,27], peptidoglycan recognition protein (spot 27) [28,29], and ubiquitin-like protein SMT3 (spot 36) [30,31]. Three proteins, PPO2, antitrypsin, and paralytic peptide binding protein, were identified as an immune adaptation against *E. bombycis* parasites in *B. mori* by Pradeep et al [8]. They all play important roles in innate immunity. Insect prophenoloxidase (PPO) is an important innate immunity protein [32]. The activation of PPO cleaving into active phenoloxidase (PO) by serine proteinase is required for a melanization cascade to isolate microorganisms from circulation and to then kill them [33]. Produced by hindgut cells, PPO is secreted into the hindgut content that induces the melanization of the hindgut content in silkworm [34]. BmCTL10 and BmMBP are the same C-type lectins (CTLs), and their amino acid sequence similarity is 99.7%; they participate in innate immune responses such as hemocyte nodule formation and PPO activation [4,35–37]. As a result of activation of the PPO cascade and nodule formation, the
parasite is blackened in the host by the deposition of melanin and encapsulation. This indicates that the expressions of immune proteins are enhanced in the silkworm resistance of \textit{E. sorbillans} parasite infestation.

4.2. Apoptosis Triggered in Response to \textit{E. sorbillans} Infestation

Parasitic infection induces autophagy and cell apoptosis in insects [6]. Cathepsin B and 14-3-3 zeta both have important roles in apoptosis [38,39]. RNAi-mediated downregulation of cathepsin B or the absence of cathepsin B induces apoptosis in cancer [38,40,41]. Downregulation of cathepsin B can induce caspase-8-mediated apoptosis and initiates a partial extrinsic apoptotic cascade in SNB19 human glioma cells [40]. Strong 14-3-3 zeta protein expression acts in cell differentiation, proliferation, transformation, and prevention of apoptosis [42]. In particular, 14-3-3 protein zeta is a major regulator of apoptotic pathways in insects and vertebrates [43–46]; downregulation of 14-3-3 zeta sensitizes cells to apoptosis [39,47]. Cathepsin B (spots 43) and 14-3-3 zeta (spot 45) were successfully identified and were significantly downregulated in the hemolymph of parasitized female silkworm pupae. Cathepsin B was transcriptionally downregulated in the host \textit{Manduca sexta} following wasp \textit{Cotesia congregata} parasitism [48]. The downregulation of cathepsin B and 14-3-3 zeta may induce cell apoptosis in the hemolymph of \textit{B. mori} following the invasion by the \textit{E. sorbillans}. The larval epithelium of \textit{B. mori} parasitized by \textit{E. bombycis} showed cellular responses, such as signs of autophagy and apoptosis [8]. Enhanced expression of autophagy 5-like (\textit{Atg5}), apoptosis-inducing Factor (\textit{AIF}), and \textit{caspase} genes coupled with the appearance of cell death symptoms indicate parasitism-induced activation of genetic machinery to modulate cell apoptosis in the epithelium [12]. Thus, cell apoptosis is triggered against the parasitism of \textit{E. sorbillans} in \textit{B. mori}.

Proteins related to growth and development, such as the ecdysteroid-regulated 16 kDa protein, were successfully identified. Ecdysteroid-regulated 16 kDa protein (ESR16) is triggered by the steroid hormone ecdysone at the onset of metamorphosis [49]. The developmentally regulated gene of ecdysteroid-regulated 16 kDa protein was downregulated in the hemolymph of \textit{B. mori} after \textit{E. sorbillans} infection, which indicates that the development of the host is affected to facilitate the growth of parasitic larvae of the \textit{E. sorbillans} maggot.

5. Conclusions

In conclusion, we successfully identified 50 differentially expressed proteins (DEPs) in the hemolymph of the day-1 female silkworm pupae and their potential roles following silkworm maggot (\textit{Exorista sorbillans}) parasitism using a proteomics-based approach. The expressions of immune proteins were enhanced, and cell apoptosis could be triggered against the parasitism of \textit{E. sorbillans} in \textit{B. mori}. To the best of our knowledge, this study is the first to report the identification of possible proteins from the \textit{E. bombycis}-parasitized silkworms at the late stage of parasitism. Our findings expand the current knowledge on resistance in silkworm to \textit{E. sorbillans} parasitization and provide a new perspective on the molecular mechanisms of dipteran parasitoid biology.

Supplementary Material: Figure S1. Tissue expression profile of genes encoding the differentially expressed proteins (DEPs). The columns represent 10 different tissues and both sexes: testis, ovary, head, epidermis, fat body, midgut, hemocyte, Malpighian tubule, anterior/median silk gland (A/MSG), and posterior silk gland (PSG) and female (F) and male (M). Gene expression levels are represented by red (higher expression) and blue (lower expression) boxes. Table S1. Peptides of the quantified proteins. Table S2. Gene Ontology (GO) annotation results of the DEPs. Table S3. Description of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. Table S4. Primers of genes for quantitative PCR (qPCR).

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