Protein Profile Analysis of *Ericerus pela* (Hemiptera: Coccoidea) Egg

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Abstract

The transformation from embryo to first instar nymph is an essential process in the insect life cycle. In order to characterize protein expression in the *Ericerus pela* Chavannes (Hemiptera: Coccoidea) egg, high-throughput proteomics and bioinformatics methods were used. A total of 678 peptides were identified and assigned to 358 protein groups. The proteins exhibited a wide range of molecular weight (3.50–495.12 kDa) and isoelectric points (3.50–13.1). Gene Ontology annotation showed that the majority of proteins were associated with cellular processes, metabolic processes, and response to stimulus processes. The predominant molecular functions of *E. pela* egg proteins included binding, catalytic activity, transporter activity, and structural molecule activity. Kyoto Encyclopedia of Genes and Genomes annotations identified 137 pathways, and most proteins were assigned to metabolism events, including many enzymes participating in energy metabolism, protein folding, sorting, and degradation. The processes and functions of the identified proteins were closely related to the physiological status of egg and embryo development. We conclude that some identified proteins are related to important egg biological characteristics, and regard them as the target proteins for future study.

Key words: *Ericerus pela* Chavannes, egg, protein profile

*Ericerus pela* Chavannes (Hemiptera: Coccoidea) is one of the oldest economic insects. It has been used successfully in commercial wax production, and reared by humans for more than a thousand years. White wax is secreted only by the male *E. pela*, and has an increasingly wide utilization; examples include candle production, printing, medicine, food, cosmetic industries, and precision machinery (Chen and Feng 2009).

The oviposition of *E. pela* occurs about in March and April each year, and incubation happens in April and May. Eggs are laid in the capsule of the female adult every day during the oviposition period; the maximum and minimum amount of eggs laid per adult are 18,047 and 12,000, respectively (Wu and Zhong 1983). When the oviposition period is over, the ventral side of the female adult body is very close to its dorsal side, and the adult will soon die. The eggs, with wax powder on their surface, will incubate in the closed capsule for at least 29 d (Wu and Zhong 1983). After the newly hatched nymphs crawl out of the capsule, the male nymphs live in the shadow of the host plant in a gregarious manner, and secret an amount of white wax to cover themselves until eclosion, while the female nymphs scatter on the host plant and do not secrete the white wax (Chen 2011).

Research on *E. pela* eggs at molecule level has not been reported up to date. There are only proteomic analyses of the male adult cuticle and the male pupal stage (Yang et al. 2011, Yang and Chen 2014), and transcriptome analyses of adults and pupae (Yang et al. 2012, 2015; Yu et al. 2016). The results of these aforementioned studies, coupled with in-depth study of biology and ecology of *E. pela* will lay the foundation for further proteomic study of *E. pela* eggs and understanding the roles of proteins at this stage. Proteomic analyses of other insect eggs or embryos have been well documented (Amenya et al. 2010; Li et al. 2010, 2011; Müller et al. 2012, 2015; Yu et al. 2016). The present study was performed to explore the protein expression profile of *E. pela* eggs, generate hypotheses about the connections between certain proteins and biological characteristics of *E. pela* eggs, and provide basic information for studying target proteins in future.

Materials and Methods

Insect Culture

*E. pela* were cultured on the branches of *Ligustrum lucidum*, in the experimental field of Research Institute of Resources Insects of the Chinese Academy of Forestry in Kunming (longitude: 102°42′E;
latitude: 25°02’N). When the first nymphs crawled out of their capsules in March, the eggs in these capsules were collected in tubes and stored at -80°C.

**Protein Extraction**

Before protein extraction, the eggs collected from different mother scale insects were mixed and randomly divided into three groups, and washed in phosphate buffer solution (pH 7.2) three times. Total protein of each group was extracted on ice in 1 ml lysis buffer (containing 10% glycerin, 2.5% SDS, 5% β-mercaptoethanol, and 62.5 mM Tris-HCl, pH 6.8) per mg of sample homogenate. The homogenate extraction was kept for 10 min at room temperature and then subjected to five rounds of sonication treatment in an ice-bath, each time for 20 s with a 20 s interval. After centrifugation at 20,000 g, the supernatant was aliquoted and stored at -20°C. Protein concentration was determined with a Bradford protein assay kit (Beyotime, Shanghai, China), BSA (Sigma, USA) was taken as standard.

**SDS–PAGE Separation and In-gel Digestion**

Before being subjected to SDS–PAGE, the extracted total protein was dissolved in SDS–PAGE loading buffer, boiled for 5 min, centrifuged at 20,000 g for 10 min, loaded on a 5% stacking gel and 12% separating gel, and was run at 15 mA for 30 min and then 30 mA for 2 hr in a mini-vertical electrophoresis system (Bio-Rad, USA). After electrophoresis, the gel was stained overnight in a solution of 0.1% (w/v) Coomassie Brilliant Blue G-250 (Sangon Biotech, Shanghai, China), 30% (v/v) methanol, and 10% (v/v) glacial acetic acid. After decolorization, the gel was analyzed for bands along with a molecular weight marker.

Thereafter, the gel containing all bands was cut into 1 mm³ particles for in-gel digestion: gel particles were washed three times in deionized water and subsequently dehydrated with 100% acetonitrile (ACN) for 10 min. The particles were incubated with 100 mM DTT for 30 min at 56°C. The resulting free thiol (~SH) groups were subsequently alkylated by incubating the samples with 200 mM iodoacetamide for 20 min in the dark. Gels were washed with 25 mM ammonium bicarbonate and dehydrated with 100% ACN sequentially. Thereafter, 10 ng/μl trypsin (Promega, USA) was added and incubated for 20 hr at 37°C for protein digestion. Supernatants were transferred to fresh tubes for mass spectrometric analysis.

**Protein Identification Using LC–MS/MS**

The resuspended extracts were separated and identified using HPLC (Easy nLC system, ThermoQuest, San Jose, CA) coupled with Q-Exactive mass spectrometer (thermo Fisher, San Jose, CA). One microliter of sample was loaded on a trapping column (Thermo scientific EASY column (2 cm × 100 μm 5 μm-C18)) each time. After flow-splitting, peptides were transferred to the analytical column (Thermo scientific EASY column (75 μm × 100 mm 3 μm-C18)) for separation equilibrated with buffer A (0.1% methanoic acid in water) and buffer B (84% ACN, 0.1% methanoic acid in water), a 280 min linear gradient was set: buffer B started from 0 to 60% at a flow rate of 250 nl/min, came to 100% subsequently, and then maintained constantly at this flow rate.

The mass spectra of the peptides were recorded on a Q-Exactive mass spectrometer. The positive ions were adopted as the mode of scanning MS spectra, the MS analysis was performed with one full MS scan (m/z 300–1800) with the resolution (R = 70,000) at m/z 200 and dynamic exclusion (40.0 s), followed by MS/MS scans on the 10 most intense ions from the MS spectrum. Collision-induced dissociation was conducted with normalized collision energy of 35% and voltage of 27 eV.

The raw data from LC–MS/MS was analyzed using maxquant 1.3, the parameters were set as: the maximum number of missed cleavages a peptide for 2, the trypsin digestion, carbamidomethyl (C) for fixed modification, oxidation (M), and acetyl (N-term) for variable modifications, proteins false discovery rate ≤0.01, and as well as peptides, specifying the string for reverse and contaminant hits. In this study, an in-house database was used for proteomic data analysis, constructed by combining the coding sequences from E. pela Illumina transcriptome sequencing databases (Yang et al. 2012) with insect sequence data downloaded from the National Center for Biotechnology Information (NCBI) Nr (nonredundant) database and the SwissProt protein database.

**Bioinformatic Analysis**

Functional analysis of the identified proteins was performed using UniProt Knowledgebase (Swiss-Prot + TriEMBL) (http://www.uniprot.org), and proteins were grouped on the basis of their biological and molecular function of Gene Ontology terms (Gala et al. 2013). The GO annotation terms were obtained from Web Gene Ontology Annotation Plotting (http://we.go.genomics.org.cn/). All the identified proteins were searched against the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg) to identify the correlated pathways. The protein enzyme commission numbers were obtained based on the best matches (E-value ≤e-15).

**Results**

**Molecular Weight and Isoelectric Point of the Identified Proteins**

In this study, about 678 unique peptides were identified, ascribed to 358 protein groups. These identified proteins exhibited a broad range of theoretical molecular weight (MW); one major group comprised proteins with MW between 10 and 30 kDa, and remarkably, there were eight proteins with MW exceeding 300 kDa. With regard to theoretical isoelectric point (pI), about 70% of the total proteins had pI in the range of 4–8, the pI of three proteins was <4, and the pI of 15 proteins was >11 (Fig. 1 and Table 1).

**Categorization of the Identified Proteins**

The identified proteins were categorized based on the biological process and molecular function as predicted from associated GO terms (Table 1). Most of the proteins were predicted to act on material conversion and transportation, and information modulation, and the proteins associated with metabolism of carbohydrates and energy, amino acid metabolism, nucleotide metabolism, transcription and translation, and other categories are shown in Table 1.

We found that a large number of proteins associated transformation of matter and energy were expressed during egg development. Many identified enzymes were related to the glycolytic pathway, pentose phosphate pathway and tricarboxylic acid cycle (Supp Table 1 [online only]). Pyruvate kinase (NP_001036906.1), malate dehydrogenase (XP_001659012.1), phosphoglyceromutase (NP_001037540.1), and other enzymes were identified, and assigned to different carbohydrate metabolism pathways and energy metabolism pathways, which suggested the carbohydrate and energy metabolism was vigorously performing in this stage.

RNA-binding protein 8A (XP_001849141.1), ribosomal protein L28 (NP_001155658.1), and other 20 proteins took part
in spliceosome pathway (ID: ko03040), ribosome pathway (ID: ko03010), and other seven pathways (Supp Table 2 [online only]), which suggested a number of transcription and translation programs were now in process, which is consistent with an embryo in a vigorously developing stage.

In the identified proteins, tubulin, actin, profilin, myosin, microtubule-associated proteins, other cytoskeleton proteins and some chaperonin proteins, such as t-complex protein 1, were identified. These proteins involved in the phagosome pathway (ID: ko04145), tight junction pathway (ID: ko04530), and gap junction pathway (ID: ko04540). The organization of the cytoskeleton plays important roles in cell morphogenesis, and the identification of these proteins might be due to the various development programs during embryogenesis (Supp Table 3 [online only]).

Another group of identified proteins were HSPs, among them, HSP 90 and HSP 70 were predominant (Supp Table 3 [online only]). Many members of this group might perform chaperone function by stabilizing new proteins to ensure correct folding or by helping to refold proteins that were damaged by the cell stress.

In addition, some proteins were protective for the embryo development. In this study, various proteasomes and ubiquitin proteins were identified (Supp Table 3 [online only]). The identified proteasomes were involved in the proteasome pathway (ID: ko03050) and antigen processing and presentation pathway (ID: ko04612). These might be necessary to regulate specific proteins and remove protein misfolding during the progress of E. pela embryo development. Moreover, some proteins associated with antioxidant system were identified (Supp Table 3 [online only]), they might play important roles in removing harmful metabolites conducted in the process of embryo development.

In the protein profile of the E. pela egg, some kinds of proteins were likely to be related to the specific differentiation and morphogenesis programs for various tissues and organs, as well as early nymphal morphogenesis (Supp Table 4 [online only]). These proteins included chitinase, cuticular protein analogous to peritrophins 3-B precursor, prophenoloxidase, similar to n-synaptobrevin CG17248-PA, muscular protein 20, transformer-2 sex-determining protein, and other proteins, which were likely to be the important proteins for cuticular, nerve tissue, and reproductive organ formation of the new nymph. From these results, it was presumed that the embryo development was in its later stage. The result was consistent with what we would expect at a stage close to egg hatching.

Biological Process and Molecular Functions of the Identified Proteins

Based on the biological process and molecular function according to the GO terms, in total, 213 proteins were found to be involved in 22 categories of biological processes (Fig. 2). Most proteins were related to cellular process (84.04%) and metabolic process (73.71%), which was consistent with the active cell division and vigorous metabolism during the course of embryo development. Proteins related to the GO term ‘response to stimulus’ showed high representation (25.82%), and proteins involved in ‘cell proliferation’, ‘multi-organism process’, and ‘growth’ made the lowest representation (0.47%) in our protein profile. Molecular function terms associated with E. pela egg proteins revealed that most of the proteins were involved in binding (49.09%), followed by catalytic activity, transporter activity, and structural molecule activity. Proteins related to other functions were represented as small groups (Fig. 3). In order to reveal the enzyme classes in E. pela egg, proteins with catalytic feature were further classified (Fig. 4). The enzyme distribution illustrated that hydrolases accounted for the largest proportion (49.28%), followed by oxidoreductases and transferases.

KEGG Pathway Analysis

When searched against KEGG reference pathway database, 129 proteins were assigned to 137 KEGG pathways, which were ascribed to five categories: organismal systems, metabolism, genetic information processing, environmental information processing, and cellular processes (Fig. 5). In the metabolism term, there were 56 pathways identified. In particular, 20, 24, and 12 proteins were, respectively, found in connection with 14 carbohydrate metabolism pathways, five energy metabolism pathways, and nine amino acid metabolism pathways. There were 18 identified biological pathways in genetic information processing, folding sorting and degradation were so complex and active that six pathways were associated with 32 proteins in this processing. Under the cellular process category, 18 proteins were involved in four pathways and linked with transport and catabolism. Environmental information processing included signal transduction (eight pathways) and signaling molecules and interaction term (three pathways), the proteins involved in the former were more numerous than the proteins involved in the latter. In the organismal systems term category, 10
Table 1. Categorization of the identified proteins of *E. pela* egg based on the GO analysis

| Protein description | ACC          | Mol. weight [kDa]/pI | Biological_process                                                                 | Molecular_function                                                                 |
|---------------------|--------------|----------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| Carbohydrate and energy metabolism |                   |                      |                                                                                     |                                                                                      |
| GF23287             | XP_001964635.1 | 56/10                | Acetyl-CoA biosynthetic process from pyruvate                                        | Pyruvate dehydrogenase (acyl-transferring) activity                                  |
| AGAP011066-PA        | XP_309579.4   | 3.5/8.1              |                                                                                     | Oxidoreductase activity                                                                |
| Isocitrate dehydrogenase [NADP] | XP_001971666.1 | 54/6.7               | Isocitrate metabolic process                                                         | NAD binding                                                                            |
| MGC80785 protein    | NP_001087022.1 | 6.5/7.7              |                                                                                     | Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor |
| Putative uncharacterized protein | XP_967960.2   | 56/7.2               |                                                                                     | Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor |
| GJ19670             | XP_002058888.1 | 80/7.6               | Carbohydrate metabolic process                                                       | Hydrolyase activity, hydrolyzing O-glycosyl compounds                                 |
| AAELO04297-PA        | XP_001648848.1 | 123/7.2              |                                                                                     | ATP binding                                                                            |
| AGAP009039-PA        | XP_319791.4   | 10/7.9               | carboxylic acid metabolic process                                                   | Carbohydrate binding                                                                  |
| Glucose-6-phosphate isomerase | NP_001020570.3 | 62/7                | Gluconeogenesis                                                                      | Glucose-6-phosphate isomerase activity                                                |
| phosphoglyceromutase | NP_001037540.1 | 28/6.8               | Glycolysis                                                                           | Phosphoglycerate mutase activity                                                      |
| GJ15342             | XP_002059015.1 | 37/9.2               |                                                                                     | ATP citrate synthase activity                                                         |
| GIL2035             | XP_002001326.1 | 6.5/7.5              | Catalytic activity                                                                   | Pyruvate kinase activity                                                              |
| pyruvate kinase     | NP_001036906.1 | 31/5.2               |                                                                                     | Coproporphyrogen oxidase activity                                                     |
| GJ17558             | XP_002052468.1 | 28/8.8               |                                                                                     |                                                                                      |
| GE24063             | XP_002098232.1 | 8.1/7.2              |                                                                                     | Holocarboxylic acid activity                                                          |
| GH22974             | XP_001995110.1 | 73/7.3               |                                                                                     | Oxidoreductase activity, acting on the CH-CH group of donors                          |
| AAELO08167-PB        | NP_001040233.1 | 52/8.6               | Fumarate metabolic process                                                          | Fumarate hydratase activity                                                           |
| ATP synthase subunit alpha |                   |                      | ATP hydrolysis coupled proton transport                                              | ATP binding                                                                            |
| Oligomycin sensitivity-confering protein | NP_001087022.1 | 6.5/7.3              | Proton-transporting ATP synthase activity, rotational mechanism                      | Proton-transporting ATP synthase activity, rotational mechanism                       |
| vacuolar ATP synthase catalytic subunit A | NP_001087022.1 | 6.5/7.3              |                                                                                     | Proton-transporting ATP synthase activity, rotational mechanism                       |
| V-type proton ATPase subunit E | P31402.1  | 7.4/9.5              |                                                                                     | Proton-transporting ATPase activity, rotational mechanism                              |
| GJ16665             | XP_002051636.1 | 93/5.9               | ATP hydrolysis coupled proton transport                                              | Hydrogen ion transmembrane transporter activity                                        |
| electron-transfer-flavoprotein beta polypeptide | NP_001040123.1 | 18/9            |                                                                                     | Electron carrier activity                                                             |
| Acyl carrier protein | XP_311483.3   | 7.6/4.7              |                                                                                     |                                                                                      |
| malate dehydrogenase, putative | XP_002432539.1 | 36/9.4              | Fatty acid biosynthetic process                                                     |                                                                                      |
| malate dehydrogenase | XP_001659012.1 | 14/9.3              | Malate metabolic process                                                            | l-Malate dehydrogenase activity                                                       |
| luciferase-like protein | BA166602.1   | 60/8.8               | Malate metabolic process                                                            | l-Malate dehydrogenase activity                                                       |
| Amino acid metabolism |                   |                      | Bioluminescence                                                                     | catalytic activity                                                                     |
| peroxidase           | XP_001867956.1 | 9.6/6.6              | Response to oxidative stress                                                         | Peroxidase activity                                                                   |
| GE13192             | XP_002090578.1 | 12/6.5               | Response to oxidative stress                                                         | Peroxidase activity                                                                   |
| AGAP009039-PA        | XP_316880.5   | 61/8.3               | Biosynthetic process                                                                | Catalytic activity                                                                     |
| phosphoserine aminotransferase | XP_001849403.1 | 30/8.6 | O-Phospho-l-serine:2-oxoglutarate aminotransferase activity |                                                                                      |
| GF18621             | XP_001955137.1 | 62/7.4               |                                                                                     | Aminopeptidase activity                                                               |
| GL23169             | XP_001999920.1 | 5.3/6.6              |                                                                                     | Aminopeptidase activity                                                               |
| prophenoloxidase     | XP_001661190.1 | 6.4/6.7              | Oxidation–reduction process                                                         | Oxidoreductase activity                                                               |
| Lipid metabolism     |                   |                      |                                                                                     |                                                                                      |
| AGAP011066-PA        | XP_309579.4   | 3.5/8.1              |                                                                                     | Oxidoreductase activity                                                               |
| aldehyde dehydrogenase (NAD+) | NP_001087022.1 | 6.5/7.3              |                                                                                     | Oxidoreductase activity                                                               |
| fatty acid synthase S-acetyltransferase | XP_001845135.1 | 249/6            |                                                                                     | Hydrolase activity, acting on ester bonds                                             |
|                      |               |                      |                                                                                     |                                                                                      |
| Protein description | ACC             | Mol. weight [kDa]/pI | Biological_process                          | Molecular_function                                      |
|---------------------|-----------------|----------------------|----------------------------------------------|--------------------------------------------------------|
| Putative uncharacterized protein | XP_971757.1     | 32/9.2               | Catalytic activity                           |                                                        |
| GK10733             | XP_002061054.1  | 11/9.6               | Fatty-acyl-CoA reductase (alcohol-forming)   activity |                                                        |
| Nucleotide metabolism | MGC130953 protein | 1/6.5                | Purine nucleotide biosynthetic process       | IMP cyclohydrolase activity                            |
| GE23527             | XP_002099157.1  | 9.2/8                | Purine nucleotide biosynthetic process       | IMP cyclohydrolase activity                            |
| guanylate kinase-1  | ACD69431.1      | 1/6.58               | Purine nucleotide metabolic process         | Guanylate kinase activity                              |
| Nudix (Nucleoside diphosphate linked moiety X)-type motif 2 | NP_001002323.1  | 7.3/5.9              | Bis(5’-nucleosyl)-tetraphosphatase activity  |                                                        |
| GMP synthase, putative | XP_002427615.1  | 76/7.1               | GMP biosynthetic process                     | GMP synthase (glutamine-hydrolyzing) activity          |
| GK17039             | XP_002061681.1  | 17/4.6               | Transcription, DNA-dependent                 | Ribonucleoside-diphosphate reductase activity, thioloduxin disulfide as acceptor |
| Ribonucleoside-diphosphate reductase | XP_001660977.1  | 30/7                 | DNA replication                              | Inorganic diphosphatase activity                       |
| AAEL003193-PB       | XP_001656515.1  | 42/6.6               | Phosphate-containing compound metabolic process | IMP dehydrogenase activity                             |
| Inosine-5’-monophosphate dehydrogenase | XP_309514.2 | 14/8.3               | GMP biosynthetic process                     |                                                        |
| GD20335             | XP_002103291.1  | 12/5.9               | Flavin adenine dinucleotide binding          | Nucleic acid binding                                   |
| transformer-2 sex-determining protein, putative Transcription and translation heterogeneous nuclear ribonucleoprotein A1 | NP_001093319.1 | 22/7.1                  | Nucleic acid binding                             |                                                        |
| Putative uncharacterized protein | XP_973561.1     | 31/10                | Nucleic acid binding                         |                                                        |
| AAEL007239-PA       | XP_001658243.1  | 44/8.3               | Nucleic acid binding                         |                                                        |
| GK13948             | XP_002073099.1  | 43/12                | Nucleotides binding                          |                                                        |
| GL19239             | XP_002014541.1  | 86/7.9               | Threonyl-tRNA aminocacylation                | Threonine-tRNA ligase activity                         |
| leucyl-tRNA synthetase, putative | XP_002422927.1  | 97/8.3                | Leucyl-tRNA aminocacylation                  | LEUCINE-tRNA ligase activity                           |
| GL25810             | XP_002018701.1  | 12/5.8               | Alanyl-tRNA aminocacylation                  | Alanine-tRNA ligase activity                           |
| 60S ribosomal protein L10A, putative | XP_002426587.1  | 25/11                | Alanine-tRNA ligase                         | RNA binding                                            |
| ACYF1006342 protein | NP_001155658.1  | 16/12                | Structural constituent of ribosome           |                                                        |
| GH23036             | XP_001995231.1  | 19/11                | Translation                                  | RNA binding                                            |
| Putative uncharacterized protein euarkyotic translation initiation factor 5 | XP_967571.1     | 24/9.8                | Translation                                  |                                                        |
| Lysine-tRNA ligase   | NP_572573.1     | 38/6.5               | Translation                                  |                                                        |
| GTP-binding nuclear protein RAN1, putative | XP_002423913.1  | 50/9.2                | Structural constituent of ribosome           |                                                        |
| RNA-binding protein 8A | XP_001849414.1  | 19/4.8               | RNA binding                                  |                                                        |
| Folding, sorting, and degradation proteasome subunit alpha type, putative | XP_002422679.1  | 4/7.5                 | RNA processing                               |                                                        |
| Proteasome subunit alpha type | NP_001040387.1  | 5.9/5.1               | Threonine-type endopeptidase activity        |                                                        |
| Proteasome subunit beta type | XP_317882.3     | 25/6.9               | Threonine-type endopeptidase activity        |                                                        |
| GK22666             | XP_002072105.1  | 16/4.8               | Regulation of catalytic activity             | Enzyme regulator activity                              |
| heat shock 70 kDa protein cognate 4 | XP_001850527.1  | 71/5.2                | Response to stress                           | ATP binding                                            |
| HSP 70 B2           | XP_001861436.1  | 70/5.5               | Response to stress                           | ATP binding                                            |
| Protein description | ACC | Mol. weight [kDa]/pI | Biological_process | Molecular_function |
|--------------------|-----|---------------------|--------------------|--------------------|
| 60 kDa HSP, mitochondrial | XP_001850501.1 | 60/5.2 | Protein refolding | ATP binding |
| GC25088 | XP_001968382.1 | 61/6.1 | Protein refolding | ATP binding |
| HSP 90 kDa alpha (cytosolic), class B member 1 | NP_001025655.1 | 77/4.7 | Protein refolding;response to stress | |
| HSP 83 | XP_001865484.1 | 82/4.6 | Response to stress | ATP binding |
| HSP 90 protein, putative | XP_002432348.1 | 83/4.7 | Protein refolding; response to stress | ATP binding |
| disulfide isomerase | XP_001866126.1 | 11/6.2 | Glycerol ether metabolic process | Isomerase activity |
| GG17350 | XP_001980787.1 | 47/4.2 | Protein folding | Calcium ion binding |
| Prksh-prov protein | NP_001087124.1 | 5.5/4.1 | N-glycan processing | Calcium ion binding |
| AGAP001424-PA | XP_321706.5 | 91/4.6 | Protein refolding | ATP binding |
| AAEL012827-PA | XP_001662951.1 | 27/4.5 | Protein refolding | ATP binding |
| 78 kDa glucose-regulated protein | XP_001845218.1 | 83/4.7 | Protein refolding; response to stress | ATP binding |
| GH11975 | XP_001861937.1 | 72/5 | Response to heat | ATP binding |
| Putative uncharacterized protein | XP_971446.1 | 45/7.6 | | |
| Thioredoxin domain-containing protein 1 | ACO12744.1 | 26/4.6 | Cell redox homeostasis | |
| GJ22764 | XP_002054516.1 | 55/9.1 | Ubiquitin-protein ligase activity | |
| Transitional endoplasmic reticulum ATPase TER94 | XP_96692.1 | 89/5.1 | Nucleoside-triphosphatase activity | |
| Transport and catabolism | | | | |
| Dnase2-prov protein | NP_001086671.1 | 9.7/6.8 | DNA metabolic process | Deoxyribonuclease II activity |
| GL12416 | XP_002019473.1 | 136/7.4 | ATP catabolic process | ATPase activity, coupled to transmembrane movement of substances |
| GG20906 | XP_001974813.1 | 53.4/8 | Microtubule-based process | GTP binding |
| Rab-protein 5 | XP_001813105.1 | 24/8.6 | Protein transport | GTP binding |
| GK13103 | XP_002073518.1 | 21/4.6 | Sphingolipid metabolic process | |
| RAC GTPase, putative | XP_002429222.1 | 21/6.8 | Small GTPase mediated signal transduction | GTP binding |
| Development and organism system | | | | |
| GD25430 | XP_002082004.1 | 13/6.5 | Oxidation–reduction process | Oxidoreductase activity |
| AAE1021262-PC | XP_001662217.1 | 45/5.2 | ATP biosynthetic process | Monovalent inorganic cation transmembrane transporter activity |
| clathrin light chain | XP_001868264.1 | 6.7/4.2 | Intracellular protein transport | Structural molecule activity |
| GL25029 | XP_002021066.1 | 10/9.5 | Fatty-acyl-CoA binding | |
| GF20350 | XP_001963345.1 | 10/8.9 | Zinc ion binding | |
| AAEL003413-PA | XP_001656777.1 | 28/6.9 | Serine-type endopeptidase inhibitor activity | |
| AGAP007452-PA | XP_001687921.1 | 299/7.1 | Regulation of Hr protein signal transduction | Rho guanyl-nucleotide exchange factor activity |
| lumbrokinase-3(1) | XP_001844812.1 | 5.4/6.7 | Proteolysis | Kinase activity |
| GK12466 | XP_002072489.1 | 9/4.7 | Proteolysis | Serine-type endopeptidase activity |
| leukocyte elastase inhibitor | NP_001083938.1 | 17/6 | Serine-type endopeptidase inhibitor activity | |
| Alpha-2-antiplasmin | NP_777095.1 | 13/5.5 | Acute-phase response | serine-type endopeptidase inhibitor activity |
| AGAP007523-PB | XP_308355.3 | 231/5.2 | | Motor activity |
| G1A13959 | XP_001360276.2 | 5.4/5.2 | | Motor activity |
| AAE1004414-PA | XP_001648499.1 | 11/6.5 | | |
| GTP-binding protein alpha subunit, gna | XP_001850518.1 | 415/5.1 | Transport | G-protein coupled receptor signaling pathway |
| GK23973 | XP_002064648.1 | 47/8 | | Signal transducer activity |
| GK17256 | XP_002061919.1 | 59/4.8 | | Voltage-gated anion channel activity |
| Peptidyl-prolyl cis-trans isomerase | XP_002134398.1 | 25/10 | | Peptidyl-prolyl cis-trans isomerase activity |
pathways were included in the immune system, and the proteins related to immune system were the most numerous, which would be consistent with the embryo requiring active protection from pathogens at this stage.

Similarity Distribution of the Identified Proteins
The identified nonredundant proteins were analyzed for their similarity distribution in the database. A majority of the annotated proteins shared similarity with proteins from arthropods (Fig. 6). *E. pela* shared maximum similarity with *Drosophila* (26.19%), followed by different mosquito fauna, beetles, *Pediculus humanus corporis*, and *Maconellicoccus hirsutus* Green (Hemiptera: Pseudococcidae), etc. Out of the identified nonredundant proteins, only about 6.09% proteins exhibited similarity with the known proteins of scale insects.

Target Proteins Selection
One aim of our study was to identify for future study proteins that may be closely connected with biological and ecological characteristics of *E. pela*. These proteins were selected through comparison and analysis using the known functional information of the most similar protein in another insect as a justification, combining this information with the biological and ecological characteristics of *E. pela* and the known information on the molecular level about *E. pela*. The protein named GK10733 (XP_002061054.1) has fatty-acyl-CoA reductase (alcohol-forming) activity and is related to cutin, suberine, and wax biosynthesis pathway according to GO and KEGG analysis (Table 1 and Supp Table 4 [online only]). We hypothesized that fatty-acyl-CoA reductase is relevant to wax secretion on the surface of *E. pela* eggs. A number of HSPs with different molecular weight were identified (Table 1 and Supp Table 3 [online only]), and we
have previously studied some HSP genes of *E. pela* (Liu et al. 2013) in an earlier study. We hypothesized that these HSPs are very likely tied to stress resistance to the environment. We analyzed the possible relationship between the genes identified in this study and the typical ecological and biological characteristics of the *E. pela* egg in part of our discussion, and will use this as a basis for further investigation.

**Discussion**

*E. pela* is one of the most economically valuable insects, belonging to the family Coccidae. There exists minimal research about *E. pela* embryo development. The protein component of the *E. pela* egg during embryo development stage has not been reported until this study. The egg is light yellow in color, and has features commonly found in an insect egg. *E. pela* embryonic development begins with cleavage by karyokinesis, goes through the formation of blastoderm and germ band, formation and disappearing of the amnion and serosa, differentiation of the germinallayer, germ band section- alization, the formation of appendage, as well as the formation of the alimentary canal, nerve tissue, dorsal blood vessel, and generative cells (Zhao and Wu 1990). The external body is well developed on the 15th day after oviposition, and the nymph crawls out of the chorion on the 18th day after oviposition. However, the duration of the egg stage varies with temperature and other factors (Zhao and Wu 1990). During the egg development process, protein expression is active, and biosynthesis and catabolism programs are performed.
Embryo development is a sequential and complex process controlled by genes. Some proteins are constitutively expressed throughout the development process—these are indispensable for egg development. The existence of proteins expressed in specific stages of the egg suggests that the different developmental stages need specific protein(s) to proceed correctly (Fang and Li 2010).

In this study, we obtained the protein profile of the *E. pela* egg at stages close to hatching. A large number of identified proteins were related to metabolism and organismal systems pathways, and the results were in accordance with the physiological development features of *E. pela* egg. The identified enzymes in the *E. Pela* egg shared maximum similarity with proteins in *Drosophila*, and the fact that *Drosophila* proteins are generally very well characterized was helpful to predict the function of *E. pela* proteins. On the basis of KEGG pathway analysis and GO analysis, we discuss the possible relationship among some identified proteins and the biological and ecological characteristics of the *E. pela* egg.

**FAR and Secreting Wax Behavior**

According to the wax ester biosynthesis pathway in organism (Cheng and Russell 2004, Doan et al. 2009, Liénard et al. 2010, Teerawanichpan and Qiu 2010, Teerawanichpan et al. 2010), fatty-acyl-CoA reductase (FAR) and wax synthase are the key enzymes. In this study, some detected proteins were predicted to be involved in white wax synthesis. Among them, a protein named GK10733 had fatty-acyl-CoA reductase (alcohol-forming) activity, and was found to be related to the cutin, suberin, and wax biosynthesis pathways according to KEGG analysis (pathway ID: ko00073). The mRNA level of *E. pela* FAR gene in nymphs has previously been analyzed using qRT-PCR, and *E. pela* FAR was assumed the key enzyme to white wax biosynthesis (Yang et al. 2012). In each capsule, all eggs, without exception, are covered with some wax powder in natural conditions. Before this study, some researchers postulated that the wax adhering to the surface of eggs was secreted by the mother (Wu and Zhong 1983), but there was not enough evidence to support this hypothesis. In the present study, we predict that FAR is likely involved in the wax formation on the surface of *E. pela* eggs, though the quantity of wax is so small that this has not generally been a priority for study by researchers. On the other hand, we also cannot rule out that the expression of FAR protein in the egg stage is possibly in preparation for the secreting wax behavior of *E. pela* nymphs.

**HSPs in *E. Pela* Egg**

HSPs are known to play a vital role in both normal cellular homeostasis and stress response, and are involved in many biological functions such as cellular communication, immune response, protein transport, cell cycle regulation, apoptosis, gametogenesis, and aging (Sarkar et al. 2011). A report documented that Hsp70 and small HSPs are probably the major players in midgut metamorphosis in *Spodoptera litura* (Ga et al. 2012); this viewpoint provides valuable insight into the roles of the HSP superfamily in insect metamorphosis. Furthermore, HSPs are documented widely as defensive response proteins to stress factors including heat shock, cold shock, and other abiotic stresses and biotic stresses in insects (Zhao and Jones 2012). The functions of various HSP often overlap but can be different between different proteins (Zhang and Denlinger 2010, Benoit et al. 2011, Michaud et al. 2011, Xu et al. 2011). In the present study, heat shock 70 kDa protein cognate 4 and HSP 70 B2 were identified, and these were primarily involved in the sphéroosome pathway, the protein processing in endoplasmic reticulum pathway, the MAPK signaling pathway, the endocytosis pathway and antigen processing and presentation pathway on the basis of KEGG analysis, which showed these proteins were associated with multiple biological processes. Moreover, some higher molecular weight proteins, HSP 90 kDa alpha (cytosolic) and HSP 83, were identified for the plant–pathogen interaction pathway, the progesterone-mediated oocyte maturation pathway, the antigen processing and presentation pathway, the NOD-like receptor signaling pathway, the PI3K-Akt signaling pathway, and the protein processing in endoplasmic reticulum pathway. In addition, other HSPs were identified, including HSP cognate 5, HSP beta-6-like isoform 1, 60 kDa HSP (mitochondrial), and a few putative small HSPs. We hypothesized that the identified HSPs likely exhibit very important role in aiding organogenesis by folding newly synthesized proteins, binding other non-native proteins, and assisting proteins in the correct folding and functional actualization.

Furthermore, synthesis of the relevant literature about *E. Pela*, particularly as regards its ecological strategy, some HSPs likely
primarily function in response to very high heat and humidity stress. We reasoned that, first, thousands of eggs are laid in relatively closed ootheca, and they need to respond to intense heat, and likely hypoxia. Secondly, temperature and humidity is very high in the source region of white wax during the period of E. pela hatching, thus the egg likely responds to this abiotic stress by using the regulatory mechanism of HSPs. The host plant is often infested by pathogens because of the scale insect colonization, and there are many pollutants on the surface of egg capsule, but surprisingly impaired eggs were not found in our investigation. For this reason, we hypothesized that there is some inhibitory mechanism, potentially HSPs, in effect to protect the eggs from these harms. Therefore, considerable further work is needed to fully understand these mechanisms, and the HSPs will be target proteins in our future work.

Amount of Metabolic Energy Required for Development

A very radical morphological transformation is exhibited from egg to nympha stage, and large amount of carbohydrate metabolism and energy production is needed to undergo extensive organogenesis during this process. In this study, according to GO and KEGG classification, some important proteins were implicated in carbohydrate metabolism and energy production. In particular, the citrate cycle (TCA cycle), glycolysis/glucoseogenesis, pyruvate metabolism, propanoate metabolism, and pentose and glucuronate interconversions were assigned 41 proteins (32%), and 24 proteins (19%) involved in energy metabolism. This suggested that, similar to other insects (Zhong et al. 2005, Li et al. 2009), large amounts of metabolic energy produced by all types of metabolism are required for E. pela embryo development.

Cytoskeletal Proteins Being Essential for Metamorphosis

Cytoskeletal proteins have a number of essential cellular functions including maintaining the stability of cell shape and structure, and play important roles in intracellular transport and cellular division (Wulffkule et al. 1998). One report showed that controlled actin assembly is crucial to a wide variety of cellular processes (Quinlan 2013), and polymerization of actin filaments against cellular membranes provides necessary force for a number of cellular processes leading to protein recruitment (Saarikangas et al. 2010, Lucas et al. 2013). Tubulins are the major constituents of microtubules, and have a range of post-translational modifications, potentially regulating the microtubule cytoskeleton (Janke and Kneussel 2010). The Tcp-1 complex belongs to Type II Chaperonin; it is a multi-subunit molecular machine that assists in the folding of 10% of newly translated cytosolic proteins in eukaryotes (Coughlin et al. 2006, Posokhova et al. 2011). In this study, the majority of identified cytoskeletal proteins were found in tubulin, actin, and myosin proteins. These proteins are associated with ultrastructure, cell division, and cellular morphology. We hypothesized these proteins are likely essential for E. pela embryo.

Protective Proteins for Embryo Development

Throughout developmental, various quantities of metabolites can be beneficial or harmful to cells and tissues. For instance, excess reactive oxygen species (ROS) can induce oxidative modification of biological micromolecules, and inhibit protein function by protein oxidation, lipid peroxidation, DNA base modifications, and strand break (Circu and Aw 2010). Aerobic organisms have developed complicated antioxidant mechanisms to protect themselves against toxic ROS, superoxide dismutases, catalases, peroxidases, thioredoxin, glutathione peroxidase, and other enzymes (Wang et al. 2008). Superoxide dismutase (XP_002048532.1), peroxidase (XP_001867956.1), manganese superoxide dismutase (AEL79287.1), glutathione S-transferase theta (ACB36909.1), aldo-keto reductase (XP_001844819.1), and other proteins were found to be expressed in the E. pela eggs stage. These proteins might participate in the protective pathways in order to provide essential protection from harmful metabolites during embryo development of E. pela.

Conclusions

E. pela is a model for scale insects. This study provided the first proteomic analysis in the eggs of E. pela near hatching, which provided a basis to elucidate the mechanism underlying embryogenesis, and illuminated candidate proteins for deeper research. Some identified proteins might be directly correlated to the biological characteristics of the eggs at the stage at which they were examined. Further research is needed to verify the functions of these important proteins.

Supplementary Data

Supplementary data are available at Journal of Insect Science online.

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