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Proteomic and Bioinformatic Analysis on Endocrine Organs of Domesticated Silkworm, *Bombyx mori* L. for a Comprehensive Understanding of Their Roles and Relations

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Three organs of silkworm larva endocrine system, including brain (Br), subesophageal ganglion (SG) and prothoracic glands (PG), were studied employing shotgun LC-MS/MS combined with bioinformatic analysis to comprehensively understand their roles and relations. Totally, 3430, 2683, and 3395 proteins were identified including 1885 common and 652, 253, and 790 organ-specific ones in Br, SG, and PG, respectively. Identified common-expressed proteins indicated the existence of intrinsic complex interactions among these parts of endocrine system. Most of the reputed organs-specific proteins were identified by this approach. KEGG pathway analysis showed 162 same pathways among the 169, 164, and 171 relating Br, SG, and PG. This analysis revealed functional similarities with exceptional resemblance in their metabolism and signaling pathways of the three organs. On the other hand, 70, 57, and 114 organ-specific enzymes related pathways were detected for Br, SG, and PG confirming their functional differences. These results reveal a cooperative mechanism among the three endocrine organs in regulating various physiological and developmental events, and also suggest that the organ-specific proteins might be the fundamental factors responsible for the functional differentiation of these organs.

Keywords: *Bombyx mori* • Insect endocrine organs • Shotgun proteomics • LC-MS/MS • Trans-Proteomic Pipeline • Gene Ontology • KEGG

Introduction

Domesticated silkworm, *Bombyx mori* is a holometabolous Lepidoptera insect. Except of economic and agricultural importance, it is perhaps the best model organism especially for lepidopteran biochemical, molecular genetic and genomic studies.[1,2] Some typical physiological events of insect such as diapause and molting have special importance in classification of silkworm varieties and silkworm breeding program. Diapause, molting and metamorphosis are mainly controlled by the brain (Br), subesophageal ganglion (SG), and prothoracic glands (PG) which are major part of endocrine system. The Br, an important neuroendocrine organ, is 'supraesophageal ganglion' which interconnected by paired circumesophageal connectives with SG.[3] It is not only the receptor and repository of environmental stimuli, but also a secretory organ that plays a central role in regulating the growth and development of insect.[4] The Br acts as the primary regulator of insect diapause, molting and metamorphosis by directly or indirectly affecting the hormone synthesis and secretion from SG and PG. One of the best-characterized factors is a cerebral neuropeptide, prothoracicotropic hormone (PTTH). It stimulates SG of female to produce diapause hormone (DH) which is a primary endocrine factor to elicit embryonic diapause in her progeny.[5] On the other hand, it is also a trigger that stimulates PG to produce ecdysteroids which maintain the individual development and initiate molting and metamorphosis of insects.[6,7] Moreover, larval and pupal diapauses are usually characterized by a shutdown of the Br−PG axis that the PG fails to synthesize the ecdysteroids.[4] In addition, the Br produces many other neurosecretory substances, such as the prothoracicostatic peptide (PTSP),[8] ecdysone receptor (EcR), ultraspiracle (USP),[9] and corazonin,[10] to orchestrate the developmental processes.

In addition to DH, SG of silkworm female synthesizes other FXPRL-amide peptides, including pheromone biosynthesis-activating neuropeptide (PBAN) and α-, β-, γ-SG neuropeptide (SGNP), from a common precursor polyprotein in neurosecretory cells of SG, and expressing DH and pheromonotropic activities at different degrees.[11]

PG is the center of ecdysteroidogenesis which is triggered by PTTH. Many factors may participate in the regulation of ecdysteroid synthesis, such as PTSP,[8] myosuppressin,[12] Start1 protein,[13] and subsets of cytochrome P450 family.[14,15] Insect molting and metamorphosis are directly or indirectly regulated by the interaction of a major biologically active insect steroid hormone, 20-hydroxyecdysone (20E), with the EcR/USP com-

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plex that recognizes specific DNA response elements and initiates the insect molting process.[16]

According to the above explanation, the different endocrine organs exhibit intrinsic relationships and complex signaling and metabolic pathways underlying the physiological events. However, previous reports paid more attention to the individual hormones and enzymes involved in these physiological processes so that the molecular basis remains poorly understood. Proteomics study provides a valuable approach to discover these exquisite relationships at the protein level. With the completion of draft sequence for the genome of several model organisms such as silkworm, the proteomics study has become the focal point in recent entomological researches. As effective tools for proteomics, the two-dimensional electrophoresis (2-DE) combined with mass spectrometry (MS) have been widely used to characterize the differential expression profiles of insect tissues and organs, such as silk gland,[17] hemolymph,[18,19] Br,[20] PG[21] and skeletal muscle,[22] during developmental processes or under different conditions.[23−25]

Concerning the complexity of biological system, a large-scale and high-throughput characterization of a profile is necessary to get a better understanding of the interactions among the components. Microarray-based approaches have globally characterized the gene expression profiles and transcriptomes of several tissues and organs in *Bombyx*,[26] *Drosophila*,[27,28] and *Arabidopsis*.[29] Shotgun proteomics, based on the in-gel or gel-free digestion of protein mixtures followed by liquid chromatography (LC) separation, MS detection and database search, provides us with a highly sensitive and high-throughput approach to understand the proteome components in a complex biological sample. In addition, the development of computer software and bioinformatics facilitates us to make the best use of the genomic resources.[30] Thousands of proteins have thus been identified in human body fluids, including blood,[31−33] urine,[34] seminal plasma[35] and tear fluid[36] by shotgun proteomics. It has also been implemented to some model insects, such as *Drosophila*,[20,37] and *Anopheles*.[38] However, the identified proteins are still much limited in number with, so far, a few reports for silkworm.

In the present study, we utilized the shotgun LC-MS/MS approach in combination with bioinformatics analysis to characterize the protein expression profiles of Br, SG and PG of silkworm for further understanding of their functions and relations in regulating insect diapause, molting, metamorphosis, and so forth. These data are expected to provide valuable hints for uncovering the mechanism of metabolism and signaling of insect endocrine system, which is currently also an active area of research because it offers the potential for disrupting the life cycle of a pest without harm to environment.

Materials and Methods

Animal Rearing and Organs Isolation. Diapaused polyvoltine silkworm strain P50 was reared on the fresh mulberry leaves under a 12 h light/12 h dark photoperiod at 26 ± 1 °C with 70−85% relative humidity. The developmental stages were synchronized after each molt by collecting new larvae. About 500 larvae (300 for LC-MS/MS and 200 for real-time PCR) were anatomized under a stereoscopic microscope at the fifth day of the final instar (2 days before the wandering period). The Br, SG, and PG were collected in ice-cold normal saline. After centrifugation, they were stored at −80 °C for further use.

Sample Preparation and SDS-PAGE Separation. Samples of Br, SG, and PG from about 300 silkworm larvae were mechanically homogenized on ice in 10 μL of lysis buffer (containing 2.5% SDS, 10% glycerin, 5% β-mercaptoethanol, and 62.5 mM Tris-HCl pH 6.8) per 1 mg of sample using a motor-driven plastic pestle. The homogenate was kept for 10 min at room temperature, and then it was subjected to continued sonication treatment in an ice-bath four times, each time 30 s with a 30 s interval. After centrifugation at 20 000*g* twice, at 25 °C for 10 min, the supernatant was aliquoted and stored at −20 °C. The sample was boiled for 2 min and centrifugated at 20 000*g* for 10 min before it was subjected to SDS-PAGE.

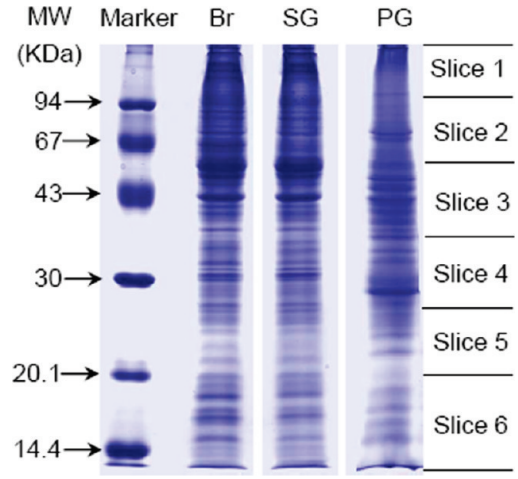

Figure 1. SDS-PAGE patterns of Br, SG, and PG of silkworm. The samples were separated by 12.5% resolving gel in quadruplicate. Each gel lane was cut into six slices.

Samples were separated by SDS-PAGE using 5% stacking gel and 12.5% resolving gel. The gels were stained with Coomassie Brilliant Blue (CBB) R250 (Sigma) after electrophoresis.

In-Gel Digestion. The CBB-stained SDS-PAGE gel lane was manually cut into 6 slices depending on protein molecular weight (MW) (Figure 1). Each slice was diced into 1 mm × 1 mm pieces, and then subjected to in-gel tryptic digestion, essentially as described by Wilm et al.[39] Briefly, the gel pieces were rinsed thrice using Milli-Q water (Millipore) and destained twice with 25 mM NH_4HCO_3 in 50% acetonitrile (ACN, Amersham) at 37 °C until the color depigmented completely. The dried gels were incubated with 50 mM Tris[2-carboxyethyl]phosphine (TCEP, Sigma) in 25 mM NH_4HCO_3 at 56 °C for 1 h to reductively cleave the disulfide bonds of proteins and then the resulting sulfhydryl functional groups were alkylated by 100 mM iodoacetamide (IAA, Amersham) in 25 mM NH_4HCO_3 at room temperature in the dark for 0.5 h. Subsequently, the proteins were digested with 20 ng/μL porcine trypsin (modified proteomics grade, Sigma) overnight at 37 °C. The resulting tryptic peptides mixture was extracted twice from the gel pieces with 5% trifluoroacetic acid (TFA, Fluka) in 50% ACN solution. The pooled extracts were evaporated in a vacuum centrifuge (Labconco, Kansas, MO), and resuspended with 0.1% methanoic acid (Sigma) prior to the LC separation and MS detection.

Shotgun LC-MS/MS Analysis. All digested peptide mixtures were separated by reverse phase (RP) HPLC followed by tandem MS analysis. RP-HPLC was performed on a surveyor LC system (Thermo Finnigan, San Jose, CA). Samples were loaded into a trap column (Zorbax 300SB-C18 peptide traps, 300 μm × 65 mm, Agilent Technologies, Wilmington, DE) first at a 3 μL/min flow rate after the split for peptides enrichment and desalting.

After flow-splitting down to about 1.5 μL/min, peptides were transferred to the analytical column (RP-C18, 150 μm $\times$ 150 mm, Column Technology, Inc., Fremont, CA) for separation with a 195 min linear gradient from 96% buffer A (0.1% methanoic acid in water) to 50% buffer B (84% ACN, 0.1% methanoic acid in water) at a flow rate of 250 nL/min. The analytical column was regenerated for 20 min with buffer A before loading the next sample. A Finnigan LTQ linear ion trap mass spectrometer equipped with a nanospray source was used for the MS/MS experiment in the positive ion mode. The temperature of the ion transfer capillary was set at 160 °C. The spray voltage was 3.0 kV and normalized collision energy was set at 35.0% for MS/MS. The MS analysis was performed with one full MS scan (m/z 400−1800) followed by 10 MS/MS scans on the 10 most intense ions from the MS spectrum with the dynamic exclusion settings: repeat count 2, repeat duration 30 s, exclusion duration 90 s. Data were acquired in data-dependent mode using Xcalibur software.

Database Construct and Search. In-house database was constructed with the FASTA protein sequences downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) containing domesticated silkworm (*B. mori*, 3892 entries) and wild silkworm (*Bombyx mandarina*, 121 entries), along with the data published by Xia et al. (21 312 entries).[40] For each experiment, MS/MS spectra were extracted from the raw data files by extract_msn program in the Biowork 3.2 (ThermoFinnigan, San Jose, CA). The searches against the in-house database were performed on a local server using TurboSEQUEST software (a part of Biowork 3.2 software package) and an open-source software X!Tandem, separately. For the SEQUEST search, the monoisotopic precursor and fragments were selected. The peptide mass tolerance was 2.0 Da and the fragment ions tolerance was used as default. The trypsin enzyme was used to cleave the amino acids bonds at both ends of protein. Two miscleavage sites were allowed. Only *b* and *y* fragment ions were taken into account. Fixed modification (Carboxamidomethyl) on cysteine and variable modification (Oxidation) on methionine were set. The results for each data set were stored in '.out' files. The parameters for X!Tandem search were similar to those for SEQUEST. And *k*-score algorithm was used in the process of X!Tandem identification.

Trans-Proteomic Pipeline (TPP) Validation Processes. The TPP software suite (v.3.4) was downloaded from the Web site (http://tools.proteomecenter.org/TPP.php) and installed with the default options. Validation of the identified peptides and proteins was carried out according to the manual of the TPP software.

PeptideProphet and ProteinProphet are two important components of the TPP that, respectively, validate peptides and proteins by computing for each a probability which can be used to discriminate between correct and incorrect results within a data set and also act as an objective criterion to compare different search engines.[41−43] The peptide and protein probability thresholds for running PeptideProphet and ProteinProphet were both set at 0.9. The search engine X!Tandem has already been integrated into the TPP software suite. The parameters and validation processes were similar to those of SEQUEST. The final results protXML file was opened and exported with an open-source Computational Proteomics Analysis System (CPAS) (LabKey2.3)[44] because of its large size. After checking the results of the two algorithms, those identified proteins with a probability greater than 0.9 were accepted. The proteins identified by both algorithms and the ones specifically identified by either algorithm were combined together.

Quantitative Real-Time PCR (qRT-PCR). Total RNA of Br, SG, and PG was extracted from each sample using TRIzol reagent (Invitrogen) according to the manufacturer's specifications and then reverse-transcribed into cDNA with oligo $(dT)_{15}$-Primer and M-MLV reverse transcriptase (Promega). The gene-specific primers were designed with online software Primer3 (v. 0.4.0, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Supplemental Table 1). The qRT-PCR was performed in 25 μL reactions with 100 ng of reverse transcription product, 200 nM each of the forward and reverse primer, and the SYBR Green Mix (SYBR Green I Dye, Applied Biosystems; *Ex Taq* HS DNA polymerase, dNTPs and optimal buffer components, TaKaRa). The cDNA was amplified in a Rotor-Gene 3000 real-time PCR system (Corbett Research, Sydney, Australia) according to the following program: initial denaturation at 95 °C for 90 s, and 40 cycles of 95 °C for 5 s and 58 °C for 30 s. Then, there was an additional 1 min at 95 °C, 1 min at 58 °C and 10 s/cycle for data collection of the dissociation curve with the increase of temperature from 58 to 92 °C at 0.5 °C/cycle. Three replications of each RNA sample were performed for qRT-PCR. Absolute quantification of the amplified transcripts was determined based on the standard curve made with the known concentration of mRNA.

InterPro Annotation and Gene Ontology (GO) Categories. Protein sequences were searched against InterPro member databases using the InterProScan[45] software to identify signatures. The meaningful matches were outputted in RAW format. The compiled RAW outputs were subjected to GO categories using the Web Gene Ontology Annotation Plot (WEGO).[46] Three groups of data set were simultaneously subjected to online analysis (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) that was convenient to compare them in one graph. The *P*-values which calculated using Pearson Chi-Square test were available.

Pathway Analysis with KEGG. The query FASTA protein sequences were searched against KEGG GENES using BLASTP program with BLOUSM62 scoring matrix (http://blast.genome.jp/). The Enzyme Commission (EC) number (if available) of the best matched protein (*E*-value $\leq$ e−15) was accepted and exported. All of these processes were performed by an in-house program. The obtained EC numbers from each data set were subjected to pathway search against KEGG reference pathway database (http://www.genome.jp/kegg/tool/search_pathway.html). Each selected pathways met the criterion that it had at least three ECs. Pairwise comparison of the pathways were undertaken and then classified according to the definition of KEGG (http://www.genome.ad.jp/kegg/pathway.html).

Results and Discussion

Global Analysis of the Endocrine Organs Proteome. The shotgun proteomics strategy, based on proteolytic digestion of complex protein mixtures, peptides LC separation and tandem MS sequencing, has been widely adopted. However, database search remains the bottleneck for many shotgun proteomics experiments other than the mass spectrometric instrumentation, especially when organism database has been not fully developed such as the silkworm. Therefore, we constructed an in-house database from different sources and carried out database search using two of the most popular algorithms, SEQUEST and X!Tandem, to increase the number of unique peptide identifications. To minimize the false positive sequence

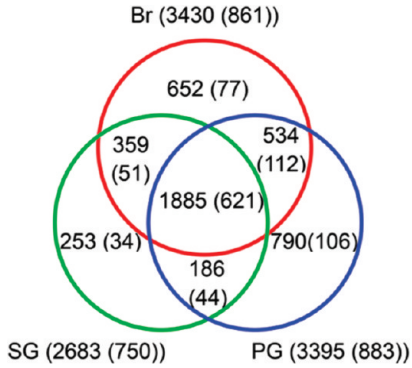

Figure 2. Venn diagram for identified protein numbers of Br, SG and PG. The overlap shows the number of common-expressed proteins between the organs. The number in parentheses denotes the proteins from NCBI.

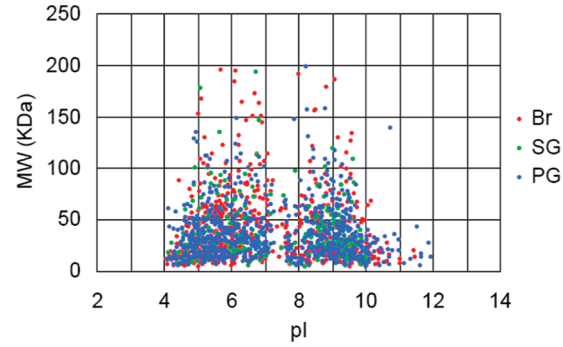

Figure 3. Theoretical 2-D (p*I*, MW) distribution of the organ-specific proteins of Br, SG and PG. The theoretical p*I* and MW of the proteins were calculated using the Compute p*I*/M_w tool (http://cn.expasy.org/tools/pi_tool.html) according to protein amino acid sequence. Proteins MW ≤ 200 KDa were selected (more than 97% of the specific proteins).

matches, the results from SEQUEST and X!Tandem were subjected to further validations utilizing the TPP under stringent criteria. For SEQUEST, 10 783, 9099, and 12 063 unique peptides from Br, SG, and PG were identified with high confidence (probability ≥ 0.9) and were assigned into 2833, 2298, and 2789 protein groups, respectively. Moreover, the false positive error rates of the results were less than 1% (Supplemental Figure 1). For the proteins assigned into one group by ProteinProphet, all were inputted in the final list, because we could not unambiguously determine which one or ones were real identifications. It might increase the risk of redundant identifications, but these data would be useful for further validation. We combined the results of SEQUEST and X!Tandem to increase the number and reliability of the total identifications (Supplemental Tables 2−4). The common part of this combination overlapped 87.95%, 87.25%, and 87.27% with SEQUEST and 96.60%, 98.93%, and 97.58% with X!Tandem results in Br, SG, and PG, separately. Totally, 3430, 2683, and 3395 proteins of Br, SG, and PG were identified with about 5% redundancy resulting from the combined database. Figure 2 shows pairwise comparison of the identified proteins of Br, SG and PG. Common-expressed proteins (excluding third-party proteins) of Br−SG, Br−PG, and SG−PG were 359, 534 and 186, respectively. The number of common-expressed proteins among the three organs was 1885, which was 54.96%, 70.26%, and 55.82% of the proteins in the Br, SG, and PG, respectively. On the other hand, the organ-specific proteins were 652, 253, and 790, which constituted 19.01%, 9.43%, and 23.27% of total proteins in the same order. The organ-specific proteins appeared much fewer than expected, especially in SG. However, many well-known organ-specific proteins (will be discussed below) were specifically identified, which increased the confidence of our results. In contrast, the common-expressed proteins were much more than expected. There are several possible reasons. First, essentially the three organs are intimately associated with each other from the time of organogenesis. For example, the Br and SG are in fact the two parts of insect 'brain'.[3] Second, because of the current limitation of silkworm proteome database, perhaps we cannot identify more unknown proteins, even more organ-specific proteins are available in these three organs. Third, there are a large number of neuropeptides in central nervous system of insect that are readily to be overlooked by shotgun proteomic approaches.[37]

Theoretical Two-Dimentional Distribution of the Identified Proteins. The general view of 2-D distribution of the identified proteins showed symmetrical distribution. While about 87% proteins were distributing in a range of p*I* 4−7 and 8−10, about 55% proteins were distributing in a range of MW 15−45 KDa (Supplemental Table 5). In the three organs, less than 7% of proteins showed p*I* 7−8. Furthermore, 179, 139, and 186 proteins with higher p*I* (more than 10), usually difficult to be separated by 2-DE, were also identified from Br, SG, and PG, respectively. The organ-specific proteins also showed nearly symmetrical distribution (Figure 3) and were similar to those of the total proteins. It revealed that the protein distributions were almost similar in the Br, SG and PG.

Profiling of Br-Specific Proteins. Total number of Br-specific proteins was 652, including 77 from NCBI (Figure 2). Among these proteins, the PTTHs, including a big PTTH, small PTTHs such as a series of Bombyxins (BBX-B5, BBX-B9, BBX-B11, BBX-C1, BBX-C2), and an undefined preproPTTH, were found uniquely expressed in Br (Supplemental Table 6). Besides, corazonin preprohormone, Retionid X receptor (RXR) type hormone receptor (including USP homologue) were also found.

Apart from these well-known Br-specific proteins, an ion transport peptide (ITP) precursor was found uniquely expressed in the Br. ITPs, highly homologous with a large family of crustacean hormones including hyperglycaemic (CHH) and moult-inhibiting hormones (MIH),[47] were first identified as antidiuretic hormones in desert locust, *Schistocerca gregaria*.[48] They have been purified from locust corpus cardiacum and shown to stimulate salt and water reabsorption and inhibit acid secretion in the ileum of *S. gregaria*. CHH-like immunocytochemical analysis revealed an increase of ITP levels in the *Manduca sexta* hemolymph just prior to ecdysis. These suggested a possible physiological role for ITP in insect ecdysis.[49] Our results and the previous researches imply that the status of ITP in silkworm may be similar to the other insects.

Two of argonaute protein family, argonaute 1 (Ago-1) and argonaute 2 (Ago-2), were also detected. Argonaute family proteins are components of RNA-induced silencing complex (RISC) which mediates RNA interference (RNAi). It has been demonstrated that Ago-2 is required for proper nuclear migration, pole cell formation, and cellularization during the early stages of embryonic development in *Drosophila*.[50]

Table 1. A Part of Br, SG and PG Common-Expressed Proteins with Annotations

protein name (arrange in name initial order)	accession no.	theor. p*I*	theor. MW (KDa)	GO no. (cellular component, molecular function, biological process)	EC no.
14-3-3 epsilon protein	gil148298752	4.90	28.10	GO:0045172, GO:0004863, GO:0006588	EC: 3.1.1.4
14-3-3zeta	gil114050901	4.66	29.67	GO:0005694, GO:0004863, GO:0000077	EC: 3.1.1.4
45 kDa immunophilin FKBP45	gil112983564	4.75	44.68	GO:0005634, GO:0003755, GO:0006457	EC: 5.2.1.8
90-kDa heat shock protein	gil112983556	4.99	82.42	GO:0030235, GO:0005515, GO:0045429	EC: 3.6.4.9
alpha-crystallin	gil12743949	6.95	9.96	—, —, GO:0009790	—
annexin B13	gil112983539	6.90	54.30	GO:0003779, —, GO:0007155	EC: 3.1.4.43
antennal binding protein	gil87248601	6.71	15.49	GO:0005575, GO:0005549, GO:0007606	—
Bombyrin	gil112983654	7.05	22.54	GO:0005576, GO:0005319, GO:0006629	—
calreticulin	gil112983032	4.49	45.80	GO:0005575, GO:0003674, GO:0008150	—
cAMP-dependent protein kinase C1	gil153792580	8.88	40.60	GO:0005952, GO:0004691, GO:0007448	EC: 2.7.11.11
casein kinase 2 alpha subunit	gil112983288	6.74	41.72	GO:0008013, GO:0047485, GO:0006468	EC: 2.7.11.1
caspase-1	gil112983104	6.45	33.34	GO:0008303, GO:0030693, GO:0006915	EC: 3.4.22.-
cdc2-related kinase	gil112983598	8.98	45.12	GO:0004693, —, GO:0006468	EC: 2.7.11.22
cell death-regulatory protein GRIM19	gil114051728	9.26	17.99	GO:0005747, GO:0003954, GO:0006120	EC:1.6.5.3, 1.6.99.3
chemosensory protein 11	gil112983052	4.97	13.52	GO:0005737, GO:0019992, GO:0008595	—
chemosensory protein 5	gil115551742	7.59	14.65	GO:0005576, —, GO:0007552	—
chemosensory protein CSP1	gil112983094	5.21	14.55	GO:0005576, —, GO:0007552	—
conventional protein kinase C	gil112983438	6.95	76.43	GO:0005576,—, GO:0007552	EC: 2.7.11.13
cytochrome P450 9a20	gil134254438	8.30	61.45	GO:0005576, —, GO:0007552	EC: 6.5.1.1
cytochrome P450 CYP4G25	gil95102948	8.74	63.29	GO:0005576, —, GO:0007552	EC: 1.14.-.-
diapause bioclock protein	gil119351373	6.12	18.29	GO:0016020, GO:0009055, —	EC: 1.15.1.1
DNA-damage inducible protein	gil114051417	5.20	43.50	GO:0016020, GO:0009055, GO:0006694	—
dopa decarboxylase	gil112984466	5.83	54.20	GO:0004058, —, GO:0006584	EC: 4.1.1.28
ecdysteroid-regulated 16 kDa protein precursor	gil151301100	5.92	15.82	GO:0005576, —, —	—
exuperantia	gil114053141	7.11	45.81	GO:0005737, —, GO:0045450	—
G protein alpha subunit Go	gil112982857	5.38	40.40	GO:0005834, GO:0003924, GO:0008356	—
G protein pathway suppressor 1	gil114051021	6.21	54.21	GO:0008180, —, —	—
glia maturation factor beta	gil153791457	5.14	16.43	—, GO:0003779, GO:0042063	—
Gq-like G protein alpha subunit	gil112982768	5.15	41.49	GO:0005834, GO:0003924, GO:0007610	—
heat shock 70 kD protein cognate	gil112984012	5.12	73.10	GO:0005783, GO:0016887, GO:0009408	EC: 1.3.1.74
heat shock protein hsp 19.9	gil112983420	6.53	19.89	—, —, GO:0009790	—
heat shock protein hsp20.1	gil112983134	5.46	20.14	—, —, GO:0009790	—
heat shock protein hsp20.4	gil112983152	6.54	20.43	—, —, GO:0009790	—
heat shock protein hsp20.8	gil148298693	5.98	20.80	—, —, GO:0009790	—
heat shock protein hsp21.4	gil112983414	5.79	21.40	—, GO:0005515, —	—
imaginal disk growth factor	gil152061158	7.64	48.16	GO:0005575, GO:0003674, GO:0008150	EC: 3.2.1.14
innexin 2	gil112982709	6.99	41.91	GO:0005921, GO:0005243, GO:0007440	—
juvenile hormone epoxide hydrolase	gil112984538	8.25	52.41	GO:0005792, GO:0004301, GO:0006719	EC: 3.3.2.9
juvenile hormone esterase	gil112983178	5.75	62.80	—, GO:0004091, —	EC: 3.1.1.1
juvenile hormone esterase binding protein	gil114052406	8.84	28.61	GO:0005739, GO:0005515, —	—
mago nashi	gil112983378	6.06	17.24	GO:0005737, —, GO:0007267	—
MAP kinse-ERK kinase	gil112982906	6.53	44.71	GO:0004707, —, GO:0007298	EC: 2.7.12.2
mitogen-activated protein-binding protein-interacting protein	gil114050829	6.82	13.44	GO:0005794, —, —	—
MLE protein	gil153792023	6.16	144.66	GO:0000785, GO:0008026, GO:0048675	EC: 3.6.1.3
p23-like protein	gil112983282	4.54	18.83	GO:0005575, GO:0003674, GO:0008150	—
p50 protein	gil112984204	6.23	52.22	GO:0005576, GO:0008367, GO:0045088	—
peripheral-type benzodiazepine receptor	gil114052781	7.74	18.87	—, GO:0003674, GO:0008150	—
PIWI	gil157674348	9.45	101.34	GO:0005737, GO:0003723, GO:0006342	—
PKG-II	gil112983098	5.79	83.98	GO:0005886, GO:0004692, GO:0007631	EC: 2.7.11.12
profilin	gil112982865	5.88	13.71	—, GO:0003779, GO:0007015	—
proliferating cell nuclear antigen	gil112984050	4.60	29.04	GO:0043626, —, GO:0019730	—
protein kinase c inhibitor	gil112983493	6.17	13.95	GO:0005575, —, GO:0008150	EC: 3.1.3.31
ras oncoprotein	gil112983398	6.33	21.83	—, GO:0019003, GO:0006916	—
ras protein	gil112983416	6.76	20.93	GO:0005811, GO:0019003, GO:0007155	—
ras-related GTP-binding protein Rab11	gil112983314	5.24	24.09	GO:0005813, GO:0005525, GO:0031532	EC: 3.6.5.2
ras-related GTP-binding protein Rab3	gil112983326	4.98	24.78	GO:0008021, GO:0007269, GO:0003924,	EC: 3.6.5.2
receptor for activated protein kinase C RACK 1 isoform 1	gil115345341	8.07	36.04	GO:0005737, GO:0005080, GO:0042335	EC: 2.4.2.-
s3a protein	gil146742935	9.69	29.81	GO:0022626, GO:0003735, GO:0000022	—
septin	gil114052056	5.50	43.30	GO:0045172, GO:0005525, GO:0007349	—
sericotropin	gil114052076	9.02	15.15	GO:0005575, GO:0005549, GO:0007606	—
sex-lethal	gil112734730	9.16	37.53	—, GO:0008083, GO:0000380	—

Table 1. Continued

protein name (arrange in name initial order)	accession no.	theor. p*I*	theor. MW (KDa)	GO no. (cellular component, molecular function, biological process)	EC no.
signal sequence receptor beta subunit	gi|114052941	6.91	20.91	GO:0005811, GO:0005048, GO:0006621	—
small GTP binding protein RAB5	gi|112983262	8.73	23.17	GO:0005768, GO:0030742, —	EC: 3.6.5.2
small GTP binding protein RAB8	gi|112982896	8.85	23.88	—, GO:0003924, —	EC: 3.6.5.2
small GTP-binding protein Rab10	gi|148298847	8.30	23.13	—, GO:0003924, —	EC: 3.6.5.2
sorbitol dehydrogenase	gi|112983008	6.31	38.68	—, GO:0003939,—	EC: 1.1.1.14
tetraspanin E	gi|114050851	7.95	25.61	GO:0016021, —, —	—
tyrosine 3-monooxygenase protein zeta polypeptide	gi|114050901	4.90	28.17	GO:0045172, GO:0004863, GO:0006588	EC: 3.1.1.4
vacuolar ATPase subunit C	gi|114050729	8.46	44.01	GO:0000221, GO:0008553, GO:0015992	EC: 3.6.3.14
vacuolar H+ ATP synthase 16 kDa proteolipid subunit	gi|95102608	8.96	15.84	GO:0000220, GO:0008553, GO:0015992	EC: 3.6.3.14
WD repeat domain 61	gi|114051355	4.95	36.25	GO:0005634, GO:0003700, —	EC: 2.4.2.-
WD40 protein	gi|114052198	6.55	35.32	GO:0008352, —, GO:0051013	EC: 2.4.2.-

Besides, seven subunits of nicotinic acetylcholine receptors (nAChRs) were found during the current research might play a central role in rapid cholinergic synaptic transmission and thus they are important targets of insecticides.[51] In the honeybee *Apis mellifera*, subsets of nAChRs are differently expressed among the brain regions and at developmental stages.[52] Furthermore, some of them may be involved in the insect olfactory associative learning through mediating synaptic transmission.[53]

Profiling of SG-Specific Proteins. The insect FXPRL-amide peptides, especially the DH and PBAN, which released from the SG of female, are deeply involved in the embryonic diapause and reproduction. Interestingly, DH can be detected in the SG of larvae who are destined to produce diapause as well as nondiapause eggs throughout larval development.[54] We found the DH and PBAN and their precursors among the SG-specific proteins (Supplemental Table 6) which is consistent with previous research results.[54] These findings indicate that FXPRL-amide peptides may have functions during postembryonic development other than the induction of embryonic diapause and activation of pheromone biosynthesis.

It is speculated that the GABA-gated chloride channel alpha subunit which is found in the SG-specific expressed proteins may extend the roles of SG. Gamma-aminobutyric acid (GABA) is a chief inhibitory neurotransmitter in the central nervous system. It has been demonstrated that GABA is possibly involved in the information transfer process from mother to progeny that shows maternal effect in the flesh fly, *Sarcophaga bullata*.[55] The GABA acts on inhibitory synapses in the Br by binding to specific transmembrane receptors. It has been proved that the GABA receptors may be involved in insect learning memory.[53]

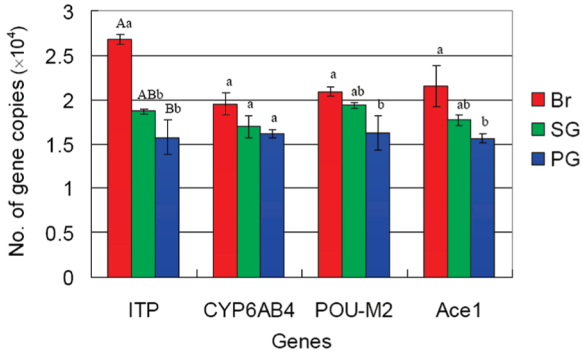

Figure 4. qRT-PCR comparison of the expression levels based on the copy numbers of genes from Br, SG and PG. The differences were analyzed by one-way ANOVA and multiple comparisons (Duncan's SSR, different capitals and lowercases show highly significant ($p < 0.01$) and significant ($p < 0.05$) differences, respectively. The same letter shows no significant difference ($p = 0.05$)). Take the ITP for example, its expression in Br was significantly higher than SG ($p < 0.05$) and PG ($p < 0.01$), while there was no significant difference between SG and PG.

DEAD box proteins are putative RNA helicases that function in all aspects of RNA metabolism, including translation, ribosome biogenesis, and pre-mRNA splicing. However, the functions of most DEAD-box proteins remain unknown. One of the well-described members is the DP103 which plays an important role in development of early embryo and modulation of steroidogenesis in mice.[56] A dead box protein 3 with unknown function was also identified in the SG.

Profiling of PG-Specific Proteins. During larval and pupal stages of insects, one of the most important steroid hormones is ecdysone, which is synthesized in the PG and plays a central role in the control of larval development. In this study, two players Cyp302a1[57,58] and Rieske-domain protein Neverland,[59] which are involved in the biosynthesis of ecdysteroids, were identified among the PG-specific proteins (Supplemental Table 6).

POU domain proteins which play an important role in transcriptional regulation of the Bom-DH-PBAN gene[60] were also found. Overexpression of POU-M2 in the silkworm cells activates the promoter of Bom-DH-PBAN, but fails to activate a promoter in which the POU-binding element is mutated. In addition, POU-M1 shows the same transcriptional activities as POU-M2.[60] These findings suggest that PG may affect the functions of SG through regulating the expression of DH and PBAN.

Samui protein which was detected in the present study is a cold-induced protein with a BAG domain similar to silencer of death domains and isolated from silkworm diapause eggs.[61] Expression of Samui will be activated after incubation in cold condition for 5−6 days. It was proposed that Samui serves to transmit the cold signal for sorbitol dehydrogenase gene expression in diapause eggs, while protecting against cold-injures in nondiapause eggs.[61] However, the role of Samui protein in insect larva is still not clear.

Interestingly, there were many immune-related proteins including BmRelA, BmRelB, gloverin-like protein 2, gloverin-like protein 3, antibacterial peptide and immulectin. This suggests that the PG of silkworm is involved in antibacterial system similar to *Locusta*.[62]

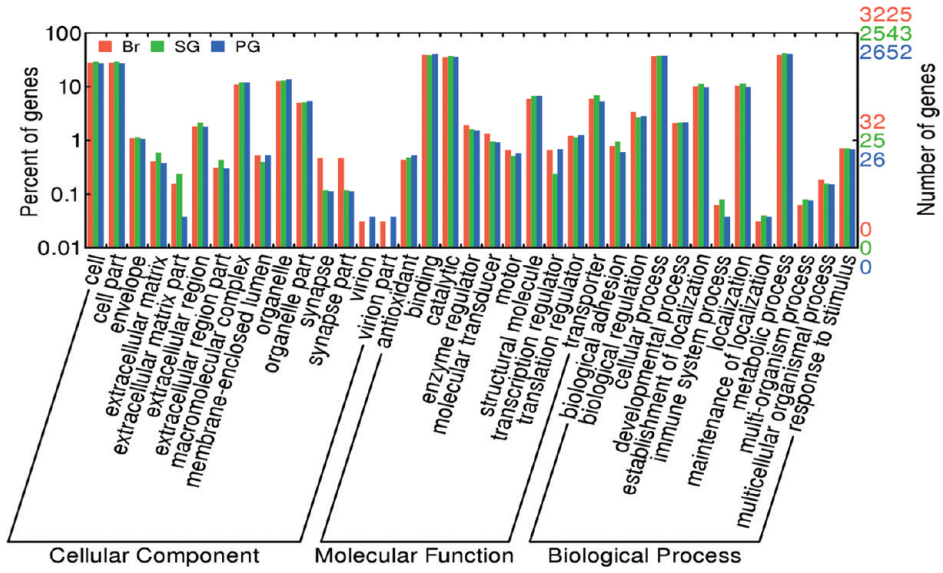

Figure 5. Gene ontology categories for the identified proteins of Br, SG and PG. The identified proteins were classified into cellular component, molecular function and biological process by WEGO according to the GO terms. The number of genes is the number of times the GO term is used to annotate genes in the cluster. The left-hand shows its proportion in total genes of related organ with GO terms.

In addition, proteins such as double-time protein, prolactin regulatory element binding protein and insulin-related peptide binding protein may enrich the possible functions of PG.

Profiling of Common-Expressed Proteins of Br−SG, Br−PG, and SG−PG. By pairwise comparisons of Br−SG, Br−PG, and SG−PG, many interesting proteins were found in each pair (Supplemental Table 7). For example, in Br and SG, Hsc70/Hsp90-organizing protein HOP was commonly expressed. The HOP coordinates Hsp70 and Hsp90 interactions during assembly of steroid receptor complexes that facilitates the progression of the receptor functional maturation.[63,64] In addition, FKBP, HSP70, HSP90, and p23 are all auxiliary factors that are necessary for the initial association of the EcR/USP heterodimer with the DNA-binding site.[65]

In the Br and PG common-expressed proteins, one of interesting proteins is the Rheb (Ras homologue enriched in brain) which is deeply involved in the regulation of cell growth and proliferation in *Drosophila*.[66,67] Reduction of Rheb activity results in a decrease both in cell number and cell size.[66]

Surprisingly, we found expression of the progesterone membrane binding protein (P_4BP) both in SG and PG. P_4BP has $GABA_A$ receptor-like properties that coimmunoprecipitated by an antibody to the alpha1 chain of the $GABA_A$ receptor.[68] Moreover, the progesterone membrane receptors usually locate on the animal brain regions and are involved in oocyte maturation, sperm hypermotility, female reproductive behaviors, and so on by interacting with the progesterone.[69,70] Combined with the result that the GABA receptor was expressed in Br and PG, it suggests that the Br, SG and PG all may be involved in these processes.

Profiling of Common-Expressed Proteins of Br, SG, and PG. On the list of common-expressed proteins of Br, SG and PG with annotation, many functional proteins were detected (Table 1) which would uncover the potential functions and relations of these organs. Among these common-expressed proteins, juvenile hormone esterase (JHE), juvenile hormone epoxide hydrolase (JHEH) and juvenile hormone esterase binding protein (JHBP) are involved in the metabolism of juvenile hormone (JH).[71,72] Besides, juvenile hormone, diol kinase (JHDK)[73] was found in the Br and PG (Supplemental Table 7). These results suggest that the Br, SG and PG may play pivotal roles in regulating insect JH titer and indirectly regulating the growth and development of larval insects.

Recently, CYP4G25, a new cytochrome P450 gene, which may be strongly associated with diapause in pharate first instar larvae, was identified between diapausing and postdiapausing pharate first instar larvae of the wild silkmoth *Antheraea yamamai*.[74] The present study showed that it was also expressed in the last instar of silkworm. Assuming a protein involving more unique identified peptides denotes the higher expression level, and therefore, the abundances of CYP4G25 in Br (18 hits) and PG (14 hits) are much higher than in SG (1 hit). Another identified important diapause-related protein was the diapause bioclock protein (TIME-EA4), a timer protein in insect eggs. It belongs to the copper−zinc superoxide dismutase (SOD) family with a high degree of sequence homology and SOD activity.[75] EA4 is a naturally glycosylated protein which may have a role in the measurement of the duration of cold exposure required for embryonic diapause development.[75,76] However, the role of EA4 in the larval development is still unknown.

Interestingly, we observed a circadian rhythm related protein, Protein Kinase G type II (PKG-II). It has been proven that the activation of PKG-II is required for the night-to-day progression of the mammalian circadian cycle, at the levels of both core clock elements and the endogenous suprachiasmatic nucleus (SCN) neuronal activity rhythm.[77]

In *Drosophila*, highly conserved proteins Mago nashi and Y14 form a stable heterodimer as core component of the exon junction complex (EJC) that strongly associates with spliced mRNA.[78] Moreover, the Mago nashi is required for germline stem cell differentiation independent of Y14,[79] although crystal structure shows that the canonical RNA-binding surface of the Y14 RNA recognition motif (RRM) is involved in extensive protein−protein interactions with Mago nashi.[80] In this study,

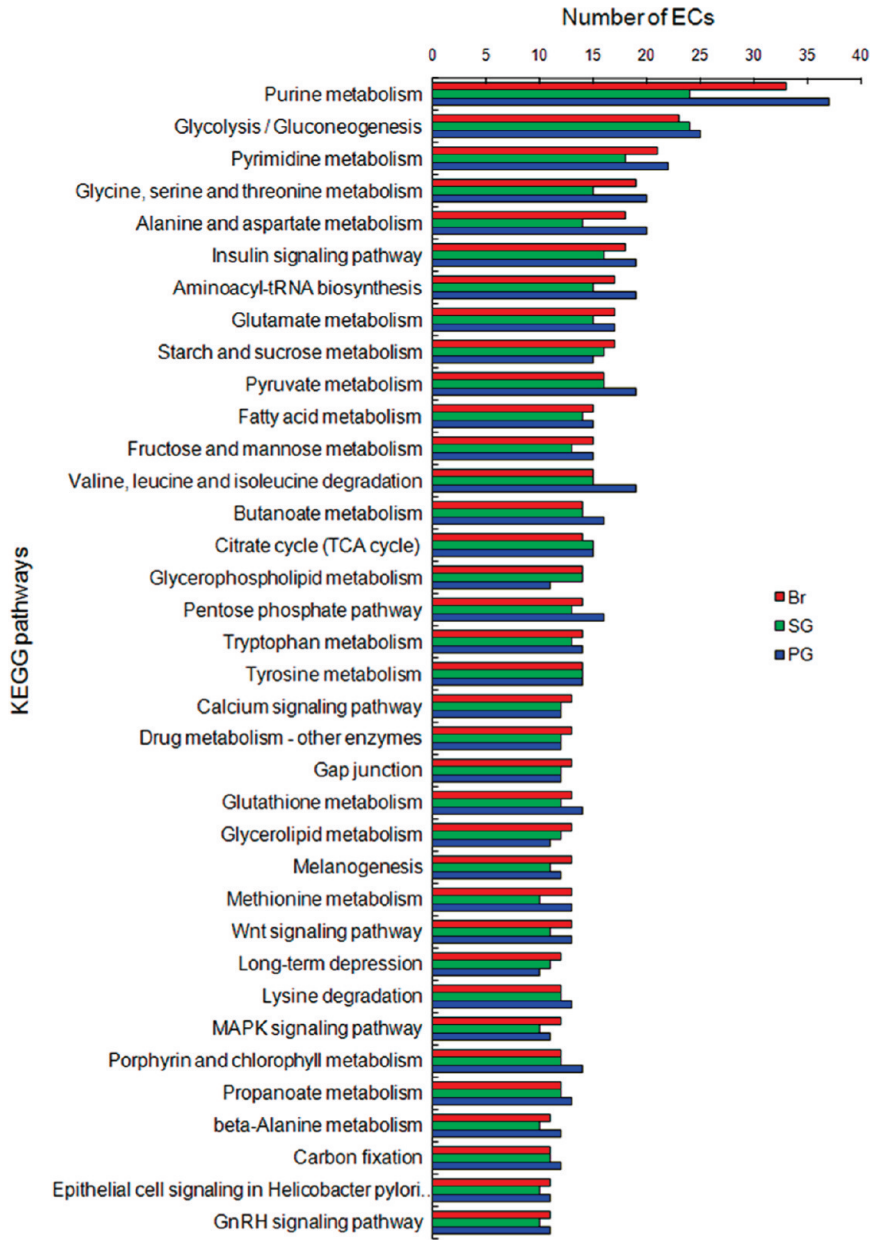

Figure 6. Comparison of the number of ECs involved in KEGG pathways in Br, SG, and PG. The pathways were arranged according to the number of identified ECs. The common pathways which containing more than or equal to ten ECs in the three organs were selected.

we found Mago nashi protein commonly expressed in Br, SG and PG, which implied their roles in silkworm sexual determination.

Quantitative mRNA Expression Analysis. To examine the gene expression levels of some interesting proteins, we selected ITP, CYP6AB4, and POU-M2, three specific-expressed genes of Br, SG, and PG, respectively, and a Br−SG commonly expressed gene of acetylcholinesterase type 1 (Ace1) for qRT-PCR analysis (Figure 4). The expression level of ITP in Br was significantly higher than that in SG and PG, which was approximately consistent with our proteomic results. While there was no significant difference on the Ace1 expression between SG and PG, it was significantly ($p < 0.05$) highly expressed in Br compared with PG. Unexpectedly, the genes of CYP6AB4 and POU-M2 expression seems inconsistent with their protein expressions. The expression of CYP6AB4 in the three organs had no significant difference and the POU-M2 in PG was expressed at low level. This inconsistency between transcription and protein expression levels was also found in our proteomics research on silkworm head (data not show) and proved that the mRNA abundance is not always consistent with the protein level,[81] which might be caused by the differences between transcriptome and proteome.

Functional Categories by Gene Ontology. Gene ontology (GO) is now widely used to describe protein function in a standardized format (http://www.geneontology.org/).[82] To further understand the functions of the proteins we identified, the protein sequences were queried against the InterPro databases and the resultant proteins were functionally categorized based on universal GO annotation terms[82] using online GO tool WEGO. Respectively, 3225, 2543, and 2652 proteins from Br, SG, and PG with annotation terms were linked to the GO cellular component, molecular function and biological process categories (Figure 5, Table 1, and Supplemental Tables

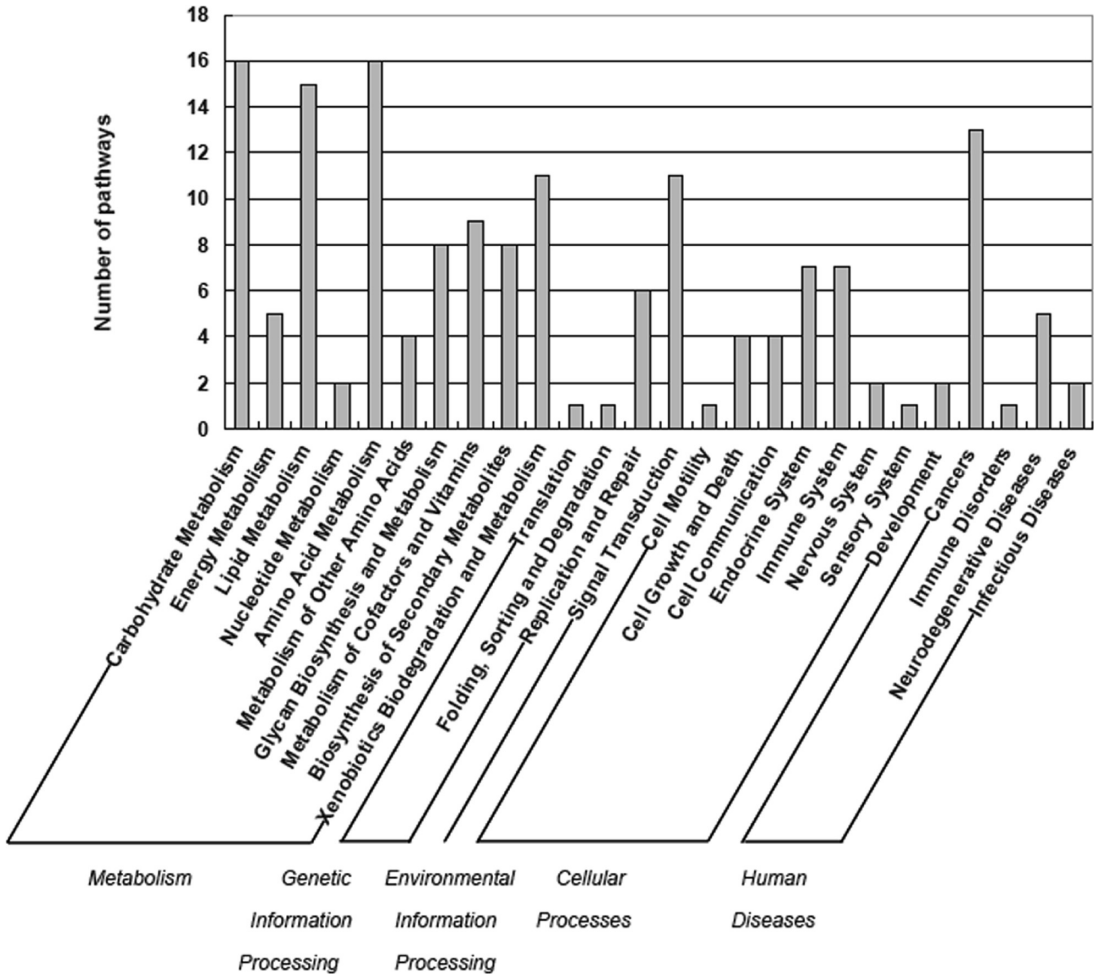

Figure 7. Categories of common-related pathways in Br, SG, and PG according to KEGG pathway taxonomy. The pathways were methodically clustered into metabolism, genetic information processing, environmental information processing, cellular processes and human diseases.

6 and 7). In the cellular component category, proteins mapping to cell, cell part, organelle and macromolecular complex related GO terms were the most abundant. In the subcategory of cell part, respectively, 308, 288, and 228 proteins of Br, SG, and PG were ascribed to membrane. In the subcategory of organelle part, 54, 48, and 45 proteins were separately assigned to organelle membrane. In addition, postsynaptic membrane proteins and synapse proteins were found in the three organs and those in Br were significantly ($p < 0.05$) more than that in SG and PG (Supplemental Table 8), which indicates the more active communications among the neurons in Br.

In the molecular function category, most proteins were addressed to binding and catalytic activity, especially the nucleotide, nucleic acid, ion binding and hydrolase, oxidoreductase, transferase activities. However, there were significant differences ($p < 0.05$) in catalytic activity, nucleic acid binding categories among Br, SG, and PG. The groups with much fewer terms include the oxygen binding, odorant binding, steroid binding, and neurotransmitter binding proteins. But the neurotransmitter binding proteins in Br were significantly ($p < 0.05$) more than those in SG and PG.

Considering the biological process category, most of proteins were involved in the metabolic process and cellular process. In the subcategories, the cellular metabolic process related proteins appeared most frequently. Besides, primary metabolic, macromolecule metabolic, and biosynthetic processes also showed active. Moreover, there were significant differences ($p < 0.05$) in the GO terms of 'generation of precursor metabolites and energy', 'secondary metabolic process', and 'regulation of cellular process' among Br, SG, and PG.

GO analysis on the identified proteins can present an overall view on the functional categories of endocrine organs proteome. The results show that the Br, SG and PG have many functional similarities. Furthermore, hydrolase, oxidoreductase and transferase showed most active in the catalytic activity category in the late larval stage. The energetic metabolism activities suggest that these endocrine organs may commit themselves to the preparation for the forthcoming metamorphosis.

KEGG Pathway Analysis. KEGG is a knowledge base for systematic analysis of gene functions, linking genomic information with higher order functional information which is stored in the PATHWAY database.[83] To uncover the metabolism and signaling pathways underlying the physiological events, the protein sequences of Br, SG and PG were searched against KEGG GENES using BLASTP program. Totally, we obtained 1375, 1158, and 1379 matched proteins with EC numbers, respectively. The identified enzymes from each data set were subjected to query of the KEGG reference pathway database. To increase the confidence of the identified pathways, only

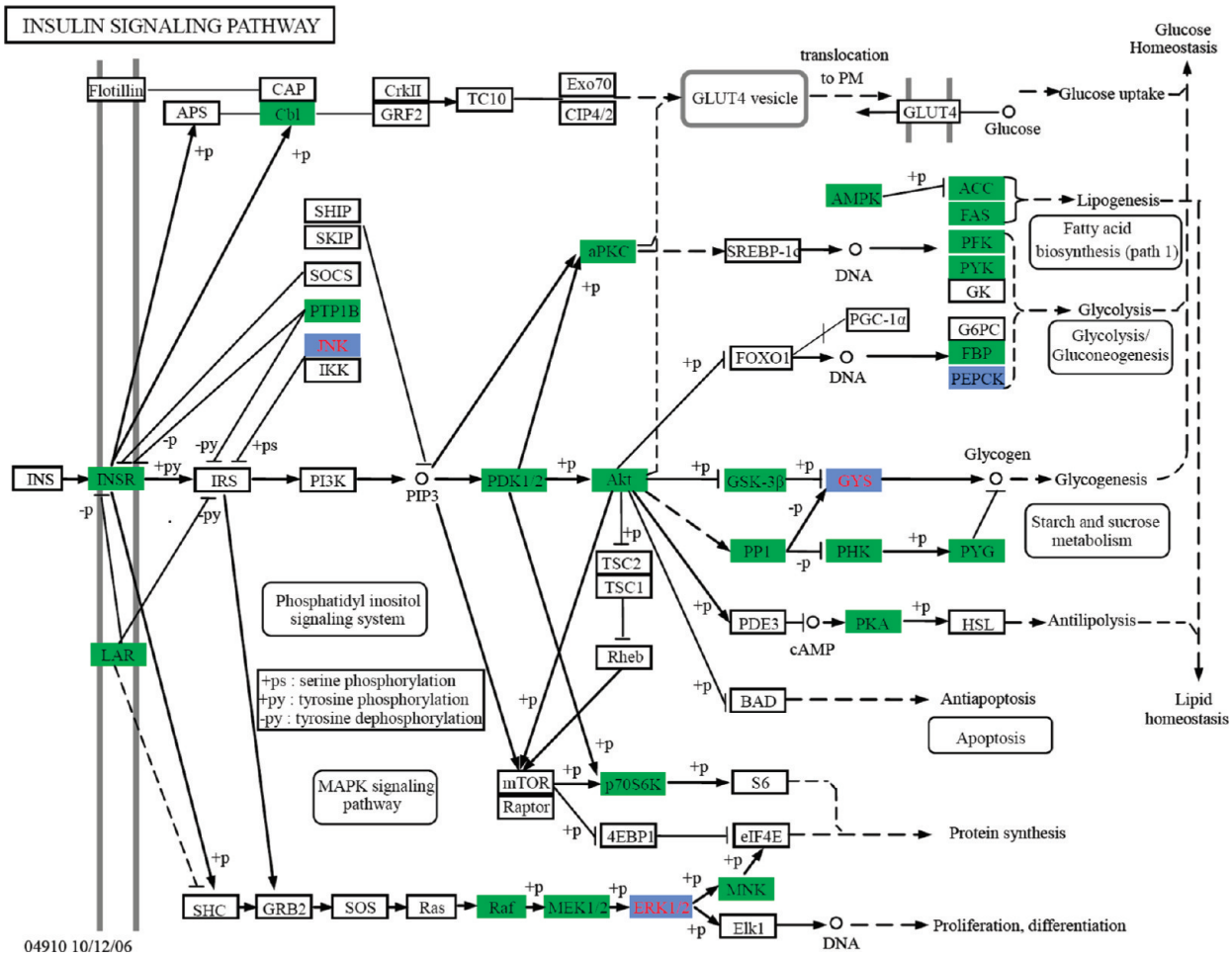

Figure 8. Insulin signaling pathway from KEGG database. The figure was manually edited. Highlighted in green are the common enzymes in Br, SG, and PG and also the enzymes of SG in this pathway. Words highlighted in red and panes highlighted in blue show the specific enzymes in Br and PG compared with the SG, respectively.

those with at least three ECs were acceptable for further analysis. The results showed that the identified proteins of Br, SG, and PG were involved in 169, 164, and 171 pathways, respectively, and among them, 162 pathways were the same including 20 signaling pathways such as insulin signaling (KEGG map04910), GnRH signaling (KEGG map04912), Wnt signaling (KEGG map04310), and MAPK signaling (KEGG map04010). However, the enzymes involved in each pathway in different organs were not completely the same (Figure 6). In addition to the typical pathways such as insect hormone biosynthesis (KEGG map00981) and apoptosis (KEGG map04210), there were several interesting common-related pathways such as Dorso-ventral axis formation (KEGG map04320), androgen and estrogen metabolism (KEGG map00150) worthy of further validation. The common pathways were ascribed to metabolism, genetic information processing, environmental information processing, cellular processes, and human diseases (Figure 7). More than a half of these pathways were involved in metabolism. Among them, the carbohydrate metabolism, amino acid metabolism and lipid metabolism were more than the other which appeared consistent with the results of GO analysis. On the other hand, the specific-expressed proteins related pathways displayed more differences. In this study, 70, 57, and 114 organ-specific enzymes related pathways were detected for Br, SG, and PG, respectively. These showed much greater differences of PG compared with Br and SG.

Insulin Signaling Pathway. Studies have shown that insulin is produced quantitatively by neurosecretory cells of the Br in response to feeding, and levels of circulating insulin produced by these cells quantitatively induce organ growth.[84,85] Satake et al.[86] proved that insect insulin-related peptide bombyxin was involved in the control of carbohydrate metabolism. In the present study, several bombyxins were detected specifically in the Br. However, more than 20 enzymes in Br, SG, and PG were involved in the insulin signaling pathway (Figure 8), such as Bmb022142 (homologous to O97137_MANSE Fasciclin II transmembrane isoform, EC 2.7.10.1, INSR), conventional protein kinase C (EC 2.7.11.13, aPKC), and Bmb030784 (homologous to glycogen phosphorylase, EC 2.4.1.1, PYG). This implied that the insulin signaling might be active in the three organs, although the involved enzymes in Br, SG, and PG had a few differences (Figure 8). The insulin signaling pathway proteins are highly conserved in insects such as *Drosophila*, where it has been extensively studied in recent years and shown to regulate a variety of fundamental processes, such as metabolism, growth, reproduction and longevity.[87] Tu et al.[88] have documented that the insulin signaling may affect JH synthesis through control of JH regulatory neuropeptides. Alternatively, it controls body and organ size of insect by signaling nutritional conditions to the growing organs.[89] After critical weight is attained, larvae stop feeding, leave the food, and search for a pupation site.

Conclusions

All aspects of an insect's life are almost associated with its endocrine system.[90] The present study provided a reliable catalogue and a complete analysis of the proteome expression profiles of the insect endocrine organs. We have shown how the results from multiple engines applied to the same data set can be used together to derive a greater number of protein identifications for the silkworm of which the protein database is still far from fully developed. On the list of identified proteins, many functionally important proteins were for the first time found in Br, SG, and PG of silkworm that may go beyond these organs' traditionally known biological functions. The inconsistency between transcriptome and proteome profiles brings on the necessary of proteomics research. For a better understanding of differential expression of the proteome among the studied organs, the quantitative proteomics approaches will be undertaken as a future research. However, the present data also hold promise for further assessment of endocrine system using a broad spectrum of approaches such as applying RNAi or antagonist for expressional interruption of the key genes in biological pathways in order to better understand their functions and relations.

Abbreviations: Br, brain; SG, subesophageal ganglion; PG, prothoracic glands; LC, liquid chromatography; MS, mass spectrometry; LC-MS/MS, liquid chromatography tandem mass spectrometry; TPP, Trans-Proteomic Pipeline; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; EC, Enzyme Commission; PTTH, prothoracicotropic hormone; DH, diapause hormone; PBAN, pheromone biosynthesis-activating neuropeptide; MW, molecular weight.

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Supporting Information Available: Tables of primers for qRT-PCR, detailed information about the total identified proteins and peptides in Br, SG, and PG, theoretical 2-D distributions of the identified proteins, a part of annotated organ-specific and common-expressed proteins, and details of the WEGO category results; figures of false positive error rates of the identifications. This material is available free of charge via the Internet at http://pubs.acs.org.

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