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Comparative Proteomic Analysis of Midgut Proteins From Male and Female *Bombyx mori* (Lepidoptera: Bombycidae)

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ABSTRACT. Many biological phenotypes of male and female silkworms (*Bombyx mori*) are quite different, and one of the major differences is the growth rate at various larval stages. Nutrient utilization by midgut varies with sexes. However, the molecular basis of this variation is not clear. To understand the molecular mechanism, comparative proteomic approach was employed to investigate the variation of midgut proteomes between male and female silkworms. Totally, 32 proteins that were grouped into four categories were differentially expressed and subsequently identified by mass spectrometry. Gene ontology analysis revealed that these proteins were attributed with biological functions such as binding, catalytic, and transporter, and these proteins were involved in biological process such as cellular process, localization, and metabolic process. Kyoto Encyclopedia of Genes and Genomes pathway analysis revealed that these proteins were involved in pathways such as glycolysis, gluconeogenesis, oxidative phosphorylation, and purine metabolism. At transcription level, the expressional variation was confirmed for six identified proteins including muscle glycogen phosphorylase, uridine 5'-monophosphate synthase, cone cGMP-specific 3',5'-cyclic phosphodiesterase subunit alpha, ATP synthase, thiol peroxiredoxin, and serpin-2. This study provides useful information for understanding the mechanisms of nutrient absorption and the protein–protein interaction in the silkworm.

Key Words: silkworm, male, female, proteomics, mass spectrometry

The silkworm, *Bombyx mori,* has been domesticated for >5,000 yr. It is a major economic resource for 30 million families from China, India, Vietnam, and Thailand. In addition, *B. mori* is a model organism for Lepidoptera insects, many of which are major pests in agriculture and forestry. Moreover, the silkworm has been developed as a bioreactor for the production of recombinant proteins (Tamura et al. 2000, Tomita et al. 2003), including some important biomolecules (Xia et al. 2009).

There are a number of biological variations between male and female silkworms, such as the quality of silk from male is better than that from female, and male larvae grow faster than female larvae at fourth and fifth instar (Shi 2011), which might be attributed to the variation of nutrient utilization by the midgut between male and female. The phenomenon that male and female grow in a different way also exists in other insects or animals, such as mosquito (Lounibos et al. 1996), butterfly (Wiklund et al. 1991, Nylin 1992, Nylin et al. 1993), or spider (Gunnarsson and Johnsson 1990). To date, there are some studies on male–female differences at molecular level. Miyagawa et al. (2005) reported that *BmAHA1* gene prominently expressed in testes of male silkworm, whereas only low expression was detected in ovary of female at day 3 of fifth instar, suggesting that *BmAHA1* protein plays a role in silkworm spermatogenesis, especially in postmeiotic differentiation. In addition, *Bmnwk* gene expressed at higher level in brain of male silkworm than in brain of female, which indicates that *Bmnwk* may be involved in the development and maintenance of the optic lobe in the brain of male silkworm (Kiya and Iwami 2011). On the other hand, Tojo et al. (1980) reported that storage proteins 1 and 2 account for 60% of total fat body proteins in females, whereas in males, they account for only 20%. Similarly, the level of vitellogenin was much higher in females than in males (Mine et al. 1983).

In this study, to investigate the mechanism underlying variation of nutrient utilization in the midgut of silkworm and difference in growth rate between male and female silkworms, we used comparative proteomic approach to detect differentially expressed midgut proteins between two sexes on the second day of fifth instar. Fifth instar stage is a transition period for larva–pupa metamorphosis and a critical period for larval development and silk spinning (Grzelak 1995). Because most of materials, energy, and nutrients used for maintaining silkworm life are from the midgut, we focused on this tissue in this study. Totally, 32 midgut proteins were identified to be differentially expressed between two sexes, and the biological processes and molecular functions these proteins may be involved in were summarized. In addition, the possible roles of some of these critical proteins were discussed.

Materials and Methods

Experimental Animals. The silkworm strain NB was used for this study. Larvae were reared on fresh mulberry leaves from hatching to spinning at 25 ± 1°C and with 75 ± 2% relative humidity. On the first day of fifth instar, female and male silkworms were separated after 3-h feeding of mulberry leaves. Two days later, male and female silkworms were dissected to isolate the midgut. The midgut was washed with 0.75% ice-cold physiological salt solution to avoid mulberry protein contamination. The midgut was immediately frozen in liquid nitrogen and stored at −80°C for later use.

Protein Preparation. The silkworm midgut was grounded in liquid nitrogen, and proteins were extracted using an extraction buffer containing 20 mM Tris-HCl, pH 7.5, 250 mM sucrose, 10 mM EDTA, 1 mM PMSF, 1 mM β-mercaptoethanol, and 1% (v/v) Triton X-100, as described before (Cilia et al. 2009). Briefly, the mixture was vortexed for 30 min and centrifuged. The supernatant was collected, and Tris-saturated phenol was added to precipitate the proteins. The phenol layer containing proteins was collected, incubated with methanol solution (containing ammonium acetate), and centrifuged to pellet the proteins. Subsequently, the protein pellet was washed with methanol acetone [containing dithiothreitol (DTT)], lyophilized, dissolved in solution containing 7 M urea, 2 M thiourea, 4% (w/v) chaps, and 1% (w/v) DTT and centrifuged. The supernatant, which contained total midgut proteins, was collected and stored at −80°C for use. The protein concentration was determined using RC DC Kit (Bio-Rad, USA).

Two-Dimensional Electrophoresis (2-DE). The 2-DE was performed with 17 cm (linear, pH 5–8) IPG gel strip (Bio-Rad, USA) according to previous study (Kim et al. 2007). One thousand two hundred micrograms of total midgut proteins were loaded onto IPG strip using active rehydration (13 h with 50 V), and the isoelectric focusing was performed at 17°C with a voltage gradient of 250 V for 0.5 h, 1,000 V for 1 h, 10,000 V for 5 h, then continued for a total of 60 kVh. The focused strip was equilibrated for 15 min with equilibration solution [6 M urea, 0.375 M Tris-HCl, 20% (v/v) glycerol, 2% (w/v) SDS] containing 2% (w/v) DTT and then equilibrated for another 15 min with equilibration solution containing 2.5% (w/v) iodoacetamide. Equilibrated strip was then sealed on the top of 12% SDS-PAGE gel for electrophoresis. The gel was visualized with 0.1% Coomassie brilliant blue R-250 and scanned with ScanMaker 9700XL (Microtek, Taiwan) at a resolution of 600 dpi. Spot analysis was performed using PDQuest (version 8.0.1, Bio-Rad, USA). Triplicate experiments were carried out for each sample. The intensity ratios of protein spots in different gels were calculated, and the spots with intensity ratios of ≥ 2 or ≤ 0.5 were defined as quantitatively different spots.

In-Gel Digestion and Mass Spectrometry (MS). In-gel digestion and MS were preformed as described before (Liang et al. 2007). Protein spots were excised from gel, washed with water, destained by sonication in 25 mM ammonium bicarbonate, 25% acetonitrile, dehydrated with acetonitrile, and dried in vacuum. The dried protein spots were treated by 10 mM DTT for 1 h at 56°C, alkylated with 40 mM iodoacetamide for 45 min at room temperature, washed with 25 mM ammonium bicarbonate, dehydrated with acetonitrile, and then incubated with 3 μl trypsin solution (20 μg/ml) at 37°C for overnight to completely digest the proteins.

The digested proteins were collected and mixed with 10 mg/ml matrix (a-cyano-4-hydroxycinnamic acid, Sigma, USA) dissolved in 50% acetonitrile containing 0.1% trifluoroacetic acid. The mixture was analyzed with matrix-assisted laser desorption/ionization-time of light MS (MALDI-TOF MS) (Bruker, Germany). Standard peptide from the manufacturer was used as external standard for calibration, and the peptide ions generated by autolysis of trypsin were used as internal standards.

Protein Identification. MS data were analyzed using MASCOT (Matrix Science, London, United Kingdom) and NCBInr eukaryotic protein sequence database. The parameters were set as following: missed cleavages was 1, fixed modification was acetylation of carbamidomethyl (C), variable modification was oxidation of methionine (M), mass tolerance was 0.3 Da, and mass value was MiH^+. A protein with a minimum ion score of 79 ($P < 0.05$) was considered to be reliably identified (Zhou et al. 2008).

Gene Ontology (GO) Analysis. The GO analysis was carried out according to the method described before (Ye et al. 2006). The sequences of identified proteins were queried against GO Database (OBO v1.2 format: http://www.geneontology.org/GO.downloads.ontology.shtml) to obtain the GO plots.

Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis. The query FASTA protein sequences were used to search against KEGG GENES (http://blast.genome.jp/) using BLASTP program with BLOUSM62 scoring matrix. If enzyme commission (EC) number was available, the best matched protein with E value $\leq$ e-15 was accepted and exported. The EC numbers from each dataset were used for KEGG pathway search (http://www.genome.jp/kegg/tool/search_pathway.html). Each selected pathway contains at least three EC numbers (Zhang et al. 2007).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Total mRNA was extracted from midguts with RNeasy Mini Kit (Qiagen, Germany). The RNA sample was digested with RNase-free DNase I at 37°C for 20 min to remove contaminated DNA. Subsequently, RNA was further purified with phenol–chloroform and precipitated with ethanol. The RNA precipitate was dissolved in DEPC-treated ddH_2O and M-MLV RTase (TaKaRa, Japan), and oligo-dT were used to synthesize cDNAs, following the manufacturer's instructions.

qRT-PCR was carried out on Bio-Rad CFX384 real-time system (Bio-Rad) and SYBR Premix Extag (TaKaRa, Japan) with SYBR Green I as a fluorescent dye. The primers were designed with Primer 5.0 software and are listed in Table 1. The mRNA of the housekeeping gene, *B. mori* silkworm translation initiation factor (*BmTIF*) was used as inner standard. The PCR amplification condition was 95°C for 30 s, followed by 40 cycles of 95°C for 10 s, 55°C for 30 s, 72°C for 30 s, and finally hold at 4°C. Three parallel experiments were performed. The result was expressed as ratio on *BmTIF* (inner standard) mRNA, and the ratio of detected gene over *BmTIF* in male silkworm was arbitrarily set as 1 (Canbay et al. 2003). The statistic significance of difference was analyzed with one sample *t*-test.

Results

Identification of Differentially Expressed Proteins. Our preliminary studies showed that 1,200 μg of midgut proteins could provide good resolution and reproducibility. PDQuest (Bio-Rad) was used to analyze protein spots stained by Coomassie brilliant blue on 2D gels. Triplicate experiments were performed for each midgut protein extract. The results showed that the 2-DE gel images were reproducible (Fig. 1, Supp Figs. 1–4 [online only]).

As shown in Fig. 1, the protein expression patterns of the midgut were different between male and female larvae. In total, 566 ± 13 protein spots were detected in the male midgut sample, and 547 ± 18 protein spots were detected in female midgut sample. The pI of most proteins was between 5.3 and 7.5, and the molecular mass of most proteins was between 20 and 100 kDa. It was found that 32 midgut proteins expressed differentially between male and female larvae (Fig. 1). Enlarged 2-DE image and spot volume analysis using PDQuest confirmed that the expression of these proteins was significantly different

Table 1. Sequences of the primers used for qRT-PCR in this study

Primer name	Primer sequence (5′→3′)	Length (bp)	T_m (°C)[a]	Product length[b] (bp)
4[c]-F[d]	GACTTTGTTGTGAGGCTGAA	20	54.1	165
4-R[e]	ACCGGGTAAAGAATGAACTG	20	54.4	
5-F	GCAAACCCATCGACAAGG	18	56.5	154
5-R	TGACCACGGGCAATAGAG	18	54.3	
7-F	TTCTTTGCGACGTAACCG	18	55.3	298
7-R	GTGAACCTGCCCATTTGA	18	54.0	
10-F	TCCCGATCTTGACTATGCC	19	55.4	242
10-R	GTCCTTGCCGATAGCGTA	18	54.2	
11-F	GACTACGGAGTGCTGGACG	19	55.9	225
11-R	CTTGGTGTCGGGCTTGAT	18	56.0	
12-F	CGCACCTTGCTGCTTTCA	18	58.7	256
12-R	TTGGCATTGGACTCTTGGAT	20	57.7	
15-F	GGTTTCACAAGCAAAGGGTT	20	56.8	235
15-R	GACAGCCGGTCAAGAGC	18	55.8	
17-F	CCACCACTGCCTATTCCC	18	55.4	153
17-R	TGCCGCATACTTGTCCAT	18	54.9	
22-F	GCCAAGTTATAGCTGAGGTCC	21	56.1	241
22-R	GAAGCATACAGATCACCGAAA	21	55.4	
24-F	TCATCGCCATTGTCAGCA	18	56.5	244
24-R	CCTCGACAGCGTCCTTCT	18	55.4	
29-F	TGGTGGCCTCACTGGACT	18	55.9	165
29-R	TTCGGGTCAGCAGCATAT	18	54.1	
32-F	CCCCATCTTGTCGGGTAA	18	56.0	168
32-R	GCGGCTTCATCACAGTTCTC	20	57.9	
BmTIF-F	TCGGACCAGCTCTGTCTCTC	20	57.2	201
BmTIF-R	CAAAGCACCAAATCTTACGG	20	56.1	

The NCBI number of each protein was used for search in NCBI database, and the resultant cDNA sequences were used to design the primer with Primer 5.0 software.

[a]T_m value was calculated by primer 5.0 software.
[b]The length of products in qRT-PCR.
[c]Spot number.
[d]Forward primer.
[e]Reverse primer.

(Fig. 2). These proteins were excised from 2-DE gels and subjected to in-gel trypsin digestion and subsequently to MALDI-TOF MS analysis. The detailed information of these proteins was listed in Table 2. These proteins could be grouped into four categories: expressed only in male larvae (EOM), expressed only in female larvae (EOF), expressed at a higher level in male larvae (EHM), and expressed at a higher level in

female larvae (EHF). Among these 32 proteins, 5 proteins (no. 1–5) belong to the EOM group, including muscle glycogen phosphorylase, isocitrate dehydrogenase, etc. Three proteins (no. 6–8) belong to the EOF group, including CoA-substrate-specific enzyme activase, ef1 alpha-like factor isoform 1, and a hypothetical protein. Seven proteins (no. 9–15) belong to the EHM group, including cone cGMP-specific
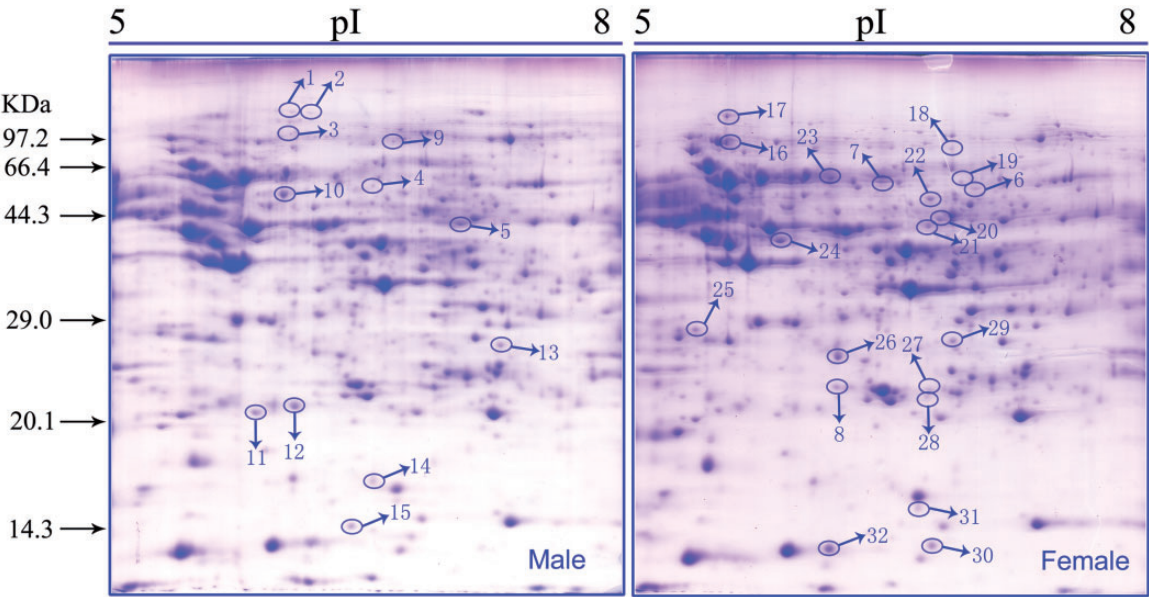

Fig. 1. 2-DE of midgut proteins from male and female *B. mori*. Midgut proteins were applied to isoelectric focusing and SDS-PAGE. The differentially expressed proteins were indicated by arrows and numerically labeled. 1–5, proteins only expressed in males; 6–8, proteins only expressed in females; 9–15, proteins expressed higher in males than females; 16–32, proteins expressed higher in females than males.
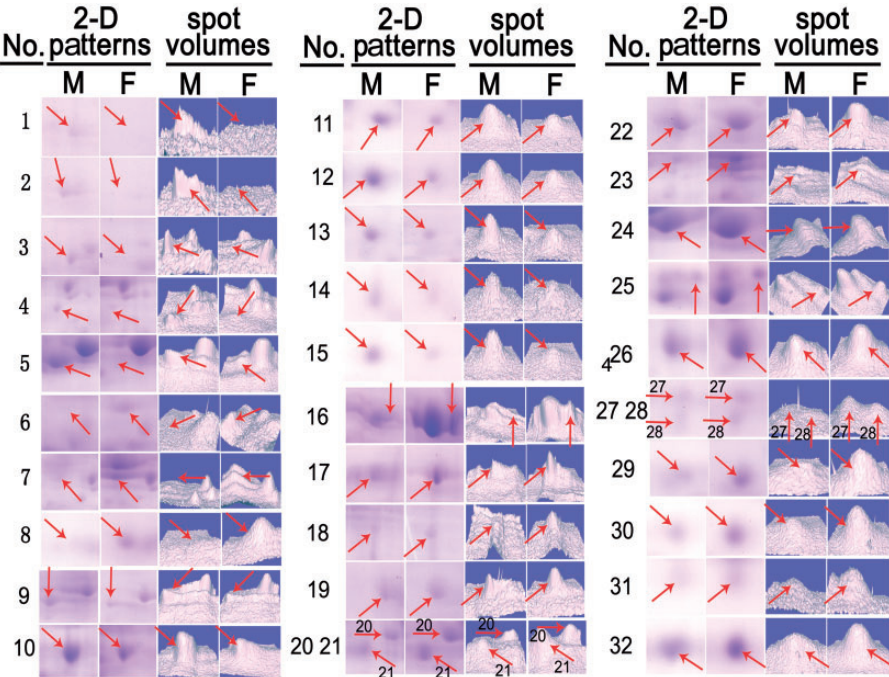

Fig. 2. Spot volume analysis of differentially expressed proteins. Protein spots and its volumes were indicated by arrows. The gel images were shown on the left panel, and the spot volumes (produced with PDQuest) were shown on the right panel. No., spot numbers; M, male; F, female; 2-DE patterns, zoomed in view of protein spots; spot volumes, volumes of proteins spots. 1–5, proteins only expressed in males; 6–8, proteins only expressed in females; 9–15, proteins expressed higher in males than females; 16–32, proteins expressed higher in females than males.

Table 2. List of the proteins differentially expressed between male and female of *B. mori*

No.	NCBI no.[a]	Protein name	pI[b]	MW[c]	AA[d]	Seq Cov[e]	Score	Ratio,[f] male:female
EOM								
1	gi|168052134	Predicted protein	9.84	42,218	375	15	91	/
2	gi|182509200	Muscle glycogen phosphorylase	5.73	96,963	841	30	110	/
3	gi|114051866	Isocitrate dehydrogenase	6.24	46,546	408	39	129	/
4	gi|114050831	Uridine 5′-monophosphate synthase	7.52	53,433	473	12	86	/
5	gi|190407685	Vacuolar ATPase B subunit	5.32	55,156	494	49	101	/
EOF								
6	gi|312792435	coA-substrate-specific enzyme activase	8.23	62,020	539	31	108	/
7	gi|114053033	ef1 alpha-like factor isoform 1	5.16	66,510	603	13	86	/
8	gi|209882377	Hypothetical protein	9.52	23,131	199	47	83	/
EHM								
9	gi|116581	Cone cGMP-specific 3′,5′-cyclic phosphodiesterase subunit alpha′	5.54	99,361	855	16	99	2.34 ± 0.493
10	gi|148298717	Vacuolar ATP synthase subunit B	5.25	54,667	490	46	184	2.03 ± 0.163
11	gi|112982996	Thiol peroxiredoxin	6.09	22,073	195	33	79	2.18 ± 0.146
12	gi|153791739	H$^+$ transporting ATP synthase subunit d	5.56	20,190	179	50	98	2.03 ± 0.211
13	gi|168058792	Predicted protein	11.41	24,439	208	24	92	2.14 ± 0.113
14	gi|153792659	Actin-depolymerizing factor 1	6.17	17,227	148	45	80	2.17 ± 0.378
15	gi|153791339	mRNA transport regulator 3	6.11	30,533	268	23	92	4.46 ± 0.877
EHF								
16	gi|295885513	Heat shock protein 90	5.01	82,598	716	21	84	0.43 ± 0.055
17	gi|195381127	GJ20828	6.85	135,615	1,182	16	86	0.41 ± 0.027
18	gi|116256119	Wall-associated receptor kinase-like 4	5.66	86,269	761	10	79	0.41 ± 0.046
19	gi|312597598	Inorganic pyrophosphatase	4.96	32,220	288	23	85	0.43 ± 0.047
20	gi|67782283	Actin 5	5.3	42,194	376	51	127	0.50 ± 0.037
21	gi|303388409	Serine palmitoyltransferase subunit 1	8.54	48,087	422	33	85	0.44 ± 0.057
22	gi|114052408	Mitochondrial aldehyde dehydrogenase	7.52	56,214	513	24	96	0.43 ± 0.047
23	gi|145546011	Hypothetical protein	5.09	58,988	494	13	93	0.18 ± 0.026
24	gi|7341329	Serpin-2	4.87	41,750	374	21	94	0.41 ± 0.029
25	gi|162952017	Annexin IX isoform C	5	36,068	323	44	103	0.41 ± 0.019
26	gi|187281708	Triosephosphate isomerase	5.67	26,933	248	61	83	0.42 ± 0.037
27	gi|151301141	Proteasome beta subunit	5.97	25,968	232	59	97	0.45 ± 0.069
28	gi|14286164	Regulator of chromosome condensation	8.14	59,441	547	22	79	0.34 ± 0.043
29	gi|112982822	Phosphoglyceromutase	6.33	28,661	255	41	83	0.30 ± 0.044
30	gi|145348862	Predicted protein	5.64	16,400	145	62	80	0.46 ± 0.059
31	gi|164448664	Antennal binding protein	6.71	15,822	140	35	81	0.31 ± 0.048
32	gi|112982671	Ribosomal protein S12	5.79	15,367	139	58	93	0.45 ± 0.024

The proteins were identified by MALDI-TOF MS.
[a] NCBI accession number.
[b] Isoelectric point.
[c] Molecular mass.
[d] Amino acid number.
[e] Sequence coveraged.
[f] Ratio: the quantifying expression ratio of male *B. mori* to female. The values are mean ± SD of triplicates. Because the expression ratios of qualitatively different proteins could not be calculated (marked with "/"), only those quantitatively different proteins were analyzed.

3′,5′-cyclic phosphodiesterase subunit alpha, vacuolar ATP synthase subunit B, and thiol peroxiredoxin. The EHF group contains 17 proteins (no. 16–32), including heat shock protein 90, GJ20828, wall-associated receptor kinase-like 4, etc. (Table 2).

GO Analysis. GO analysis is commonly used in proteomic study to explore the physiological roles of numerous proteins identified by 2-DE and MS. The protein sequences were queried against the InterPro database, and the resultant proteins were analyzed in terms of biological process, cellular component, and molecular function. Most proteins are annotated with at least one GO term, except spots 1 and 8 (predicted protein and hypothetical protein, Table 2).

To evaluate which GO term was more pervasive in males or females of the silkworm, the differentially expressed proteins were divided into two groups: 1) more in males and 2) more in females. As shown in Fig. 3, "more in males" proteins were mainly distributed in cell, cell part, macromolecular complex, and organelle. "More in females" proteins had a similar location profile. However, the percentage of each GO term in "more in females" was lower than that of "more in males" proteins. In addition, there were "more in males" proteins located in envelope and organelle part, whereas no "more in females" proteins were located in this category. For molecular function, a major part of both "more in males" and "more in females" proteins were distributed

to binding and catalytic categories. On the other hand, some of "more in males" proteins belonged to transporter, and some of "more in females" proteins were distributed to enzyme regulator and translation regulator. For biological process, a major part of both "more in males" and "more in females" proteins were involved in cellular process and metabolic process. Some of the "more in males" proteins were specifically involved in biological regulation, establishment localization, and localization, whereas some of the "more in female" proteins specifically involved in cellular component organization and response to stimulus.

KEGG Pathway Analysis. KEGG pathway analysis is widely used to characterize genes and proteins and illustrate the connection between the genome (DNA) and functions (protein) categorized in PATHWAY database (Kanehisa and Goto 2000). The sequences of differentially expressed proteins were used to search KEGG GENES using BLASTP program. The resultant enzymes or factors for proteins were queried against the KEGG reference pathway database. Only those pathways that at least three enzymes are involved were accepted for further analysis. Among the 32 identified proteins, 16 met the above-mentioned standards. Although some of the proteins participated in more than one pathway, these identified proteins were involved in 20 pathways (Fig. 4).The protein number corresponding to each of the pathways was shown on y-axis. For the "more in males" proteins, their numbers were
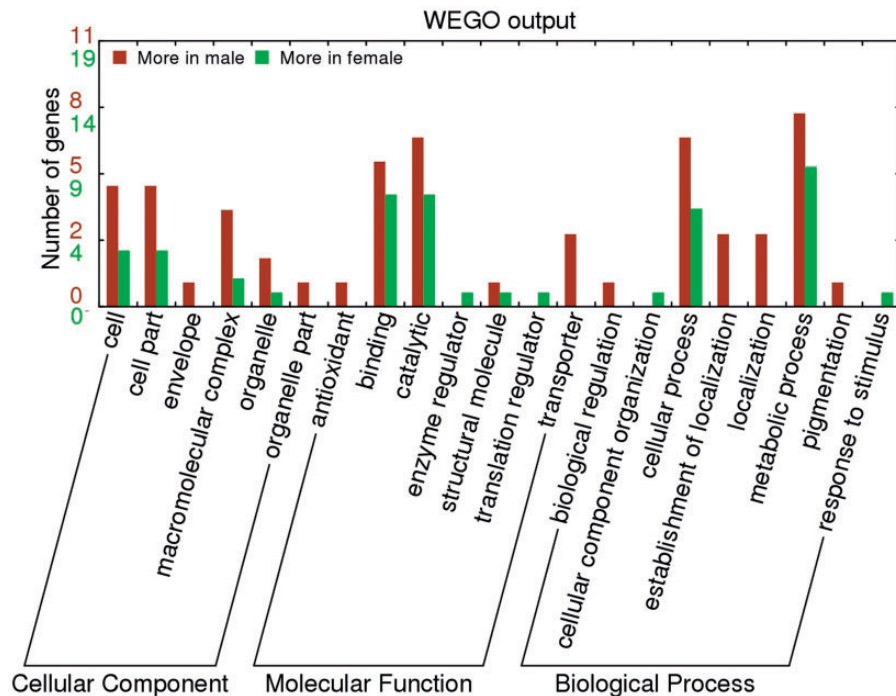
Fig. 3. GO analysis. Proteins differentially expressed between male and female *B. mori* were divided into two groups: more in males, including the proteins that were male-specific and expressed higher in males; more in females, including the proteins that were female-specific and expressed higher in females. The GO terms of differentially expressed proteins were classified in cellular component, molecular function, and biological process. The right axis indicates the number of proteins for each GO annotation, and the left one represents the proportion of proteins for every GO annotation.

defined as positive numbers, and for those "more in females" proteins, their numbers were defined as negative numbers. The "more in males" proteins participated in many metabolism pathways, including starch and sucrose metabolism, synaptic vesicle cycle, oxidative phosphorylation, pyrimidine metabolism, purine metabolism, and drug metabolism-other enzymes. The "more in females" proteins participated in other metabolism pathways, such as arginine and proline metabolism, glycolysis/gluconeogenesis, etc. Interestingly, some of "more in males" and "more in females" proteins were involved in the same KEGG pathways, such as synaptic vesicle cycle and oxidative phosphorylation.

Validation of Protein Expression by qRT-PCR. qRT-PCR was used to verify the variation of transcript expression of 12 proteins, which were most significantly differentially expressed among the 32 identified proteins. Finally, 6 out of the 12 selected proteins were confirmed by qRT-PCR to have similar expression patterns as the protein profiles, including uridine 5'-monophosphate synthase (spot 4), thiol peroxiredoxin (spot 11), H$^+$ transporting ATP synthase subunit d (spot 12), mRNA transport regulator 3 (spot 15), serpin-2 (spot 24), and phosphoglyceromutase (spot 29). The mRNA levels of the six proteins showed significant differences ($P < 0.01$) between male and female larvae (Fig. 5), which was consistent with the 2-DE results (Fig. 1). The qRT-PCR results of other six transcripts were not consistent with the 2-DE results, suggesting that posttranscriptional regulation (de Groot et al. 2007) or mRNA stability (F.Weaver 2002) might impact the variation of protein expression.

Discussion

In this study, 32 proteins were identified to be differentially expressed in midgut between male and female silkworms. Though spot 5 and 10 proteins (Table 2) had different NCBI accession numbers and different locations on the 2-DE gels, however, protein alignments showed that they were the same protein. That two protein spots were identified as the same protein may be due to that the two proteins were

differently modified (Giavalisco et al. 2005) or partially degraded (Zhou et al. 2008).

Muscle glycogen phosphorylase (spot 2) expressed only in male silkworm (Table 2). In rat, it was found that when a rat was fasted, activity of glycogen phosphorylase decreased. However, when the animal was refed, the glycogen phosphorylase activity was increased again (Maddaiah and Madsen 1966). Based on the relationship between glycogen phosphorylase level and nutrition status, it was proposed that a higher level of glycogen phosphorylase in male silkworm indicates a better nutrition status in the male. In insects, phosphorylase plays a major role in mediating fat body metabolism (Gäde 1990). In addition, Insects store energy reserves in forms of glycogen and triglycerides, and these two components together with lipids are stored in the adipocytes (Kerkut and Gilbert 1986). Glycogen phosphorylase catalyzes the degradation of glycogen to glucose-1-phosphate, which is involved in synthesis of trehalose, the major hemolymph carbohydrate in lepidopteran insects (Bailey 1975). Thus, the specific expression of glycogen phosphorylase in male silkworm may result in more glycogen to convert to glucose-1-phosphate to meet the requirement of vigorous energy metabolism, which modulates the rate of insect growth (Mirth and Riddiford 2007). In addition, the lipids, which are stored together with glycogen in adipocytes, are essential for insect growth (Arrese and Soulages 2010). The higher energy metabolism and lipid level may allow male silkworms to grow faster than females during the fifth instar stage. Boston et al. (1997) found that there is a male-specific leptin-independent pathway for regulation of insulin by proopiomelanocortin neurons. Whether or not the glycogen phosphorylase-mediated pathway is specific for male silkworm needs to be further investigated.

In addition to glycogen metabolism, glycogen phosphorylase was also found to be related to stress. It was reported that the insecticides fenitrothion and ethion significantly elevated glycogen phosphorylase and trehalase in the fat body of fifth-instar silkworm, suggesting that the organophosphorus insecticides provoke an increase in the activity
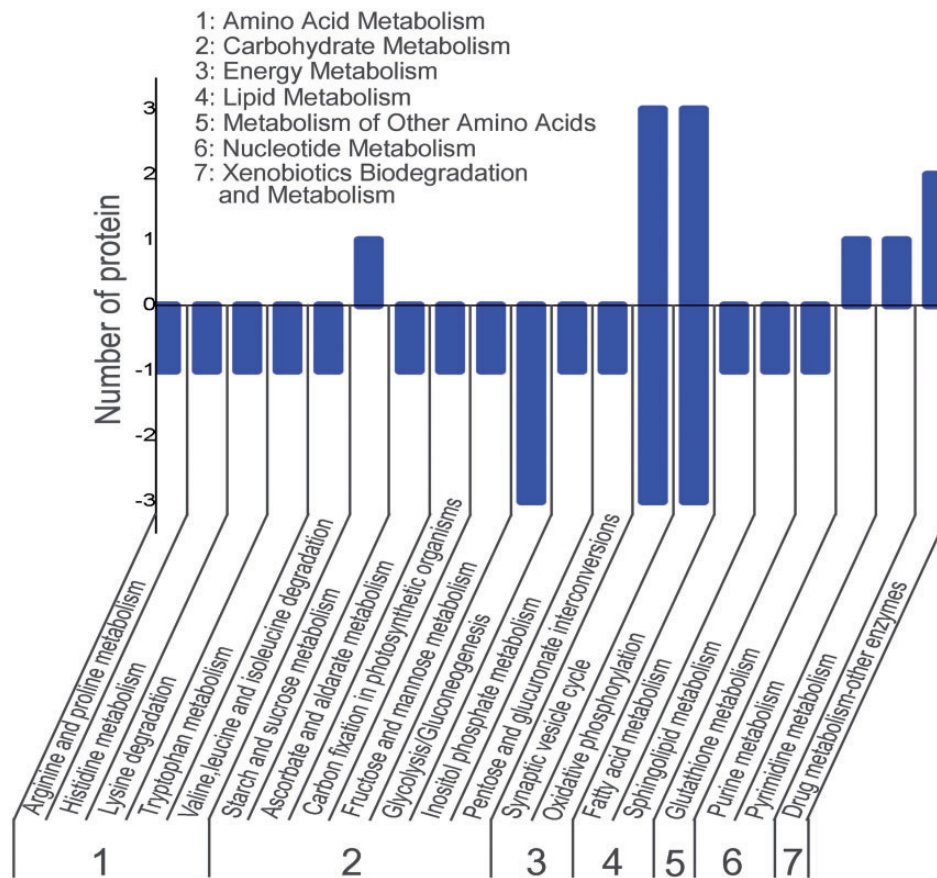
Fig. 4. KEGG pathway analysis. The sequences of differentially expressed proteins were queried against KEGG GENES using BLASTP program and classified into KEGG pathways. KEGG pathways were grouped into seven categories: amino acid metabolism (1), carbohydrate metabolism (2), energy metabolism (3), lipid metabolism (4), metabolism of other amino acids (5), nucleotide metabolism (6), and xenobiotics biodegradation and metabolism (7). For those proteins that were expressed more in males (including qualitative and quantitative different proteins), their numbers were defined as positive numbers. For those expressed more in females, their numbers were defined as negative numbers.
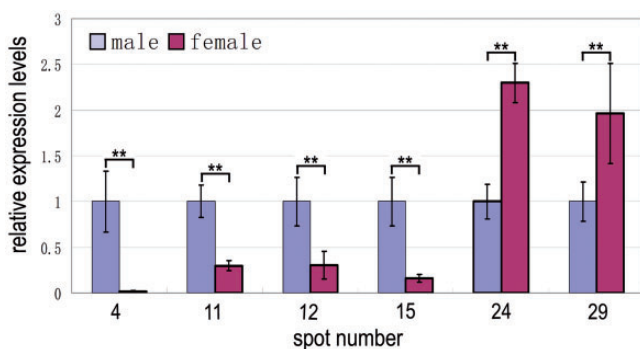

Fig. 5. qRT-PCR verification of the proteins differentially expressed between male and female *B. mori*. Assays were performed using the RNA sample extracted from the same batch of male and female midguts used in the 2-DE study, followed by qRT-PCR quantitation. mRNA of housekeeping gene BmTIF was used for normalization. The result was expressed as ratio of BmTIF (inner standard) mRNA, and the ratio of detected gene/BmTIF in male silkworm was arbitrarily set as 1 (Canbay et al. 2003). x-axis represents spot number, and y-axis represents the relative expression level of each proteins. "**" represents that difference in protein expression between two groups was significant ($P < 0.01$). Data show mean ± SD of three repeat experiments, and the standard deviations were represented by error bars.

of glycogen phosphorylase (Surendra Nath 2002). When silkworm was subjected to insecticide stress, it may need more glycogen to meet higher energy demands to cope with insecticide (Surendra Nath 2002). In Pacific oyster (*Crassostrea gigas*), glycogen was needed for coping with temperature stress or pathogens. When glycogen is not enough, glucose may be used as a substitute (Samain et al. 2007). In our study, glycogen phosphorylase level was higher in male silkworms (Table 2), which may result in a higher glucose level, allowing the male insects more resistant against insecticides and bacterium.

Uridine 5′-monophosphate synthase (spot 4) was detected only in male silkworm (Table 2). It is a critical enzyme in pyrimidine synthesis (Jones 1980, Nasr et al. 1994). Another protein, cone cGMP-specific 3′, 5′-cyclic phosphodiesterase subunit alpha (spot 9), was expressed higher in male insect. It is important for purine biosynthesis (Naciff et al. 2007). These results implied that pyrimidine and purine synthesis probably was more vigorous in male larvae than in females. Because pyrimidine and purine are required for biosynthesis of DNA/RNA, some amino acids, phospholipids and polysaccharides (Santoso and Thornburg 1998), more vigorous pyrimidine, and purine synthesis in male silkworm may result in more vigorous DNA/RNA synthesis and related metabolism and subsequently allowing male larvae to grow faster than females in the fifth instar.

In this study, two ATP synthase subunits, vacuolar ATPase B subunit (spot 5 and 10, they are same protein as mention above) and H⁺ transporting ATP synthase subunit d (spot 12) expressed more highly in male silkworm than female. ATP synthase is a novel family of

ATP-dependent proton pumps in various membrane traffic pathways (Finbow and Harrison 1997, Forgac 1999). This enzyme also functions in acidifying vacuolar compartments in eukaryotic cells (Stevens and Forgac 1997). Acidification of vacuolar compartments plays an important role in a number of cellular processes (Forgac 1989), such as receptor-mediated endocytosis and coupled transport of small molecules (Stevens and Forgac 1997). The higher expression level of ATP synthase in male midgut (Table 2) suggested a higher level of energy and nutrient absorption ability. In human being, enhanced ATP synthesis can enable a person to promote greater muscular hypertrophy by increasing myosin heavy chain expression, possibly due to an increase in myogenic regulatory factors (Buford et al. 2007). It is interesting to explore whether higher ATP synthase level in male silkworm makes it more robust than female.

In silkworm, thiol peroxiredoxin (spot 11) has oxidoreductase activity (Xu et al. 2011). In other species, oxidative stress responsive proteins also protect aerobic organisms against oxidative stress by degradation of peroxides and other substrates (Fujii and Ikeda 2002). The peroxiredoxin family is known to participate in signal transduction and cell proliferation (Wood et al. 2003). This feature can affect the growth of an organism. In addition, peroxiredoxins protect organisms from oxidative stress, which can also influence organisms' growth patterns (Rho et al. 2006). For example, under aerobic conditions, *Saccharomyces cerevisiae* lacking peroxiredoxins has significantly reduced growth rates (Chae et al. 1993). Peroxiredoxin has a high degree of functional homology among different species. For example, human peroxiredoxin can protect *S. cerevisiae* from oxidative stress induced by paraquat (Tien Nguyen-nhu and Knoops 2003). In this study, thiol peroxiredoxin expressed higher in male silkworm (Table 2), this may be another reason for male larvae growing faster than females.

In arthropod, most of serpins can function as serine protease inhibitors (Kanost 1999). In silkworm, serine protease-2 expressed only in midgut tissue and can reduce the infectivity of BmNPV virus (Nakazawa et al. 2004, Qin et al. 2012). In black tiger shrimp *Penaeus monodon* species, serine protease could interact with virus (Sriphaijit et al. 2007). Therefore, serine protease has been considered as an immunity factor and an antivirus protein in insects. In this study, serpin-2 (spot 24) expressed at a higher level in female silkworms than males (Table 2). This possibly results in a lower serine protease activity in female larvae than in males, leading females less robust than males. Serpins are also relevant in tissue growth and nutrition absorption. In locusts, serpins play role in the metabolism of proteins by regulating proteinase activity in protein reserves mobilization, tissue growth and repairing, and nutrient absorption in nutritional stress (Hinks et al. 1993). Whether or not these identified serpins have similar functions in silkworm needs further investigation.

In this study, we compared proteomic profiles of the midgut of two silkworm sexes. The results showed that 32 proteins were qualitatively and quantitatively different between male and female silkworms. The cellular component, molecular function, biological process, and the participated pathways these proteins are involved in were summarized and discussed. This study provides hints of the possible molecular mechanisms underlying the different traits in the midgut between male and female silkworms. Given the complexity of the mechanisms underlying male–female differences, further studies of functional genomic, systematic biology, and protein function analysis are necessary for better understanding the differences between male and female silkworms.

Acknowledgments

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