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Antennal Proteome Comparison of Sexually Mature Drone and Forager Honeybees

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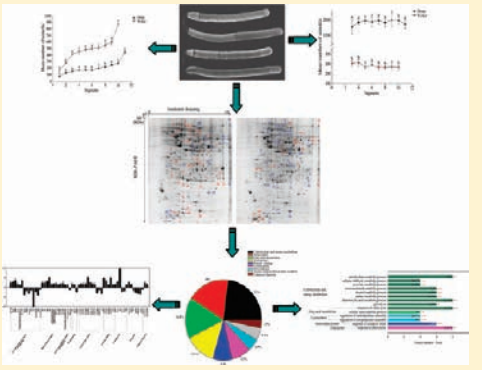
Supporting Information

ABSTRACT: Honeybees have evolved an intricate system of chemical communication to regulate their complex social interactions. Specific proteins involved in odorant detection most likely supported this chemical communication. Odorant reception takes place mainly in the antennae within hairlike structures called olfactory sensilla. Antennal proteomes of sexually mature drone and forager worker bees (an age group of bees assigned to perform field tasks) were compared using two-dimensional electrophoresis, mass spectrometry, quantitative real-time polymerase chain reaction, and bioinformatics. Sixty-one differentially expressed proteins were identified in which 67% were highly upregulated in the drones' antennae whereas only 33% upregulated in the worker bees' antennae. The antennae of the worker bees strongly expressed carbohydrate and energy metabolism and molecular transporters signifying a strong demand for metabolic energy and odorant binding proteins for their foraging activities and other olfactory responses, while proteins related to fatty acid metabolism, antioxidation, and protein folding were strongly upregulated in the drones' antennae as an indication of the importance for the detection and degradation of sex pheromones during queen identification for mating. On the basis of both groups of altered antenna proteins, carbohydrate metabolism and energy production and molecular transporters comprised more than 80% of the functional enrichment analysis and 45% of the constructed biological interaction networks (BIN), respectively. This suggests these two protein families play crucial roles in the antennal olfactory function of sexually mature drone and forager worker bees. Several key node proteins in the BIN were validated at the transcript level. This first global proteomic comparative analysis of antennae reveals sex-biased protein expression in both bees, indicating that odorant response mechanisms are sex-specific because of natural selection for different olfactory functions. To the best of our knowledge, this result further provides extensive insight into the expression of the proteins in the antennae of drone and worker honeybees and adds vital information to the previous findings. It also provides a new angle for future detailed functional analysis of the antennae of the honeybee castes.



KEYWORDS: honeybee, drone, worker, antennae, proteome, sex, olfaction

1. INTRODUCTION

Honeybees live in large eusocial colonies in which a single queen is responsible for reproduction. There are three castes (individual female queen, workers, and male drones) in a colony; the sterile female worker bees perform numerous tasks inside and outside the colony, and several hundred male drones are available for mating.[1,2] To regulate the complex social interactions, honeybees have evolved an intricate system of chemical communication.[3,4] Consequently, specific proteins involved in odorant detection are most likely supporting such diversity.[5]

In honeybees, like other insects, odorant reception takes place mainly in the antennae, within hairlike structures called olfactory sensilla.[5,6] These morphofunctional units enclose the olfactory receptor neurons, which are surrounded by a protein-enriched lymph[7] and accordingly respond to various odor stimuli, including the major component of the queen sex pheromone 9-keto-(*E*)-2-decenoic acid (9-ODA).[8,9] Odors enter through sensillar pores and bind to hydrophilic proteins called odorant binding proteins (OBPs), in the sensillum lymph. These small (<20 kDa), soluble proteins are present at high concentrations in the nasal mucosa of vertebrates[10] and the antennal sensillum lymph of insects.[11] Different investigations of the honeybee antennae have mainly focused on the soluble binding proteins, which belong to two large families of OBPs and chemosensory proteins (CSPs).[12] In the honeybee genome, 21 odorant binding proteins and 6 chemosensory proteins are encoded, but only 11 OBPs and 2 CSPs are expressed specifically in the antennae.[13] Six subclasses of antenna-specific proteins (ASPs), ASP1−ASP6, have been identified.[14,15] In fact, the ASP3 subclass belongs to the CSP family.[16,17] ASP1 is thought to be associated with queen pheromone detection because of the apparent association of its

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general odorant binding proteins (GOBPs) and pheromone binding proteins (PBPs) with neurons sensitive to general odors and to sex pheromone, respectively.[14] Analysis of differentially expressed protein also shows three candidate proteins (D-AP1, W-AP1, and Amwat) are involved in caste and/or sex-specific olfactory processing among the antennae of worker, queen, and drone honeybees.[18]

Although previous findings are sources of insightful information, how the proteome varies between the drone and the adult worker antennae and how they influence the olfactory behaviors of both bees are virtually unknown. On the other hand, sexually mature drones are groups of bees that are highly valuable for the success mating and adult forager workers in collecting important resources from the field. Therefore, the investigation of sensational mechanisms deployed during drone−queen pursue and the worker bee field activities makes vital contributions to the efforts of uncovering the honeybee intricate social organization and further triggers questions about whether there are differences among different race, age, and caste groups. Hence, in this study, we show differentially expressed proteins of sexually mature drone and forger worker antennae using global proteomic analysis and highlight their specific functions in each caste.

2. MATERIALS AND METHODS

2.1. Chemical Reagents

Urea, Tris base, sodium dodecyl sulfate (SDS), sodium bicarbonate (NH_4HCO_3), dithiothreitol (DTT), iodoacetamide, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Biolyte and immobilized pH gradient (IPG) strips were from Bio-Rad (Hercules, CA). Acrylamide, *N*,*N*′-methylenebisacrylamide, ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), glycerol, bromophenol blue, Coomassie Brilliant Blue (CBB) G-250, and α-cyano-4-hydroxycinnamic acid (CHCA) were from Bruker Daltonics (Billerica, MA). Trypsin was from Roche (modified, sequencing grade, Roche, Mannheim, Germany), and trifluoroacetic acid (TFA) and acetonitrile were from J. T. Baker (Phillipsburg, NJ). All the chemicals used for RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR) were from Bio-Rad. Chemicals used but not specified here are noted with their sources in the text.

2.2. Biological Samples

As sexually mature drones are highly valuable for successful mating and adult forager workers for collecting important resources from the field, these age groups were targeted. To ensure the exact age of the sexually mature honeybee drones (*Apis mellifera* L.) to be sampled, we confined the queen bee to a single wax comb frame containing drone cells for 18 h with a cage made of a queen excluder, through which workers but not the queen could pass. Subsequently, the queen was removed, and the workers and the drone eggs contained in the frame were maintained in the honeybee colony for further development. Newly emerged (<12 h; $N > 2000$) drone bees from five colonies were marked on their thoraxes and placed back into the colonies. Then, 1000 marked drone bees were collected on day 15 and their antennae dissected. Similarly, 1000 foraging workers of the correct age (with pollen loaded) were also collected from the same five colonies at the entrance of the hive at 8−10 a.m. and their antennae dissected. Antennae collected from both bees were randomly appropriated to three replicates, with an equal number of antennae in each replicate. Then the antennae were washed with phosphate-buffered saline (PBS) to remove the contaminants on the surface and stored at −80 °C until later use.

2.3. Morphometric Measurements of Antennae

Ultrastructure photographs of the antennae from the drone and the worker bees were taken using a scanning electron microscope (SEM). Antennae were washed with PBS and then sonicated for 15 s to remove contaminates on the surface. The antennae were then dehydrated in an ethanol series (50, 70, 80, and 90% for 30 min and 100% for 30 min twice); after being dried in air, the antennae were placed on stubs and sputter coated with palladium−platinum. Images were processed with a Hitachi S-3400N camera (at 350× magnification, 15 kv). Sensilla were counted on the image of the antennae segment by segment.

2.4. Protein Extraction and Two-Dimensional Gel Electrophoresis (2-DE)

The antennae were homogenized with liquid nitrogen and then mixed in PBS (1 mg/10 μL). The mixture was homogenized for 5 min on ice and sonicated for 2 min and also centrifuged at 12000*g* for 10 min at 4 °C and recentrifuged at 15000*g*. The supernatant was collected and the pellet resuspended in PBS (1 mg/2 μL) and centrifuged at 15000*g* for 10 min at 4 °C; the supernatant was collected and pooled with the first collection. This specimen was termed the PBS-soluble antenna protein extract. The pellet containing PBS-insoluble proteins was mixed in lysis buffer (LB) [8 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris base, 30 mM DTT, and 2% Biolyte (pH 3−10), 1 mg/10 μL]. The lysate was sonicated for 2 min and centrifuged at 15000*g* for 10 min at 4 °C. The supernatant was collected and mixed with PBS-soluble protein extract. Trichloroacetic acid was added to the samples constituting 10% of the final volume of the supernatant. The sample was kept on ice for 10 min to allow protein precipitation and desalting. Subsequently, the sample was centrifuged twice at 15000*g* for 10 min at 4 °C. The supernatant was discarded and the pellet resuspended in LB (1 mg/4 μL). The sample was homogenized for 5 min on ice and sonicated for 2 min. The pH of the samples was adjusted to 7.0 with 2 M NaOH, and the protein concentration was determined using the Bradford method.[19]

The protein sample (550 μg) described above was suspended in 84 μL of LB and mixed with 336 μL of rehydration buffer [8 M urea, 2% CHAPS, 0.001% bromophenol blue, 45 mM DTT, and 0.2% Biolyte (pH 3−10)]. The mixture was loaded onto a 17 cm IPG strip (immobilized pH gradient, pH 3−10, linear, Bio-Rad). Isoelectric focusing (IEF) was performed (Protean IEF Cell, Bio-Rad) at 18 °C according to the following program: 14 h at 50 V, 250 V for 30 min (four times), 1000 V for 60 min, 9000 V for 5 h, 9000 V for 60000 V h.

Prior to SDS−PAGE (sodium dodecyl sulfate−polyacrylamide gel electrophoresis), the IPG strips were first equilibrated for 15 min in equilibration buffer 1 [6 M urea, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, 2% SDS, and 2% DTT] and later in equilibration buffer 2 [6 M urea, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, 2% SDS, and 2.5% iodoacetamide] for 15 min. After equilibration, the strip was transferred to an SDS−polyacrylamide, 12% T separating gel (1.00 mm). Two-dimensional electrophoresis, SDS−PAGE, was performed in a Protean II Xi Cell (Bio-Rad) at 25 mA/gel for 6 h.

2.5. Image Acquisition and Statistical Analysis

Gels were fixed overnight in 50% (v/v) ethanol with 10% (v/v) acetic acid, washed in water, and stained with Flamingo fluorescent dye (Bio-Rad) for image analysis and then further dyed with CBB G-250 to visualize spots for mass spectrometry (MS) analysis. Three independent 2-DE gel images from triplicate samples of each honeybee caste were digitized with Image Scanner III (GE Healthcare) at 16 bit and 300 dpi resolution. Image filtration, background subtraction, spot detection, spot matching, and quantitative intensity (all the pixels making up the spot) analyses were performed using PDQuest (version 8.0.1, Bio-Rad). All gels were matched with one of the selected reference gels. The match analysis was performed in an automatic mode, and further manual editing was performed to correct the mismatched and unmatched spots. The expression level of a given protein spot was expressed in terms of the volume of the spot. To compare spot quantities between gels accurately, we normalized the spot volumes as percentages of the total volume of all of the spots in the gel. The means and standard deviations from the triplicate experiments were calculated, and the statistical significance of the level of expression of the protein and mRNA between the antennae of drone and worker was assessed with one-way ANOVA (SPSS version 16.0, SPSS Inc.); a Duncan's multiple-range test was used to compare the difference between the means of the expression level of the two honeybee castes. A $p < 0.05$ error probability was considered to be statistically significant with respect to at least 1.5-fold changes.

2.6. Trypsin Digestion for MS

The CBB-stained spots were manually excised from each gel of drone and worker samples and destained for 30 min using 100 mL of acetonitrile (50%) and 25 mM NH_4HCO_3 (pH 8, 50%) three to four times until the gels were transparent. The gels were dehydrated for 10 min with acetonitrile (100%) and dried for 30 min using a Speed-Vac system. To prepare the trypsin solution, 2.5 mL of 25 mM NH_4HCO_3 was added to 25 μg of trypsin (final concentration of 10 ng/μL, Roche). The trypsin solution (10 μL) was pipetted onto each dried protein spot, and the dishes were incubated for 60 min at 4 °C. To avoid trypsin autodigestion, the excess trypsin was removed. Then the sample was incubated for 14 h at 37 °C.

To extract the peptide fragments from the tryptic digests, 20 μL of 5% (v/v) TFA was added and incubated for 60 min at 37 °C. Thereafter, 20 μL of 50% (v/v) acetonitrile [containing 2.5% (v/v) TFA] was added to gel pieces and incubated for 60 min at 30 °C. After each step, the supernatants were pooled and dried using a Speed-Vac system.

2.7. Identification of Proteins by MS

The matrix was prepared by dissolving CHCA in a 50% acetonitrile/0.1% trifluoroacetic acid mixture. The solution (10 μL) was added onto the dried digests and vortexed for 30 min. A total of 1.5 μL of the reconstituted in-gel digest sample was spotted initially on an Anchorchip target plate (600/384F, Bruker Daltonics), followed by addition of 1 μL of matrix solution. The dried sample on the target was washed twice with 1 μL of 0.1 mM TFA and left for 30 s before the solvent was removed. Mass spectra were recorded on an Ultra flex MALDI TOF-TOF/MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) instrument (Bruker Daltonics) in reflectron, positive mode with a mass range of 700−4000 Da. Spectra were calibrated by a protonated mass signal from a standard peptide calibration mixture consisting of eight peptides covering a mass range of 700−3100 Da (Bruker Daltonics) for MALDI TOF-TOF/MS.

MS spectra were analyzed with MASCOT distiller (version 2.3.2.0, Matrix Science) using default parameter settings. All known contaminants (trypsin autoproteolysis and known keratin peaks) were excluded during the process. The measured tryptic peptide masses were transferred through MASCOT daemon (version 2.3.0, Matrix Science) as inputs to search against the nonredundant database of the National Center for Biotechnology Information (NCBInr, release date, January 22, 2010) using MASCOT 2.2 (Matrix Science). Search parameters were as follows: taxonomy, *A. mellifera*; trypsin cleavage; allow up to one missed cleavage; peptide mass tolerance, 0.2 Da; fixed modification, carbamidomethyl (C); variable modification, oxidation (M). A total of 10348164 sequences and 3529470745 residues in the database were actually searched.

When the identified peptides matched multiple members of a protein family or a protein appears under the same name and accession number, the match was considered in terms of a higher Mascot score, the putative function, and differential patterns of protein spots on 2-DE gels. Protein identification was accepted when the established probability was greater than 95% and contained at least two identified peptides having maximal peptide coverage.

2.8. Bioinformatics Analysis

The identified proteins were searched against the Uniprot database and combined Flybase search results and grouped on the basis of their biochemical functions. Hierarchical clustering of the expression profiles of the identified proteins was performed by the expression values of protein spots based on the average distance (software calculated) using cluster software (Gene cluster, version 3.0).

The protein−protein interaction of the identified proteins was predicted using Pathway Studio (Ariadne Genomics). Briefly, the protein lists were run against the *Drosophila* database that was equipped with functional relationships from other scientific literature. The filters that we applied included "all shortest paths between selected entities". The information received was narrowed to our proteins of interest. Each link was built on the basis of evidence from at least three publications. Different symbols were assigned by the software to define proteins, i.e., "red elipse" for proteins, "sickle" for kinases, "rhomb" for ligands, "stick" for receptors, "O-vertex" for transcription factors, "2 triangles" for phosphates, "grey elipse" for cell objects, and "orange hexapon" and "yellow rectangle" for the cell process.

The functional enrichment of the identified proteins with respect to specific functional terms was analyzed with CluoGo applied to the *Drosophila* database downloaded from the Gene Ontology database (release date, January 10, 2010).[20] Ontology was selected as a biological process, and the enrichment analysis was conducted by a right-side hypergeometric statistic test. Its probability value was corrected by the bonferroni method. The results were represented visually in a graph.

2.9. qRT-PCR

Total RNA was extracted from both drone and worker antennae using TRIzol reagent (Takara bio). Reverse transcription was performed using an RNA PCR kit (Takara bio), according to the manufacturer's instructions. The analysis of qRT-PCR was conducted on 10 differentially expressed proteins selected from six major functional groups (carbohydrate

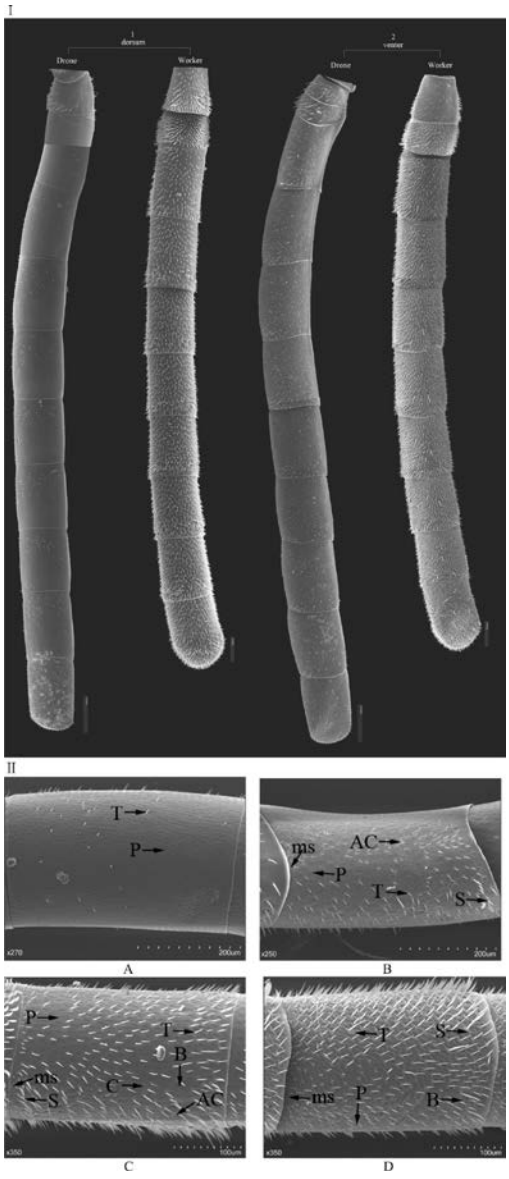

Figure 1. Images of sexually mature honeybee drone and forager worker antennae. Panel I compares whole antennae of the dorsal side and ventral side of sexually mature honeybee drones and forager workers. Panel II shows magnified images of segments: (A and B) dorsal side and ventral side of drone's flagellum, respectively, and (C and D) the dorsal side and ventral side of the worker's flagellum, respectively. The letters in the image indicate the different kinds of sensilla. AC denotes ampullaceal or coeloconic sensilla, B basiconic sensilla, C coelocapitular sensilla, ms margin sensilla, P poreplate sensilla, S seta sensilla, and T trichoid sensilla.

metabolism and energy production, protein folding, transporter, cytoskeleton, fatty acid metabolism, and antioxidation) based on their connectivity in the network. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference (Table S1 of the Supporting Information). Real-time PCR was performed using an iQ5Multicolor Real-Time PCR Detection System (Bio-Rad) in a 25 μL reaction system containing 1 μL of cDNA, 5 pmol of forward and reverse primers, 12.5 μL of SYBR Green Supermix (Bio-Rad), and water. The fold change was calculated using the $2^{-\Delta\Delta Ct}$ method.[21]

3. RESULTS

3.1. Ultrastructure and Protein Expression of Antennae

We targeted sexually mature drone and adult forager workers as they are important for ensuring the continuity of the generation and play field activities for maintenance of the colony, respectively. Accordingly, the difference in the morphology of antennae of the drone and worker bees and the logic behind it at the protein level were compared using SEM and proteomic examination. The SEM results revealed that the surface of the antennae was covered with many sensilla, including hairlike sensilla and poreplate sensilla (Figure 1A,B). More hairlike sensilla and poreplate sensilla were located independently on the dorsal surface and the ventral surface. On the drone antennae, the total poreplate sensilla of ventral and dorsal sides had a significantly larger number (16662), which was almost 7.5 times more than the number for the worker bees (2223) (Figure 2B). In contrast, the forager bees had more hairlike sensilla (9648), more than twice as many as the drones (4489) (Figure 2A).

Furthermore, the 2-DE-resolved proteins from both the drone and the worker antennae showed differential expression (Figure 3). Consequently, more than 400 protein spots were visualized in the antennal proteomic image of the drones and the foragers. A total of 90 protein spots showed differential expression (>1.5-fold change; $p < 0.05$). Among these, 61 protein spots were successfully identified by MS (Table 1 and Figure 3). The remaining proteins were not identified either because of their weak ability to produce enough spectra or because the database search scores were not higher than the 95% required to yield unambiguous results.

3.2. Qualitative and Quantitative Comparisons of Differentially Expressed Proteins

The 61 differentially expressed proteins were classified into nine functional groups: carbohydrate metabolism and energy production (23%), antioxidant activities (18%), fatty acid metabolism (16.4%), molecular transporter (13.1%), protein folding (8.2%), cytoskeleton (8.2%), amino acid and nucleotide metabolism (4.9%), development (4.9%), and unknown function (3.2%) (Figure 4). Proteins associated with carbohydrate metabolism and energy production, antioxidant system, fatty acid metabolism, and molecular transporter contributed to the major proportion of the differentially expressed proteins (Figure 4).

Interestingly, the upregulated protein numbers were 67.2% in the antennae of the drones and 32.8% in the antennae of the worker bees. In particular, the drone overexpressed more proteins in the fatty acid metabolism, antioxidant system, protein folding, metabolism of amino acid and nucleotides, development, and cytoskeleton groups than the worker bees (Figure 5). On the other hand, the worker bees upregulated more proteins related to carbohydrate metabolism and energy production and molecular transport than the drones. Specifically, from the total 61 upregulated protein spots in antennae from both castes, 41 were strongly expressed in the drone and 20 in the workers. Among the 41 proteins upregulated by the drone antennae, the most represented functional groups were those related to fatty acid metabolism (10), followed by antioxidant system (8), metabolism of carbohydrates and energy (6), protein folding (5), cytoskeleton (3), molecular transporter (3), amino acid and nucleotide metabolism (2), development (2), and unknown function (2). Similarly, among the 20 upregulated proteins in the forager bees, the carbohydrate metabolism and energy production group was the most represented (8), followed by

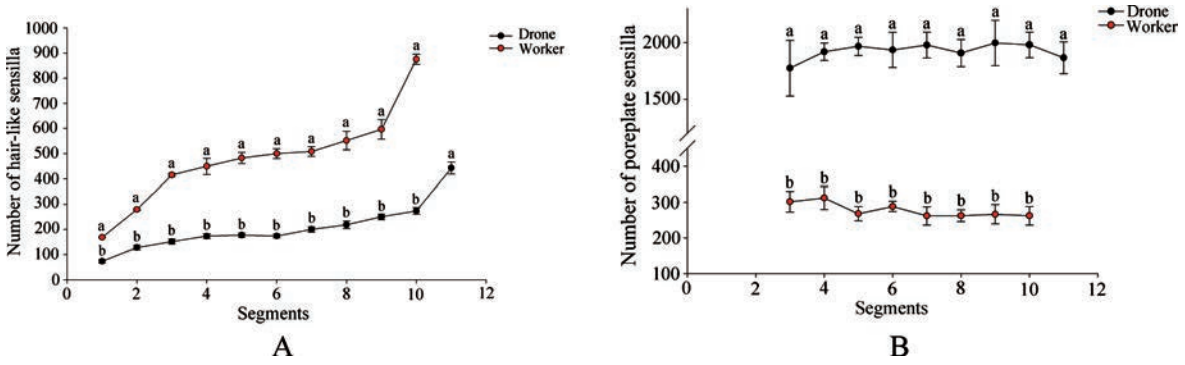

Figure 2. Comparison of the number of sensilla in sexually mature honeybee drones and forager workers. Number of hairlike sensilla (A) and poreplate sensilla (B) from antennae of the two honeybee castes.

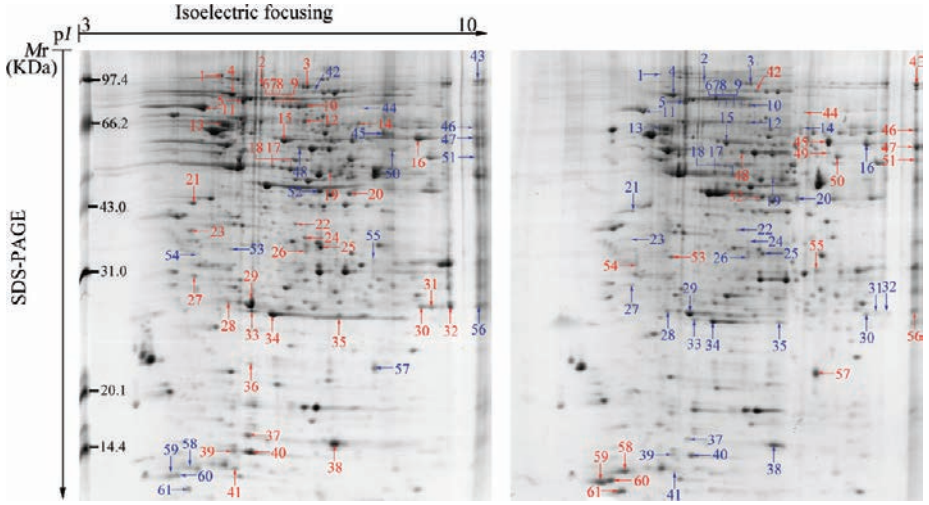

Figure 3. 2-DE profiles of sexually mature honeybee drone and forager worker antennae. Each sample (550 μg) were subjected to 2-DE and stained by CBB G-250. The red and blue labels indicate the proteins that were upregulated and downregulated, respectively.

molecular transporter (5), antioxidation (3), cytoskeleton (2), metabolism of amino acid nucleotides (1), and development (1) groups (Figure 5).

Using the ANOVA log ratio and qualitative protein expression comparisons ($|\log_{1.5}$ ratio$| \geq 1$), protein expression intensity comparisons between the two bee castes were also made. The p values for the differentially expressed proteins were calculated as the ratio of the protein abundance (drones to worker), and only protein spots with p values of ≤ 0.05 were analyzed (Table 1 and Figure 6). In general, the computed results established that all the upregulated proteins in antennae of either of the two castes were strongly expressed (Figure 6). In this view, the number of proteins with a higher level of expression was 41 in the drones as compared with 20 in the workers. However, the level of expression of cytoskeleton proteins (tsr, spot 36) in particular was >10-fold greater in the drones than in the workers. On the other hand, the level of expression of carbohydrate metabolism and energy production, particularly, Ald (spot 49), was >10-fold greater in the workers than in the drones (Figure 6).

3.3. Bioinformatics Analysis

Differentially expressed proteins were mapped to a fixed canonical pathway and biological process (BP) using Pathway Studio. Apparently, 36 proteins identified were linked to the BIN through the shortest path based on diverse linkage relationships like protein−protein interactions, modifications, regulation of expression, etc. Of the 36 proteins linked to the BIN, 10 (28%) proteins were related to the carbohydrate metabolism and energy production pathway; five proteins, ARY (spot 20), Men (spot 12), Aldh (spot 15), scs-fp (spot 10), and malate dehydrogenase (spot 22), were upregulated in the antennae of the drones, and five proteins, vha55 (spot 47), 1(1)G0156 (spot 52), l(1) g0255 (spot 50), l(1) g0030 (kdn, spot 51), and ald (spot 49), were upregulated in the antennae of the workers. Likewise, in the BIN, three (16.6%) proteins belonging to the molecular transporter group, vib (spot 25), obp99a (obp14, spot 37), and obp56a (obp5, spot 41), were upregulated in the drones, while three others, obp56d (obp21, spot 58), porin (spot 55), and obp57c (obp2, spot 59), were upregulated in the workers. Two (11%) proteins related to the cytoskeleton, tsr (spot 36) and tm2 (spot 21), were upregulated in the drones, and two proteins, tm1 (spot 23, 61) and mlc2 (spot 54), were upregulated in the workers. Four (11%) proteins in the protein folding group, hsp60 (spots 4 and 5), Gp93 (spot 1), hsp83 (spot 2), and pdi (spot 13), were all upregulated exclusively in the drone antennae. Two (11%) antioxidatant proteins, gstd1 (spots 30−32 and 56) and gsts1 (spots 28 and 29), were unregulated in the drone, while two proteins, phgpx (spot 57) and cat (spot 46), were unregulated in

Table 1. Identification of Differentially Expressed Proteins in Antennae of Sexually Mature Drone and Forager Worker Honeybees (*A. mellifera* L.)[a]

spot number	experimental pI/M_r (kDa)	theoretical pI/M_r (kDa)	sequence coverage (%)	matched/ searched	Mascot score	protein name	accession number	mean ± standard deviation: drone	mean ± standard deviation: worker	p value
						Carbohydrate and Energy Metabolism				
10	79.65/6.63	73.27/6.75	39	24/76	121	succinate dehydrogenase (Scs-fp, EC 1.3.5.1)	gi|66505480	2998 ± 241[a]	1115 ± 94[b]	0.001899
12	70.95/6.61	60.23/5.84	52	27/72	237	malic enzyme (Men)	gi|110761561	2068 ± 379[a]	637 ± 45[b]	0.019956
15	61.00/6.19	55.94/6.69	49	22/57	190	aldehyde dehydrogenase (Aldh, EC 1.2.1.3)	gi|66530423	8468 ± 264[a]	1930 ± 452[b]	0.000159
20	48.95/7.41	36.87/6.67	52	20/74	175	aldehyde reductase (ARY, EC 1.1.1.21)	gi|110763386	1985 ± 330[a]	942 ± 96[b]	0.038398
22	43.45/6.43	36.19/6.25	41	12/39	120	malate dehydrogenase (EC 1.1.1.37)	gi|66506786	1226 ± 16[a]	767 ± 63[b]	0.002136
24	41.38/6.60	29.93/6.35	47	12/41	132	carbonic anhydrase 1 (CAH1, EC 4.2.1.1)	gi|48095863	1392 ± 37[a]	293 ± 42[b]	1.05×10^{-5}
43	90.87/10.00	86.68/8.67	29	22/50	162	aconitase (Acon, EC 4.2.1.3)	gi|48098039	1473 ± 757[b]	6506 ± 418[b]	0.00434
44	76.53/7.65	70.52/7.55	17	10/20	102	malic enzyme (Mdh)	gi|66524798	211 ± 37[b]	412 ± 41[b]	0.022145
47	58.49/10.00	55.39/5.41	43	25/54	219	vacuolar H^+-ATPase 55 kDa B subunit (Vha55)	gi|66531434	1502 ± 1216[b]	14641 ± 2590[b]	0.003
48	58.59/6.50	40.18/5.51	32	9/31	100	enolase (Eno, EC 4.2.1.11)	gi|110761968	1221 ± 184	2273 ± 75[a]	0.006185
49	58.51/8.05	39.96/7.57	43	11/33	118	aldolase (Ald, EC 4.1.2.13)	gi|110748949	0 ± 0[b]	779 ± 263[a]	
50	57.30/8.22	48.65/8.11	37	16/44	131	lethal (1) G0255 [l(1)G0255, EC 4.2.1.2]	gi|110767168	1087 ± 209[b]	2639 ± 255[a]	0.009235
51	55.83/10.00	52.13/9.07	23	12/29	95	citrate synthase (kdn, EC 2.3.3.1)	gi|66521738	2340 ± 156[b]	11357 ± 2964[a]	0.038467
52	49.57/6.89	39.68/7.52	50	17/86	144	isocitrate dehydrogenase subunit α [l(1)G0156, EC 1.1.1.41]	gi|48102814	2108 ± 209[b]	3409 ± 210[a]	0.011796
						Fatty Acid Metabolism				
6	79.02/6.02	64.20/6.12	34	18/42	143	juvenile hormone esterase (Jhe, EC 3.1.1.59)	gi|58585076	1856 ± 189[a]	521 ± 195[b]	0.005831
7	78.79/6.16	64.20/6.12	49	27/67	204	juvenile hormone esterase (Jhe, EC 3.1.1.59)	gi|58585076	1418 ± 260[a]	538 ± 9[b]	0.027617
8	78.62/6.31	64.20/6.12	40	24/51	170	juvenile hormone esterase (Jhe, EC 3.1.1.59)	gi|58585076	1893 ± 236[a]	494 ± 100[b]	0.001697
9	78.33/6.47	64.20/6.12	36	17/46	125	juvenile hormone esterase (Jhe, EC 3.1.1.59)	gi|58585076	3231 ± 648[a]	470 ± 29[b]	0.002163
11	78.20/4.71	62.02/4.81	42	28/63	199	type B carboxylesterase (EC 3.1.1.1)	gi|66560187	29521 ± 1196[a]	15356 ± 2409[b]	0.006228
14	68.84/7.59	83.33/5.94	24	20/78	128	long-chain fatty acid-CoA ligase (EC 6.2.1.3)	gi|110762211	959 ± 39[a]	437 ± 10[b]	0.000209
19	53.43/7.07	46.83/8.51	44	18/58	129	probable medium-chain-specific acyl-CoA dehydrogenase (MCAD, EC 1.3.99.3)	gi|66499429	1582 ± 183[a]	672 ± 8[b]	0.007696
38	17.76/7.04	15.14/6.37	74	16/51	141	fatty acid binding protein (FABP)	gi|58585202	14499 ± 1001[a]	8395 ± 538[b]	0.005803
39	15.71/5.17	15.54/5.46	72	11/37	128	FABP-like protein	gi|58585214	2562 ± 267[a]	1185 ± 135[b]	0.0068
40	15.73/5.54	15.540/5.46	73	12/51	135	FABP-like protein	gi|58585214	11141 ± 1222[a]	4621 ± 906[b]	0.012798
						Amino Acid and Nucleotide Metabolism				
3	91.75/6.65	76.63/6.09	24	19/47	140	nucleotidyltransferase (EC 2.7.7.7)	gi|110755367	1853 ± 165[a]	1103 ± 31[b]	0.011043
27	34.72/4.75	26.82/4.83	44	9/37	94	proteasome subunit α type 5 (Prosama5, EC 3.4.25.1)	gi|66541426	1679 ± 193[a]	1010 ± 102[b]	0.037741
53	38.73/5.15	46.44/5.36	25	8/45	85	serine-type endopeptidase (HtrA2, EC 3.4.21)	gi|110758534	841 ± 81[b]	1421 ± 167[a]	0.034982
						Development				
16	61.16/8.69	59.76/9.00	45	29/57	234	bellwether (blw)	gi|48100966	7887 ± 345[a]	3581 ± 548[b]	0.002662
17	53.67/5.95	48.98/6.97	33	19/92	100	skpA-associated protein (skpA, EC 6.2.1.5)	gi|66548261	2636 ± 43[a]	1484 ± 151[b]	0.00183

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Table 1. Continued

spot number	experimental pI/M_r (kDa)	theoretical pI/M_r (kDa)	sequence coverage (%)	matched/ searched	Mascot score	protein name	accession number	mean ± standard deviation: drone	mean ± standard deviation: worker	p value
45	62.16/8.07	49.00/8.06	35	15/25	186	imaginal disk growth factor 4 (Idgf4)	gi|66514614	3281 ± 819[b]	8783 ± 1714[a]	0.044255
						Antioxidant System				
28	31.52/5.12	17.69/5.40	39	10/38	126	glutathione *S*-transferase S1 (GstS1, EC 2.5.1.18)	gi|66534655	2428 ± 44[a]	1447 ± 207[b]	0.009739
29	31.17/5.49	17.69/5.40	39	7/29	103	glutathione *S*-transferase S1 (GstS1, EC 2.5.1.18)	gi|66534655	12156 ± 45[a]	5595 ± 178[b]	3.64×10^{-6}
30	31.46/8.76	31.44/6.01	54	17/45	157	glutathione *S*-transferase 1-1 (GstD1, EC 2.5.1.18)	gi|110763730	4075 ± 621[a]	1951 ± 140[b]	0.028926
31	31.48/8.98	31.44/6.01	45	8/19	98	glutathione *S*-transferase 1 (GstD1, EC 2.5.1.18)	gi|76262439	6505 ± 1937[a]	44.9 ± 327[b]	0.003544
32	31.30/9.30	20.34/5.60	39	11/24	110	glutathione *S*-transferase 1-1 (GstD1, EC 2.5.1.18)	gi|110763730	3673 ± 312[a]	2137 ± 74[b]	0.000652
33	30.32/5.59	21.94/5.65	49	10/22	129	thioredoxin peroxidase 1 (Jafrac1, EC 1.11.1.15)	gi|66548188	3418 ± 12[a]	1236 ± 76[b]	9.34×10^{-6}
34	30.27/5.98	21.94/5.65	66	16/84	141	thioredoxin peroxidase 1 (Jafrac1, EC 1.11.1.15)	gi|66548188	11332 ± 1855[a]	5118 ± 353[b]	0.030169
35	30.18/7.19	21.94/5.65	68	13/92	101	thioredoxin peroxidase 1 (Jafrac1, EC 1.11.1.15)	gi|66548188	3607 ± 475[a]	1934 ± 291[b]	0.039825
46	66.21/10.00	58.18/8.39	40	23/51	228	catalase (Cat, EC 1.11.1.6)	gi|25990773	2175 ± 191[b]	8286 ± 1896[a]	0.032669
56	31.36/10.00	31.44/6.01	43	11/29	129	glutathione *S*-transferase 1-1 (GstD1, EC 2.5.1.18)	gi|110763730	3164 ± 442[b]	10548 ± 2409[a]	0.00343
57	24.33/7.82	19.37/7.63	70	14/68	142	glutathione peroxidase (PHGPx)	gi|66546646	4581 ± 380[b]	7997 ± 726[a]	0.01403
						Protein Folding				
1	99.62/5.01	92.15/4.96	41	40/68	245	glycoprotein 93 (Gp93)	gi|110758921	1610 ± 222[a]	199 ± 57[b]	0.001147
2	93.59/5.69	83.79/4.98	33	29/77	147	heat shock protein 90 (hsp90)	gi|229892248	1107 ± 57[a]	295 ± 51[b]	0.000454
4	70.84/5.27	60.55/5.64	31	16/32	142	heat shock protein 60 (hsp60)	gi|66547450	2833 ± 218[a]	1494 ± 357[b]	0.005933
5	80.30/5.27	60.55/5.64	43	26/69	166	60 kDa heat shock protein (hsp60)	gi|66547450	3020 ± 889[a]	282 ± 81[b]	6.76×10^{-5}
13	67.59/5.03	24.44/4.74	45	12/67	91	protein disulfide isomerase (Pdi, EC 5.3.4.1)	gi|110768510	24421 ± 2959[a]	13666 ± 1824[b]	0.036446
						Cytoskeleton				
21	46.25/4.74	32.25/4.73	52	17/111	102	tropomyosin 2 (Tm2)	gi|66522386	37515 ± 339[a]	1998 ± 78[b]	0.007245
23	41.64/4.71	29.24/4.70	53	17/61	133	tropomyosin 1 (Tm1)	gi|66500390	1624 ± 290[a]	391 ± 248[b]	0.008193
36	24.59/5.51	17.05/6.17	70	11/32	150	protein twinstar (tsr)	gi|110751158	1328 ± 119[a]	0 ± 0[b]	
54	37.53/4.81	23.53/4.78	62	17/104	105	myosin regulatory light chain 2 (mlc2)	gi|66555437	444 ± 105[b]	1068 ± 178[a]	0.038898
61	11.20/4.65	33.00/4.74	46	18/61	140	tropomyosin 1 (Tm1)	gi|48094441	3082 ± 81[b]	6293 ± 526[a]	0.003796
						Transporter				
25	40.05/6.92	31.76/6.20	62	23/75	204	vibrator (vib)	gi|66557640	4855 ± 407[a]	2780 ± 9[b]	0.007012
37	18.12/5.47	15.60/5.71	66	11/34	130	odorant binding protein 14 (obp14)	gi|94158822	5193 ± 547[a]	1585 ± 216[b]	0.000346
41	13.57/5.18	16.950/6.30	62	12/39	101	odorant binding protein 5 (obp5)	gi|58585124	2563 ± 421[a]	1376 ± 184[b]	0.038
42	87.48/6.76	80.030/6.77	39	25/99	166	transferring (tsf1)	gi|58585086	672 ± 51[b]	1260 ± 148[a]	0.019626
55	37.23/7.84	30.630/8.25	59	11/34	132	porin	gi|66521459	755 ± 142[b]	2311 ± 161[a]	0.001906
58	13.55/4.69	15.54/4.76	44	6/15	100	odorant binding protein 21 (obp21)	gi|94158718	1661 ± 522[b]	6141 ± 87[a]	0.001062
59	12.19/4.39	14.03/4.44	86	14/80	99	general odorant binding protein Asp2 (obp2)	gi|78100778	2636 ± 299[b]	10567 ± 1950[a]	0.01586
60	12.29/4.48	15.54/4.55	31	6/16	87	odorant binding protein 16 (obp16)	gi|94158709	2627 ± 39[b]	4251 ± 121[a]	0.000219

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Table 1. Continued

spot number	experimental pI/M_r (kDa)	theoretical pI/M_r (kDa)	sequence coverage (%)	matched/ searched	Mascot score	protein name	accession number	mean ± standard deviation drone	mean ± standard deviation worker	*p* value
					Unknown Function					
18	53.65/6.31	43.86/5.37	53	24/117	106	CG31974	gi|110758754	3091 ± 69^a	1173 ± 201^b	0.000834
26	39.32/6.63	34.89/6.52	26	9/29	83	CG11550-PA	gi|66499360	1595 ± 197^a	797 ± 74^b	0.019173

[a] Spot number corresponds to the number of the protein spots in Figure 3. The theoretical molecular weight (M_r) and isoelectric point (pI) of the identified proteins were retrieved from the protein database of NCBInr. Experimental M_r and pI values were calculated with PDQuest and internal standard molecular mass markers. Sequence coverage is the ratio of the number of amino acids in every peptide that matches with the mass spectrum divided by the total number of amino acids in the protein sequence. Matched peptide is the number of paring an experimental fragmentation spectrum to a theoretical segment of protein, and searched is the total searched peptide. The Mascot score was searched against the NCBInr database. Protein name is given when proteins were identified by MALDI-TOF/MS. The taxonomy is *A. mellifera*. Accession number is the unique number given to mark the entry of a protein in the NCBInr database. The mean ± standard deviation is the mean value of the relative protein amount. *p* values of ≤0.05 were considered as differentially expressed proteins where the value marked with a superscript a was significantly higher than that marked with a superscript b.

Figure 4. Classification of proteins altered in their expression in antennae of sexually mature honeybee drones and forager workers. Color codes represent different protein functional groups.

Figure 5. Comparisons of the numbers of upregulated proteins in antennae of sexually mature honeybee drones and forager workers. Green and red represent the number in drones and forage workers, respectively.

the worker bees. In addition, three (8.4%) proteins from the fatty acid metabolism group, rfabp (spots 38−40), type B carboxylesterase (spot 11), and MCAD (spot 19), were all upregulated in the drone antennae. Two (5.5%) development proteins, blw (spot 16) and idgf4 (spot 45), were upregulated in the drones and worker bees, respectively. One (5.5%) amino acid and nucleotide metabolism protein, HtrA2 (spot 53), was overexpressed in the workers, and prosma5 (spot 27) was expressed in the drone; one (3%) unknown functional protein, cg31974 (spot 10), was expressed in the drones (Figure 7).

Functional enrichment analysis was conducted on the proteins from antennae of both castes using CluoGo. Significantly overrepresented Gene Ontology (GO) terms were examined to determine the hypotheses for the biological events behind the data and to provide a broad overview of the principal characteristics of

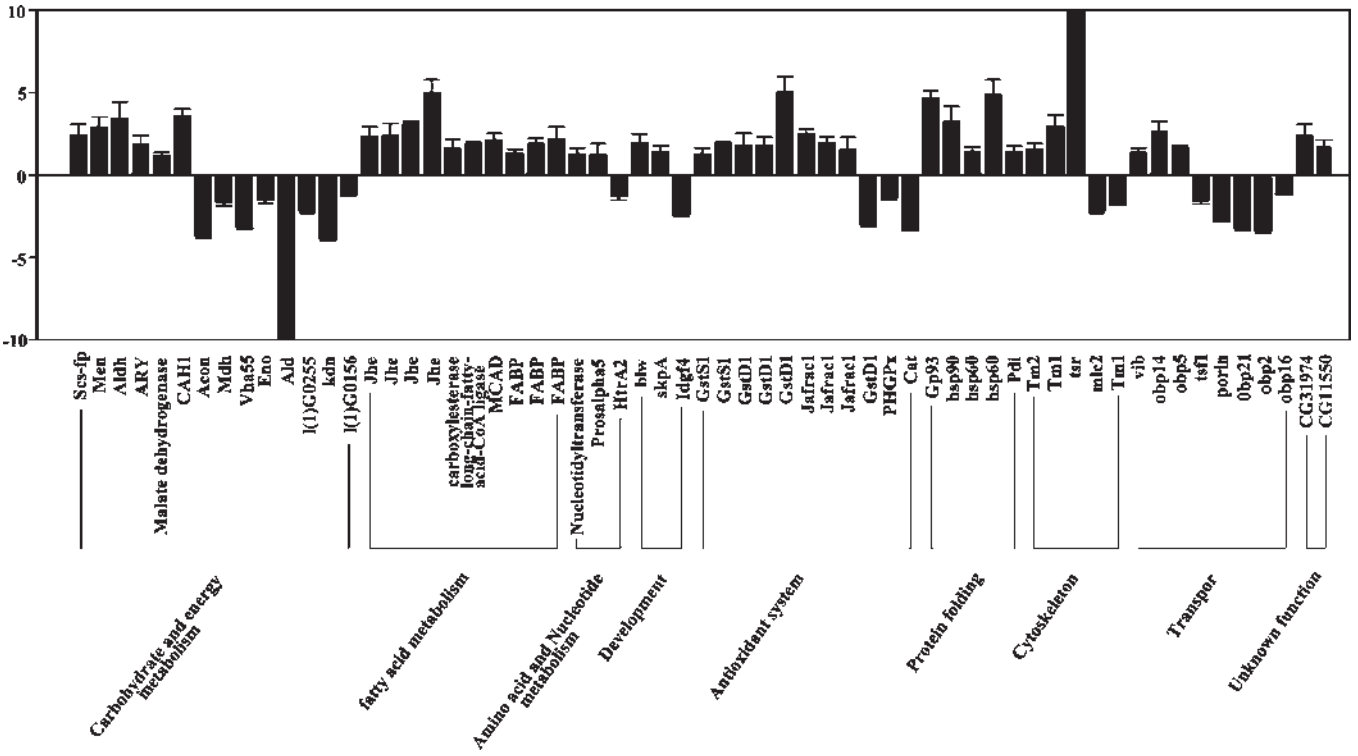

Figure 6. Quantitative comparison of differentially expressed proteins in the antennae of sexually mature honeybee drones and forager workers. The ratios of protein abundance (drone to forager) were transformed, and the protein spots with $|\log_{1.5}\text{ ratio}| \geq 1$ and $p \leq 0.05$ were selected as the differentially expressed proteins. Protein names (in abbreviations) and protein numbers are listed. Positive values indicate drone expression over forger worker and negative values forger worker over drone. The $|\log_{1.5}\text{ ratio}|$ of the uniquely expressed proteins is limited to 10.

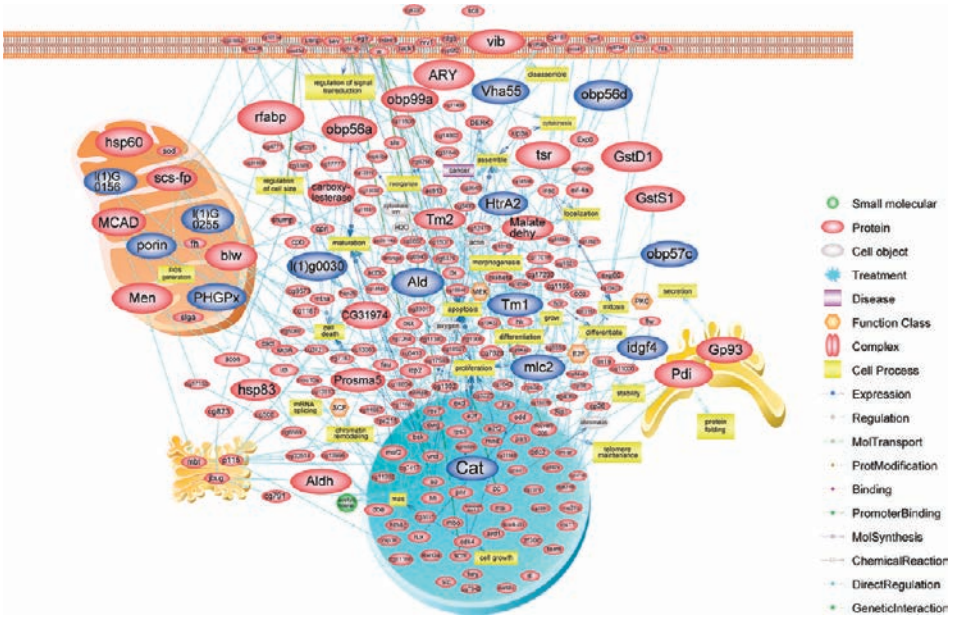

Figure 7. Network analysis of all the pathways and interactions connected to all of the identified proteins. Those highlighted in blue represent the key node proteins identified in this study and validated by the network software program. Protein entities that belong to distinct functional groups were represented in different shapes according to the default settings of the software as described in the legend.

a proteome. Therefore, functional enrichment analysis showed that the identified proteins were significantly enriched in five major functional groups (Figure 8). These were carbohydrate metabolism and energy production, fatty acid metabolism, cytoskeleton, antioxidant activity, and molecular transporter. The leading term (a functional term with the lowest statistical p value) in carbohydrate metabolism and energy production was the dicarboxylic acid metabolic process in which malate dehydrogenase (spot 22),

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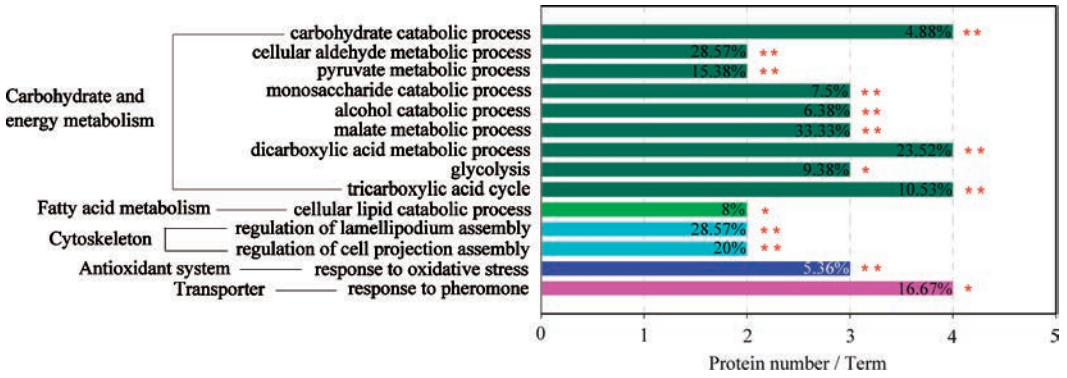

Figure 8. Functional enrichment analysis of the differentially expressed proteins using ClueGO. One or two asterisks indicate significant enrichment at the $p < 0.05$ and $p < 0.01$ statistical levels, respectively.

Mdh (spot 44), Men (spot 12), and l(1)G0255 (spot 50) were significantly enriched, whereas in fatty acid metabolism, the leading term was the lipid catabolic process in which MCAD (spot19) and Jhe (spots 6−9) proteins were significantly enriched. The leading term in the cytoskeleton group is regulation of lamellipodium assembly and significantly enriched with Tm2 (spot 21) and tsr (spot 36). The leading term in the antioxidation was response to oxidative stress in which GstS1 (spots 28 and 29), Jafrac1 (spots 33−35), and PHGPx (spot 57) were significantly enriched. The leading term in molecular transporter was response to pheromone in which Obp56a (obp5, spot 41), Obp56d (obp21, spot 58), Obp56h (obp16, spot 60), and Obp99a (obp14, spot 37) were significantly enriched (Figure 8).

At the transcript level, confirmations of the differentially expressed proteins showed that 9 of 10 genes [hsp90 (spot 2), aldh (spot 15), Tm2 (spot 21), FABP (spot 38), FABP-like protein (spots 39 and 40), obp14 (spot 37), obp5 (spot 41), obp21 (spot 58), and obp2 (spot 59)] were consistent with their mRNA expression. However, a diverse pattern between mRNA and protein expression in one gene [PHGx (spot 57)] (Figure 9) might be due to the lack of a direct relationship between mRNA and protein expression or attributed to post-transitional effects and/or other regulatory mechanisms, such as a lack of synchronization.

4. DISCUSSION

This study reveals that there is a remarkable dimorphism between the drones and the workers with respect to the relative number of sensilla. There is a significantly higher number (7.5 times) of poreplate sensilla on the antennae of the drones than on the antennae of the workers, in agreement with previous studies.[22] This enormous difference between the antennae of two bee castes is mainly attributed to sensilla placodea, which number ∼18600 in the drones and cover almost all the male antennomeres.[22] Sensillae placodea, which appear in the form of poreplates, are the main olfactory antennal structures in the honeybee,[23,24] and its receptor neurons respond to a variety of plant and flower odorants and honeybee pheromones.[4,23] The higher number of hairlike sensilla (2-fold) in the worker antennae versus the drone antennae in this study might be attributed to the differences in the sensory equipment on the mainly sensory cells of the antennae, which number ∼65000 in the worker and 340000 in the drone.[23] Hence, this difference primarily suggests task-specific responses to various odors in which the antennae of the workers are designed to respond to activities like plant and flower odorants and honeybee pheromones for colony maintenance and social organization, while the antennae of the drone are designed to respond to 9-ODA, the main component of queen mandibular pheromone to pursue the queen for mating.

The molecular insight from 2-DE showed more protein species and proportions (67.2%) upregulated in the antennae of the drones, which signifies a high density, and the large volume of the poreplate promotes its improved performance probably to gain success in the rigorous competition of mating.[23,25,26]

A higher level of expression of proteins associated with carbohydrate metabolism and energy production, antioxidation, fatty acid metabolism, and molecular transporters in the antennae of both castes of bees implies high energy source requirements for the performance of chemical communication with each other and the queen for the normal social organization.[18,25,27] In particular, the worker bees use these energy source proteins for the laborious foraging activities (sensory detection and collection of nectar and pollen) through their olfaction systems.[26] For this reality, important carbohydrate metabolism and energy production metabolic proteins like ald (spot 49), malic enzyme (spot 44), eno (spot 48), citrate synthesis (spot 51), isocitrate dehydrogenase (spot 52), and vha55 (spot 47) were strongly expressed in the forager bees.[25] Specifically, proteins like ald (spot 49) showed the highest expression intensity that could justify the metabolic processes are important regulators of behavioral development for foraging activity. In addition, the composition of metabolic enzymes possessed by the honeybees in general and the forager bees in particular reflects their dietary specialization (nectar and pollen) as they rely strongly on the energy produced by carbohydrate metabolism to provide the energy for foraging flights.[25]

In contrast, proteins related to carbohydrate metabolism and energy production were less upregulated in the antennae of the drones than in those of the worker bees. This might be attributed to their lack of involvement in foraging activities and the other social organization of the colony unlike the worker bees. Furthermore, the protein species required for the drone sex pheromone processing during mating flight is most likely different from that of the foraging flight needed by the forager bees. aldh (spot 15), ARY (spot 20), malate dehydrogenase (spot 22), Men (spot 12), and carbonic anhydrase (spot 24) were among the upregulated proteins in this functional group in the drone antennae. aldh is the group of enzymes that catalyze the oxidation of aldehydes to carboxylic acids, and it is suggested that it involves the synthesis

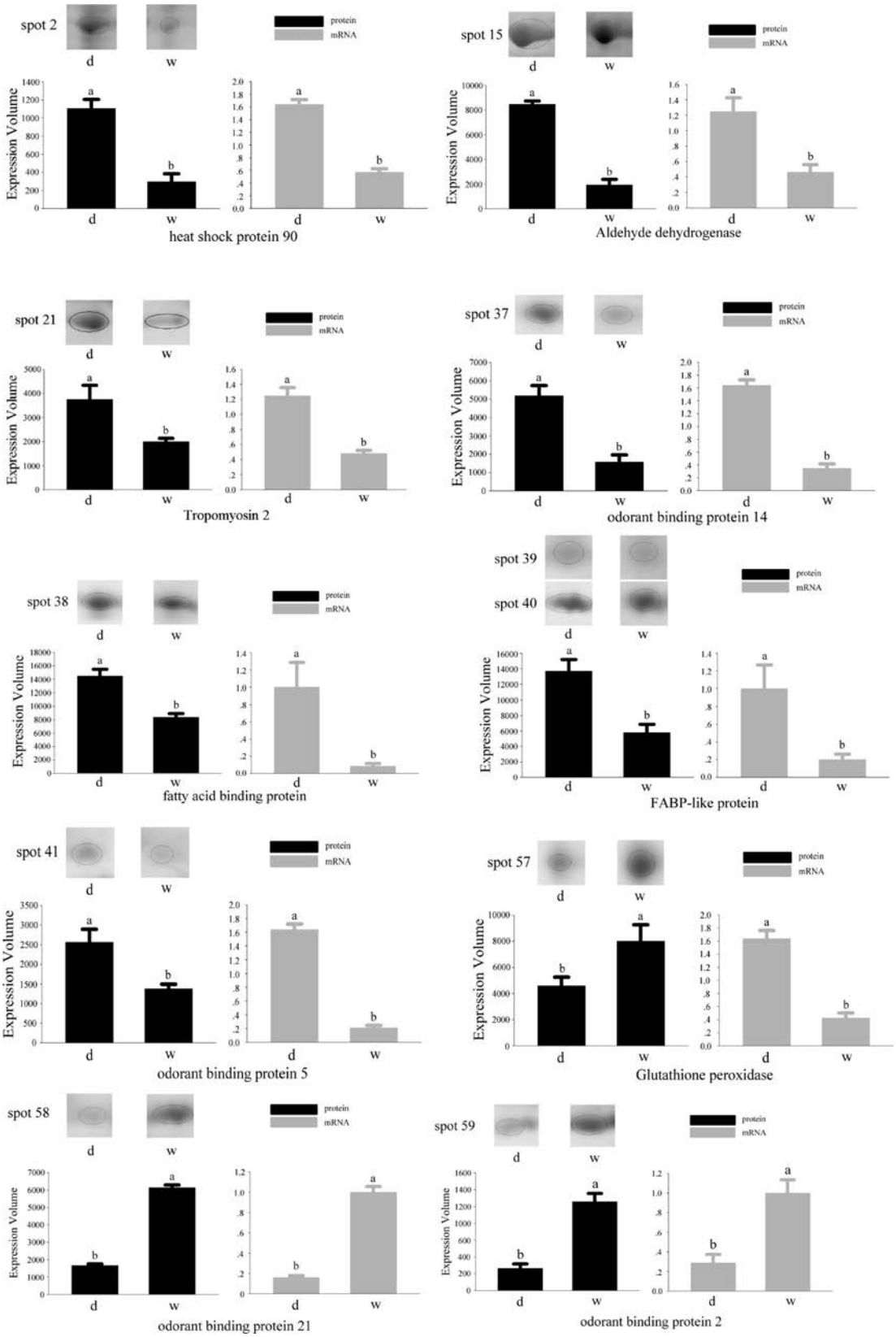

Figure 9. Ten differentially expressed proteins between the antennae of the honeybee drone (d) and forager worker (f) validated at the mRNA level. The magnified images of protein spots from the 2-DE gels are shown in the top part of each panel. The values of protein abundance are the average percent volume of spots in three replicated gels. The lowercase letters (a and b) above the bars indicate significant differences between the antennae of the honeybee drone and forager worker ($p < 0.05$), where values marked with a are significantly higher than values marked with b.

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or degradation of pheromone compounds in sexually mature drones.[15,28] Therefore, aldh in the antennae of sexually mature drones might play a role in pheromone transformation and consequently promote queen recognition during the mating flight. It is reported that aldh may also serve as a biotransformation enzyme for pheromones and possibly xenobiotics in a manner similar to that of the antenna-specific GST of *Manduca sexta*.[29] Malic enzyme activity is important for the accumulation of storage lipid in the drone antennae and supports the hypothesis that this enzyme is important in the generation of nicotinamide adenine dinucleotide phosphate for lipid biosynthesis.[30] Carbonic anhydrase enzymes produce hydrogen ions located in the mitochondria to be utilized as fuel for the ion pump that maintains the integrity of the cell wall membrane and also as fuel for all metabolic activities.[31]

Proteins related to fatty acid metabolism were exclusively upregulated in the drone antennae, suggesting their vital importance for the drone mating flight compared to their involvement in the foraging flight of the worker bees. Drone sensory neurons respond to odor stimuli, particularly to the major components of honeybee queen sex pheromone of fatty acids (9-ODA and 9-HDA).[9,32] Here Jhe (spots 6−9), type B carboxylestrase (spot 11), LC-FAC (spot 14), MCAD (spot 19), FABP (spot 38), and FABP-like protein (spots 39 and 40) were expressed abundantly. Jhe plays an important role in controlling the circulating hormone levels in insect development and reproduction in general by degrading the juvenile hormone.[33] The expression of carboxylesterases, in particular, carboxylestrase (CES) (spot 11) as one of the vital proteins in fatty acid metabolism in the drone antennae, might be associated with the drone-preferential D-AP1 (drone antennae protein 1), which is a secretary carboxylesterase.[18] The physiologic function of this enzyme is to degrade sex pheromones after they bind to the receptor to prevent the accumulation of residual stimulus[18,34] and hence sensory adaptation.[11,35] Some recent studies have also suggested that pheromone-degrading enzymes that belong to the carboxylesterase family could play a role in the dynamics of the olfactory response to acetate sex pheromones in insects[7] signaled in the drone antennae by the rapid degradation of these compounds.[18] Rapid degradation of female sex pheromone in male antennae is believed to play an essential role during male flight in pheromone trial.[11,36] Hence, the unique expression of this protein in the drone antennae implies its vital importance for the drone mating flight. Moreover, the presence of FABPs is linked to their function as a transporter of energy from mitochondria to high-energy demand areas for the development of honeybee brain, thorax, compound eyes, and antennae.[37]

Protein involved in antioxidant activity was more highly upregulated in the drone than in the worker bees. They are reported to terminate oxidation reactions that damage cells by removing free radicals and stopping and restraining other oxidation reactions by being oxidizing themselves.[28] The overexpression of metabolizing peroxide gstd1 (spots 31 and 32 in the drone and spot 56 in the worker) and cellular detoxificant gsts1 (spots 28 and 29 in the drone) signifies their contribution in averting oxidation reactions because of high metabolic activities in the drone for the process of sex pheromones while performing other social metabolic activities in the workers.[18,28,38] GSTs are multifunctional proteins essential for xenobiotic metabolism and protection against peroxidative damage.[39] Rapid degradation of odorants is necessary for insects to detect and respond to changes in the concentration of an odorant.[39] The significantly stronger expression of an olfactory-specific gsts1 in the drone could be associated mainly with its important role in the degradation of odorants and, on the other hand, with the protection of the olfactory system from harmful xenobiotics.[29]

The functions of proteins as molecular transporters in honeybees are mostly linked with odorant binding transport, sensory perception of chemical stimulus, and pheromone response. The expression of OBPs in both sexually mature drones and forager bees is expected as the antennae are specialized for olfactory proteins.[34] However, more proteins were upregulated in the antennae of the workers than in those of the drones. In particular, proteins such as Tsf1 (spot 42), porin (spot 55), obp21 (spot 58), Asp2 (obp2, spot 59), and obp16 (spot 60) were exclusively upregulated in the worker antennae, suggesting a sex-specific role in sensory perception of chemical stimulus and pheromone response during field activities and activities in the hive, whereas the exclusive upregulation of proteins as vib (spot 25), obp14 (spot 37), and obp5 (spot 41) as male sex biased in the drone antennae suggests reaction to queen pheromone response during the mating flight.[40] Workers communicate with a large number of different pheromones, cuticular hydrocarbons, and plant volatiles.[23] Such diversity is probably supported by variations in specific proteins involved in odorant detection.[14] Hence, the upregulation of most of these OBPs proteins in the workers is probably directly related to their essential role in the foraging activities, particularly detection of various flowering plant odors[41,42] and the response to the queen pheromone in colony organization.[40,43] Generally, the variations in OBP expression intensity between the two bee castes suggest polymorphism due to the social organization of honeybees and may be a result of their sex-biased activities.[14,44]

Hsps primarily function as a molecular chaperone under normal physiological conditions, facilitating protein folding, preventing protein aggregation, or targeting improperly folded proteins in specific degradative pathways.[45,46] Therefore, the exclusively unregulated hsp60 (spots 4 and 5), hsp83 (spot 2), gp93 (spot 1), and pdi (spot 13) proteins in the drone antennae indicate their important roles in protein−protein interactions such as folding and assisting in the proper protein conformation and prevention of unwanted protein aggregation during high-level oxidation of fatty acids and processing of complex pheromones during the mating flight.

Proteins as cytoskeleton were expressed more in the antennae of the drones than in those of the workers. The upregulation of tsr (spots 36) and tm2 (spot 21) in the antennae of drones and mlc2 (spot 54) and tm1 (spots 23 and 61) in the antennae of workers suggests their important role in assisting proper cellular divisions and regulating cell shapes in the more segmented antennae of the drone.[38,44] This is supported by the 10-fold greater intensity of expression of tsr (spot 36) in the antennae of the drones compared with that in the antennae of the workers. As one of the main regulators of actin cytoskeleton remodeling,[47] tsr is required for many different processes in the cell, including cell motility,[48] cell polarity during migration,[49] endocytosis,[50] axon guidance,[51] and cytokinesis.[52] However, the higher level of expression of tm1 (spots 23 and 61) in the antennae of the workers implies its association with the stretch-activated indirect flight muscle (IFM) because the antennae can be used as an instrument to measure flight speed, detect odors, and detect even the direction of the odor.[53,54]

Development proteins were expressed in the antennae of both the drones and the workers. blw (spot 16) and idgf4 (spot 45) were the major proteins in the drones and workers, respectively.

This implies that as is the case with other tissues and/or organs, honeybee antennae require the presence of growth factors to ensure their full development. Within this group, blw is an allele of an essential gene that encodes the α subunit of the mitochondrial ATP synthase,[55] and idgf4 cooperates with insulin-like peptides to stimulate the proliferation, polarization, and motility of imaginal disk cells.[56] The idgfs function as cofactors of the insulin-like molecule, stimulating imaginal disk cell growth and 10 pairs of imaginal discs, which give rise to the eyes/antennae, the legs, the halters, the wings, various head structures, and a genital disk in *Drosophila* larvae.[56,57] In addition, the overexpression of blw and idgf4 in this study suggests that the antennae of both drone and forager bees need growth factors to attain and maintain the required growth process and perform efficient olfactory functions.[57]

Proteins serve as fundamental parts of protein complexes in the living cell rather than existing independently. As a result, 36 proteins that were part of the constructed BIN of the antennal proteome of both sets of bees were recognized. The majority (20) were proteins that were expressed in the antennae of the drones and (16) in the antennae of the workers, suggesting the specificity of each bee antennae in performing specific and sex-related tasks. The fact that the principal amount of proteins (45%) was related to carbohydrate metabolism, energy production, and molecular transporter further emphasizes the significance of these groups of proteins for the construction and proper function of the antennae of both sets of bees. Similarly, the analysis of functional enrichment additionally confirmed the importance of carbohydrate metabolism and energy production, and other related proteins play a central role in the antennal olfactory functions of the drones and the workers.

The consistency of expression of nine proteins with mRNA indicates a prospective opportunity for reverse genetics through manipulation of potential genes for optimizing olfactory functions like better worker organization in the hive and field and successful drone mating, which eventually contribute to the improvement of colony management.

5. CONCLUSION

The number and protein differential expression of respective antennal poreplate and hairlike sensilla were compared between the sexually mature drones and the forager bees using SEM in combination with 2-DE MS, bioinformatics, and qRT-PCR. Overall, the antennae of both bees showed morphological and functional differences in the number and protein expression of poreplate and hairlike sensilla in relation to sex. The study confirmed that antennae of drones possess significantly more porplate sensilla (7.5 times) than antennae of workers. Furthermore, the differential expression of proteins in the antennae of two bee castes showed a considerable and wide spectrum of proteins upregulated in the antennae of drones versus those of the workers. Proteins in the fatty acid metabolism, antioxidation, protein folding, cytoskeleton, development, and amino acid and nucleotide groups participated in building and maintaining the high-level antennal olfactory functions of drones in response to sex pheromones. On the other hand, the antennae of the workers strongly expressed carbohydrate metabolism and energy production and the molecular transporter proteins as an indication of the strong demand for energy and OBPs for their foraging activities and other olfactory functions. As opposed to previous similar works on honeybee antennae, this global proteomic study investigated wide ranges of proteins involved in the construction and function of antennae of both sets of bees and detailed how proteins identified in antennae of each caste are molecularly related to their daily activities. Furthermore, the study provides additional information about those previous studies, contributes to our understanding of honeybee antennae, and encourages us to set up additional investigations of antennal proteomes affected by the differences in sex, caste, and division of labors of workers to pursue a more complete and better understanding of chemical communication in the honeybee community.

■ ASSOCIATED CONTENT

Supporting Information

Primer sequences used for qRT-PCR of genes differentially expressed in antennae of sexually mature drone and forager worker honeybees. This material is available free of charge via the Internet at http://pubs.acs.org.

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