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Proteome Analysis of the Hemolymph, Mushroom Body, and Antenna Provides Novel Insight into Honeybee Resistance against *Varroa* Infestation

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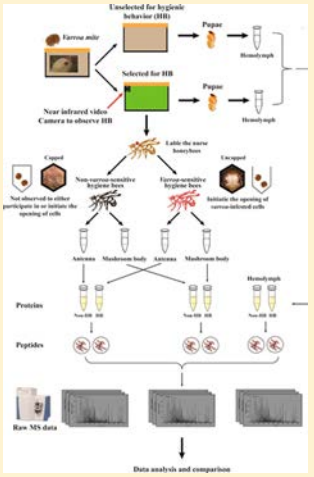
Supporting Information

ABSTRACT: *Varroa destructor* has been identified as a major culprit responsible for the losses of millions of honeybee colonies. *Varroa* sensitive hygiene (VSH) is a suite of behaviors from adult bees to suppress mite reproduction by uncapping and/or removing mite infested pupae from a sealed brood. Despite the efforts to elucidate the molecular underpinnings of VSH, they remain largely unknown. We investigated the proteome of mushroom bodies (MBs) and antennae of adult bees with and without VSH from a stock selected for VSH based on their response to artificially *Varroa*-infected brood cells by near-infrared camera observation. The pupal hemolymph proteome was also compared between the VSH-line and the line that was not selected for VSH. The identified 8609 proteins in the hemolymph, MBs, and antennae represent the most depth coverage of the honeybee proteome (>55%) to date. In the hemolymph, the VSH-line adapts a unique strategy to boost the social immunity and drive pupal organogenesis by enhancing energy metabolism and protein biosynthesis. In MBs, the up-regulated proteins implicated in neuronal sensitivity suggest their roles to promote the execution of VSH by activation of synaptic vesicles and calcium channel activities. In antennae, the highly expressed proteins associated with sensitivity of olfactory senses and signal transmissions signify their roles by inputting a strong signal to the MBs for initiating VSH. These observations illustrate that the enhanced social immunities and olfactory and neuronal sensitivity play key roles in the combat against *Varroa* infestation. The identified candidate markers may be useful for accelerating marker-associated selection for VSH to aid in resistance to a parasite responsible for decline in honeybee health.



KEYWORDS: *Apis mellifera, Varroa destructor, Varroa sensitive hygiene, hemolymph, antennae, mushroom body*

1. INTRODUCTION

The honeybee (*Apis mellifera*) has evolutionarily adapted to habitats across the world since they were split from an ancient lineage of cavity-nesting bees in Asia around 300,000 years ago.[1] Approximately 70% of plant species cultivated for human consumption are at least partly dependent on insect pollination,[2] of which *Apis mellifera* is the most important pollinator species. The ectoparasitic mite, *Varroa destructor*, is the most destructive parasitic pest of the Western honeybee (*Apis mellifera*), and it is considered to be one of the major causes of colony losses in recent decades.[3,4] The *Varroa* mite infests honeybees by sucking out the hemolymph of larvae and adults, and thus it the bees' body resources and suppresses their immunities.[5] The mite is also a major vector for the transmission of pathogens.[6,7] The honeybees live in a highly social community, in which their high population density and trophallactic feeding behavior pose a high risk of pathogen transmission. In the honeybee genome, however, homologues can be found for only one-third of genes implicated in immune response in nonsocial insects such as fruit flies, mosquitos, or moths.[8] the eusocial bees have evolved other defense mechanisms to guarantee their survival.

A wide repertoire of mechanisms, which operate on different levels, has been reported to be involved in the honeybee social defense system. Individual immunity functions by means of mechanical, physiological, behavioral (autogrooming), and immune defenses.[8−10] Allo-grooming can be seen to present a pairwise level of defense, whereas colony-wide mechanisms include the spread of antibiotics in the form of plant-derived resins (propolis) or the secretion of H_2O_2-producing enzymes into honey.[8,10,11] Also among these communal defense strategies, hygienic behavior (HB) is a collective response by adult bees to detect and remove the diseased and parasitized brood. Specifically, *Varroa* sensitive hygiene (VSH), as opposed to classic HB, has received much attention since it has been shown to defense against *Varroa* mites.[12,13] This behavior is now regarded as a genetically

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based quantitative trait.[14−16] Therefore, VSH offers a sustainable means of controlling mite parasitism, other factors such as grooming behavior, environmental conditions, and beekeeping management, contributing to a stable parasite-host relationship.[17−19] has been performed which seek to understand the mechanism regulating VSH, including the analysis of gene expression in the pupae and adult brains of VSH and non-VSH bees drawn from different breeding programs.[20−22] Some of these studies report that the differentially expressed genes may lead to a higher olfactory sensitivity in hygienic bees,[22] whereas others do not support this finding.[20,21] Recently, rapid progress in mass-spectrometry-based proteomic technologies has greatly advanced to a state-of-the-art method with which thousands of proteins can be identified in a single sample (tissue, organ, organism), such as the honeybee embryo,[23,24] hypopharyngeal gland (HG),[25] and brain.[26] To date, however, only two works have reported on the proteome differences between hygienic and nonhygienic bees by sequencing the proteomes of larval integument and adult antennae. They identified several protein markers and partially explained the mechanisms that underpin the biology associated with HB.[27,28] Only 1300 proteins, however, were identified in these two works, representing only about 8.5% of the honeybee proteome. Moreover, although several studies have made efforts to elucidate the regulating mechanism of HB at both the gene and protein levels using brain, antenna, and larval tissue, most of these have worked with bees taken from colonies that have been selected for the removal of freeze-killed brood, instead of those of genuinely infested larvae/pupae.[20,21,27,29−31] Also, the bees used in these works are sampled more or less randomly from their colonies (seen as "hygienic" in their entirety), regardless of whether the individual insects display HB or not. The level of expression of HB varies between individual colonies and populations. These variations are also reflected in individual bees within the same colony, partially because colony members are offspring from different drones with whom the queen bee mated.[21] Hence a "hygienic" colony does not consist entirely of individual bees showing HB. To further extend our knowledge of honeybee VSH, it is of great importance to establish a highly detailed proteome map of the important tissues and organs related to VSH, such as the MBs and antennae using a more exact approach to sample bees, which individually express VSH. Antennae are the primary sensory organs of adult bees and can sense the presence either of the *Varroa* itself or of a nest-mate damaged by *Varroa*.[27,28,32] The MBs are a high-order center in the brain which processes the information received from olfactory sensory neurons in the antennae through the neurons projecting into the glomeruli of the antennal lobe, and they are associated with regulating the VSH.[20,21,33,34] of the biochemical foundations of VSH, we utilized a near-infrared video system installed in beehives to observe the uncapping of artificially *Varroa*-infested brood cells from a line that has been bred for *Varroa* resistance since 1997, and we compared the proteomes of MBs and antennae from adult bees which individually showed or did not show VSH. Moreover, the hemolymph of honeybees nutrient transport and immune defense, but also as an important indicator that reflects the physiological state of the honeybees.[35] To investigate social physiological change upon the VSH selection, the hemolymph proteome of pupae from the selected line and reference line was also investigated by state-of-the-art proteomics. This will be novel knowledge bees fight against parasitism and in finding protein markers to facilitate the selection of resistant bees.

2. MATERIALS AND METHODS

2.1. Chemical Reagents

Unless otherwise specified, all chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were of analytical grade or better. Trypsin (modified sequencing grade) was bought from Promega (Madison, WI, USA).

2.2. Honeybee Samples

Honeybee colonies (*Apis mellifera carnica*) used in the sampling of adult workers which either did or did not show VSH were taken from a breeding line selected for the uncapping of *Varroa*-infested brood since 1997 by the Institute for Bee Research, Hohen Neuendorf e.V, in Berlin, Germany. The main selection criterion for this line was the initiation of artificially *Varroa*-infested brood, as measured by near-infrared video observations of individually number-tagged worker bees. This system was installed in the hives, and can observe and record the behaviors of bees on computer. VSH and non-VSH bees observed during 6−8 days postemergence were identified by the number on their thorax during colony check, and then collected as samples. The bioassay used for the measurement of the hygienic trait is described in detail by Spötteret al.[36] Several repetitions of the video experiment, involving at least 1,500 bees each time, were run every year, and the frequency of VSH individuals among the worker offspring was used as a selection criterion of mother queens. In addition, the breeding values for two auxiliary criteria, the removal of pin-killed brood, and the development of the *Varroa*-infestation rate within colonies were estimated by a software, described by Bienefeld et al.[37]

Individual bees (6−8 days postemergence) in the colonies observed initiating the opening of *varroa*-infested cells in the video experiment were regarded as VSH bees, whereas bees moving around brood cells (*Varroa*-infested or not) and not observed to either participate in or initiate the opening of cells were regarded as non-VSH bees. Generally, 20 of each VSH and non-VSH bees were sampled from five hygienic selected colonies, and then the MBs and antennae were dissected. To investigate the physiological stage of hygienic bees upon selection, we also sampled the hemolymph of pupae from the VSH-line and their control line. The control line is the normal *A. m. carnica*-breeding line of the institute, bred for gentleness, honey production, low swarming drive and calmness, but not *Varroa* resistance for many years. 50 pupae from 10 selected colonies (5 pupae per colony) and 50 pupae from 10 control colonies were sampled and the hemolymph was processed by inserting a disposable microcapillary glass pipet into one side (2/3 down from head) of the pupal body. Then, the hemolymph was drawn in by capillary action as previously described.[38,39]

2.3. Protein Preparation and Digestion

Prior to protein extraction, the samples of antenna, MB, and hemolymph were processed by pestle homogenization. The homogenates were mixed with a lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris-base, 30 mM dithiothreitol (DTT), 1 mg/10 mL) in ice for 30 min, and then centrifuged at 15,000g at 4 °C for 20 min. The supernatant was recovered and three volumes of ice-cold acetone were added; then they were kept in ice for 30 min for protein precipitation and desalting.

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Afterward, protein pellets were centrifuged at 15, 000g at 4 °C for 20 min. The protein samples were resuspended in 100 μL of 5 M urea, followed by dissolution in four volumes of 40 mM NH_4HCO_3 and concentration measurement using a Bradford assay based on our previously published protocols.[25,40] Protein samples were reduced with DTT (final concentration 10 mM) for 1 h to prevent reformation of disulfide bonds, and finally, alkalized with iodoacetamide (final concentration 50 mM) for 1 h in the dark. The protein samples were digested using sequencing grade modified trypsin at 37 °C overnight.

A system of LC-MS/MS was used to analyze three independently digested peptide samples of each tissue. LC-MS/MS was run on a Q-Exactive mass spectrometer coupled to a two-column EASY-nLC 1,000 nanoflow system (Thermo Fisher Scientific). Before the analytical separation, the peptide samples were loaded onto a trap column (5.0 μm Aqua C18 beads, 2 cm long, 100 μm inner diameter fused silica, Thermo Fisher Scientific) for 2 min in buffer A (0.1% acetic acid) at a flow rate of 5 μL/min. Peptides were separated from the analytical column (15 cm long, 75 μm inner diameter fused silica trap column filing with 3.0 μm Aqua C18 beads, Thermo Fisher Scientific) using a 120 min gradient. The elution conditions were: 100% buffer A (0.1% formic acid) to 8% buffer B (0.1% formic acid, 80% acetonitrile) for 5 min, 8% to 20% buffer B for 55 min, then 20% to 30% buffer B for 10 min, followed by an increase to 100% buffer B for 5 min and 100% buffer B for an additional 15 min. The eluting peptides were directly infused into a Q-Exactive mass spectrometer (Thermo Fisher Scientific) via ESI. MS and MS/MS information was retrieved in a data-dependent mode. The MS/MS data were obtained using the following settings: one full scan (resolution 70,000 at 400 m/z; 300−1,800 m/z) followed by top 20 MS/MS scans using higher energy dissociation in the linear ion trap mass spectrometer (resolution: 17,500, isolation window: 2 m/z, normalized collision energy: 27) and using dynamic exclusion (charge exclusion: unassigned 1, > 8; peptide match: preferred; exclude isotopes: on; dynamic exclusion: 10 s).

2.4. Protein Identification and Abundance Level Quantitation

The MS/MS data in RAW were retrieved using Xcalibur (version 2.2, Thermo Fisher Scientific) and searched using the PEAKS search engine (version 7.0, Bioinformatics Solutions Inc.) against the protein database of *Apis mellifera* (downloaded February, 2015) and incorporated with common contaminants, totaling 21,778 entries. The search was done with the following parameters: precursor ion and MS/MS tolerances: 15 ppm and 0.05 Da; enzyme specificity: trypsin; maximum missed cleavages: 2; fixed modification: carbamidomethyl (C, + 57.02); and variable modification: oxidation (M, + 15.99). The fusion strategy of target and decoy sequence, an enhanced target-decoy approach that makes more conservative false discovery rate (FDR) estimations,[41] was used to control at FDR ≤ 1% at both the peptide and protein level for protein identification. Only a protein identified by at least one unique peptide with the identification of at least two spectra was considered to be identified. The LC-MS/MS data have been deposited to the ProteomeXchange Consortium (http://Proteomecentral.Proteomexchange.org) via the PRIDE parter repository with the data set identifier PXD004467.

To quantify the level change of protein abundance between the VSH and non-VSH samples, triplicates of each sample were analyzed by the quantitation module of the PEAKS software via a label-free strategy. Feature detection was performed separately on each sample by using the expectation-maximization algorithm. The features of the same peptide from different samples were reliably aligned using a high-performance retention time alignment algorithm.[41] Peptide features and proteins were considered to have changed significantly between different samples when they contained both a P value of <0.05 and a fold change of ≥1.5. The expressional profile of differentially expressed proteins between different samples was created by the PEAKS software using an uncentered Pearson correlation and average linkage.

2.5. Quantitative Real-Time PCR

The hemolymph of pupae from the genotypes of both those selected and unselected for HB, was sampled to test the expression trends between proteins and their encoding genes. The total RNA of the five individual pupal hemolymphs from each of the VSH selected and reference colony was isolated as a pool sample using the RNeasy Mini Kit (QIAGEN China [Shanghai] Co. Ltd.), following the manufacturer's protocol. Twenty colonies from VSH selected or reference colonies were labeled as 2 groups: VSH group and non-VSH group (10 samples of each group). The RNA concentration, purity, and integrity were evaluated with an Agilent 2100 BioAnalyzer (Agilent Technologies Inc., Palo Alto, CA). Then, cDNA was generated using a reverse transcription kit (PrimeScript RT reagent kit, RR037, Takara Biotechnology [Dalian] Co. Ltd., China). The primer sequences of selected genes used for PCR are stated/listed in Table S1. The PCR amplification and data collection were conducted using an iQ5Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The PCR program was conducted in a 20 μL system as follows: 95 °C for 30 s; each circle at 94 °C for 3 s, 60 °C for 30 s, 40 circles, and with melt curve tracking from 55 to 95 °C. After verifying the amplification efficiency of the selected genes and reference gene *GAPDH* in almost equal levels, the differences in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method.[42] The statistical analysis of gene expression was performed with an independent two-sample t-test (SPSS version 18.0, SPSS, Inc. Chicago, IL, USA). An error probability of $P < 0.05$ was considered statistically significant.

2.6. Bioinformatics Analysis

To interpret the biological implications of the proteins identified, the unique identifiers of GI number of bee proteins identified in the hemolymph, MB, and antennae were used as an input for functional category and pathway enrichment analyses using ClueGO v2.1.7, a Cytoscape plug-in (http://www.ici.upmc.fr/cluego/).[43] The enrichment was done by comparing the number of proteins identified from the samples to the number of functionally GO annotated proteins in the entire *Apis mellifera* genome. The significantly enriched functional GO categories and pathways in biological processes were reported using a right-sided hypergeometric test. FDR was calculated using a Bonferroni step-down test to correct the P-value. The GO terms were considered as significantly enriched only when the corrected P-value was <0.05, which was functionally important for specific samples. The nodes in functionally grouped networks were linked based on their kappa score level (0.4) in ClueGO. GO Tree Levels ranged from levels 3−8, GO term restrictions were a minimum of 20 genes, and covered minimum of 20% genes. For comparison, groups sharing 60% of terms were merged.

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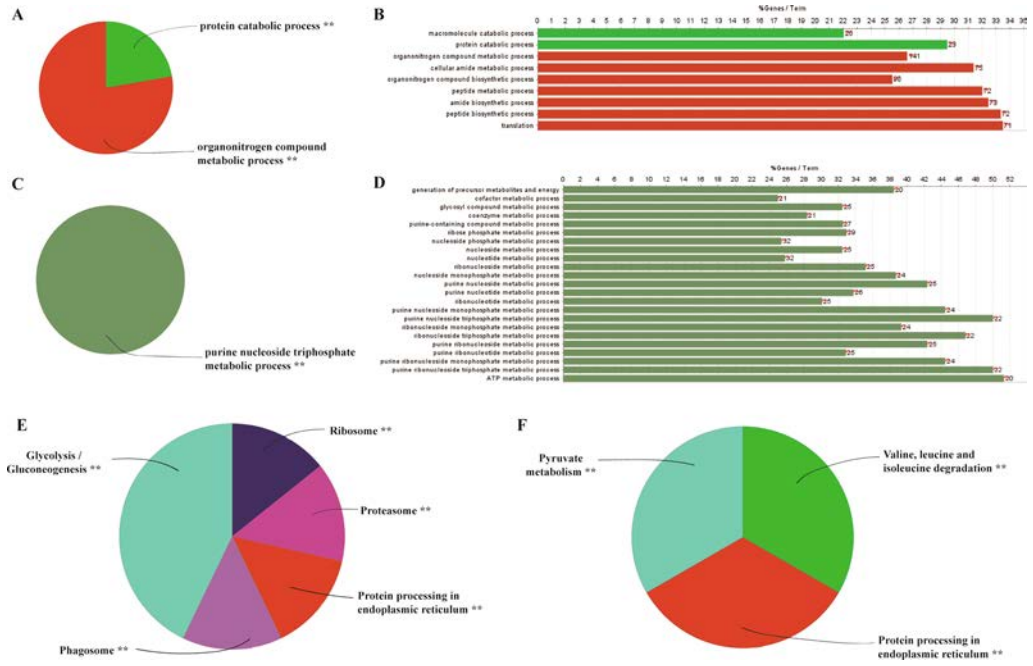

Figure 1. Qualitative comparison of identified hemolymph proteins between VSH bees and non-VSH bees using ClueGO, a Cytoscape plugin software. The proteins identified in VSH bees and non-VSH bees are analyzed by ClueGO to compare the functional classes and pathways specifically enriched by the two data sets. The detailed parameter settings are available in the methodology section. (A) Pie chart overview of the significantly enriched unique functional classes in VSH bees. (B) Bar chart indicating the specific terms in each functional class corresponding to A in VSH bees. (C) Pie chart overview of the significantly enriched unique functional classes in non-VSH bees. (D) Bar chart indicating the enriched specific terms of each functional class corresponding to C in non-VSH bees. (E) The significantly enriched unique pathways in VSH bees. (F) The significantly enriched unique pathways in non-VSH bees. Note: The name of each functional class is assigned by the lowest *P* value of the term in this class. The same color represents terms belonging to the same functional class. *, $P < 0.05$; **, $P < 0.01$. Seen also Table S-4.

3. RESULTS

To gain novel knowledge underlying *Varroa* tolerance, we selected three tissues involved in the bee-*Varroa* interaction: the hemolymph of red-eyed pupae, and the MBs and antennae of nurse-aged bees. Overall, 8609 proteins were identified in three tissues/organs, covering more than a 55% depth of the honeybee proteome (15323, http://www.uniprot.org/proteomes/UP000005203). The protein expression profiles of these three tissues were compared, and large qualitative and quantitative differences were found between the VSH and non-VSH bees.

3.1. Proteome Comparison of the Hemolymph between Pupae from Breeding Lines Selected and Not Selected for VSH

Of the 2320 proteins identified in the hemolymph, 1924 and 1897 were found in VSH and non-VSH pupae, respectively (Tables S2 and S3). Four functional groups, homeostatic processes, purine nucleotide metabolic processes, cellular macromolecular assembly, and nucleoside triphosphate metabolic processes (Figure S1A and Table S4), and one pathway, oxidant phosphorylation, were found to be significantly enriched in the two hemolymph groups (Figure S1B and Table S4). In VSH bees, however, two major groups, including 9 GO terms, were uniquely enriched, and these were led by protein catabolic process and the organonitrogen compound metabolic process (Figures 1A and B, and Table S4). In non-VSH bees, only one major group containing 23 GO terms was significantly enriched as a unique representation, of which the purine nucleoside triphosphate metabolic process was the leading term (lowest *P* value) (Figures 1C and D, and Table S4).

Evaluation of the abundance level alteration of the 2320 proteins in the two groups showed that the expression levels of 249 proteins varied, of which 174 and 75 proteins were up-regulated in VSH and non-VSH pupae, respectively (Figure 2A and Table S5). Regarding the 174 proteins up-regulated in pupae from the VSH stock, they were significantly enriched in functional classes and pathways associated with ribosomes, lysosomes, amino sugars, and nucleotide sugar metabolism, and the aminoglycan metabolic process. Of these, those associated with ribosomes was the largest group, and that contained most of the up-regulated proteins, followed by those associated with amino sugars and nucleotide sugar metabolism (Figure 2B, Figure S2, and Table S6). However, the 75 up-regulated proteins in the non-VSH stock were not enriched in any functional groups or pathways.

3.2. Proteome Comparison of MBs between VSH and Non-VSH Bees

Among the 3983 and 3753 proteins identified in the MBs of VSH and non-VSH bees, they represented a total of 5052 proteins (Tables S7 and S8). Four categories, organophosphate biosynthetic process, inorganic cation transmembrane transport, cellular component assembly, and cytoplasmic transport, and one pathway of protein processing in the endoplasmic reticulum (ER) were significantly enriched and overlapped in the two hygienic phenotypes (Figure S3 and Table S9). Noticeably, eight functional classes, ATP metabolic process, cellular macromolecular complex assembly, nucleoside triphosphate metabolic process, protein transport, small molecule metabolic process, peptide biosynthetic process, protein catabolic process, and oxidation-reduction process, and four pathways, phagosome, proteasome, ribosome, and oxidative

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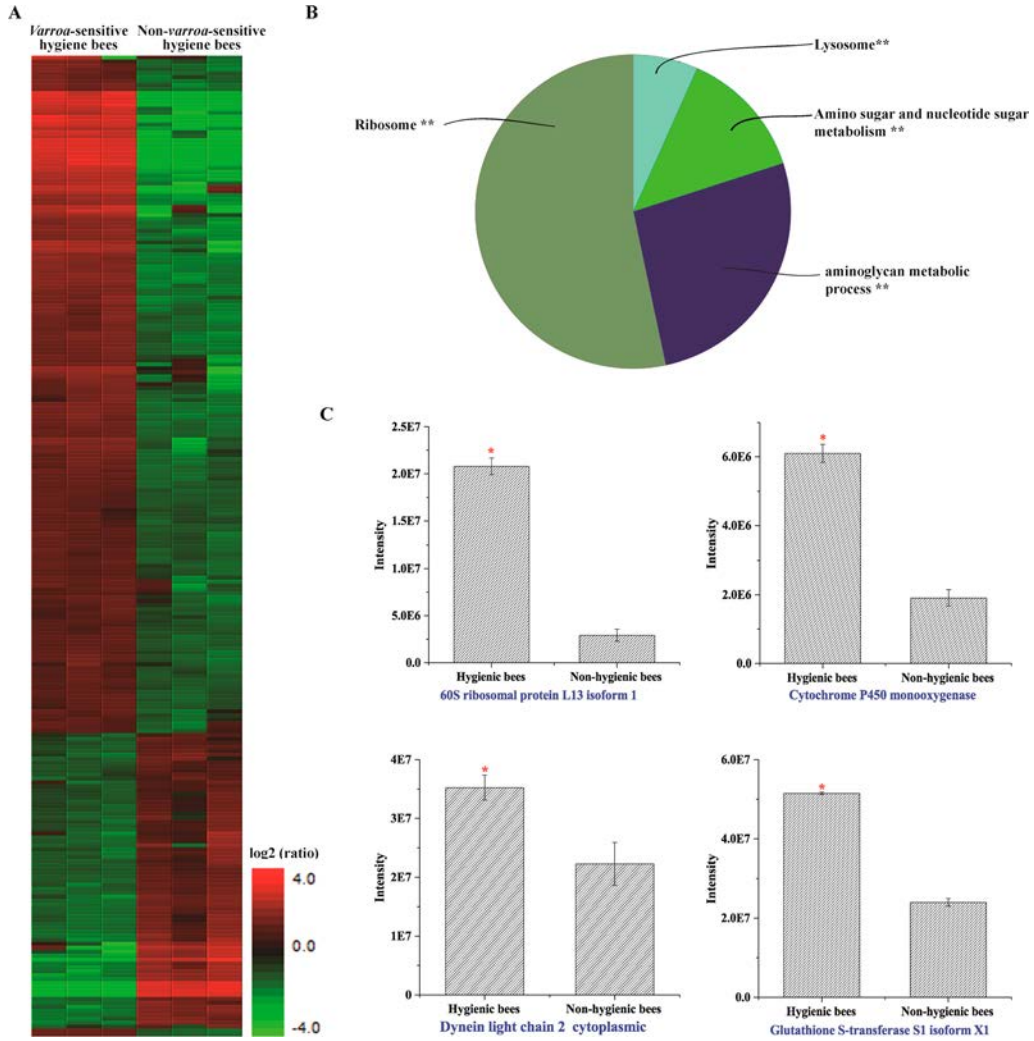

Figure 2. Quantitative comparison of hemolymph proteins between VSH bees and non-VSH bees. (A) The unsupervised hierarchical clustering analysis of the differentially expressed proteins (fold change >1.5 and $P < 0.05$); the columns represent the replicates in each of two honeybee lines, and the rows represent the individual proteins. The up- or down-regulated proteins are indicated by red and green, respectively. The color intensity changes with the protein expressional level as noted on the key bar. (B) The significantly enriched functional classes and pathways of up-regulated proteins in VSH bees. The up-regulated proteins in non-VSH bees are not enriched in any functional classes or pathway. For the details of the enrichment analysis results, see Figure S2 and Table S6. (C) Representative protein expressional level between VSH bees and non-VSH bees. *, $P < 0.05$; **, $P < 0.01$.

phosphorylation, were exclusively enriched in the VSH bees (Figure 3A and B, and Table S9). However, only one functional class and two pathways were significantly enriched in the non-VSH bees: cellular macromolecule catabolic process, endocytosis, and citrate cycle (TCA cycle) (Figures 3C and D, and Table S9).

Of the 5052 proteins observed in the MBs, 1195 proteins altered their expression levels between the two groups. Among these, 755 proteins were up-regulated in VSH bees and 441 were up-regulated in non-VSH bees (Figure 4A and Table S10). The up-regulated proteins in VSH bees were significantly enriched to seven functional groups, purine nucleoside monophosphate metabolic process, organonitrogen compound metabolic process, generation of precursor metabolites and energy, protein transport, cellular macromolecular complex assembly, cellular homeostasis, and oxidation-reduction process, and four pathways, oxidative phosphorylation, ribosome, endocytosis, and protein processing in the ER (Figure 4B, Figure S4A, and Table S11). The up-regulated proteins in non-VSH bees were significantly enriched in functional groups and pathways of cellular protein localization, RNA transport, phagosome, protein processing in the ER, and proteasome (Figure 4C, Figure S4B, and Table S11).

3.3. Proteome Comparison of Antennae between VSH and Non-VSH Bees

With regard to the antennal proteome, 3398 and 3130 proteins, representing a total of 3758 proteins, were identified in the antennae of two hygienic phenotypes (Figure 5 and Tables S12 and S13). There were seven functional categories and three pathways significantly enriched and overlapping in the two groups: purine nucleoside metabolic process, ATP metabolic process, cellular protein localization, nucleoside phosphate metabolic process, cellular protein catabolic process, nucleoside metabolism and process, and regulation of protein metabolic process; and fatty acid degradation, ribosome, and phagosome (Figure S5 and Table S14).

Among those 3758 proteins identified in the antennae, 126 proteins altered their expression levels between the two groups,

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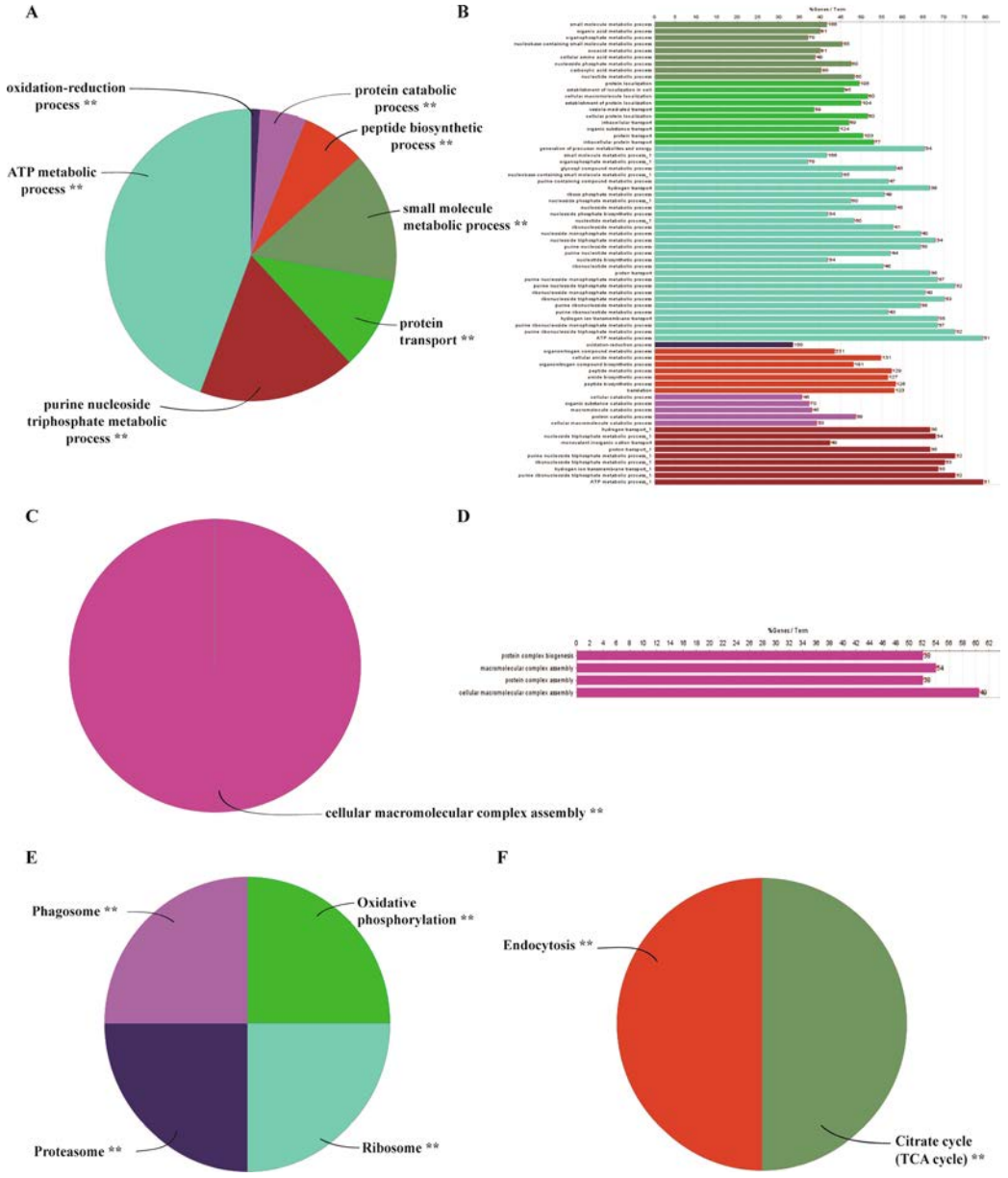

Figure 3. Qualitative comparison of identified mushroom body proteins between VSH bees and non-VSH bees using ClueGO, a Cytoscape plugin software. The proteins identified in VSH bees and non-VSH bees are analyzed by ClueGO to compare the functional classes and pathways specifically enriched by the two data sets. The detailed parameter settings are available in the methodology section. (A) Pie chart overview of the significantly enriched unique functional classes in VSH bees. (B) Bar chart indicating the specific terms in each functional class corresponding to A in VSH bees. (C) Pie chart overview of the significantly enriched unique functional classes in non-VSH bees. (D) Bar chart indicating the enriched specific terms of each functional class corresponding to C in non-VSH bees. (E) Significantly enriched unique pathways in VSH bees. (F) Significantly enriched unique pathways in non-VSH bees. Note: The name of each functional class is assigned by the lowest *P* value of the term in this class. The same color represents terms belonging to the same functional class. *, $P < 0.05$; **, $P < 0.01$. Seen also Table S9.

of which 78 proteins were up-regulated in VSH bees and 48 were up-regulated in non-VSH bees (Figure 6A and Table S15). The up-regulated proteins in VSH bees were related to the three functional groups, anion transport, intracellular protein transport, and cofactor biosynthetic process, and two pathways of proteasome and oxidative phosphorylation (Figure 6B, Figure S6A, and Table S16). Again, the up-regulated proteins in non-VSH bees were associated with two functional groups, hydrogen transport and phagosome, and one pathway, oxidative phosphorylation (Figure 6C, Figure S6B, and Table S16).

3.4. Candidate Protein Biomarkers for Breeding Hygienic Bees

To identify potential protein predictors for the marker-assistant selection (MAS) of VSH, we chose proteins with high abundance levels and fold changes among the differential proteins in the two phenotypes of VSH. In all, 32 proteins were selected as potential biomarkers to predict the expression of VSH from the proteomic results of the pupal hemolymph, MBs, and antennae of the adult bees. They were involved in functional groups and pathways such as translation, peptide biosynthetic and metabolic process, organonitrogen compound biosynthetic and metabolic process, intracellular protein

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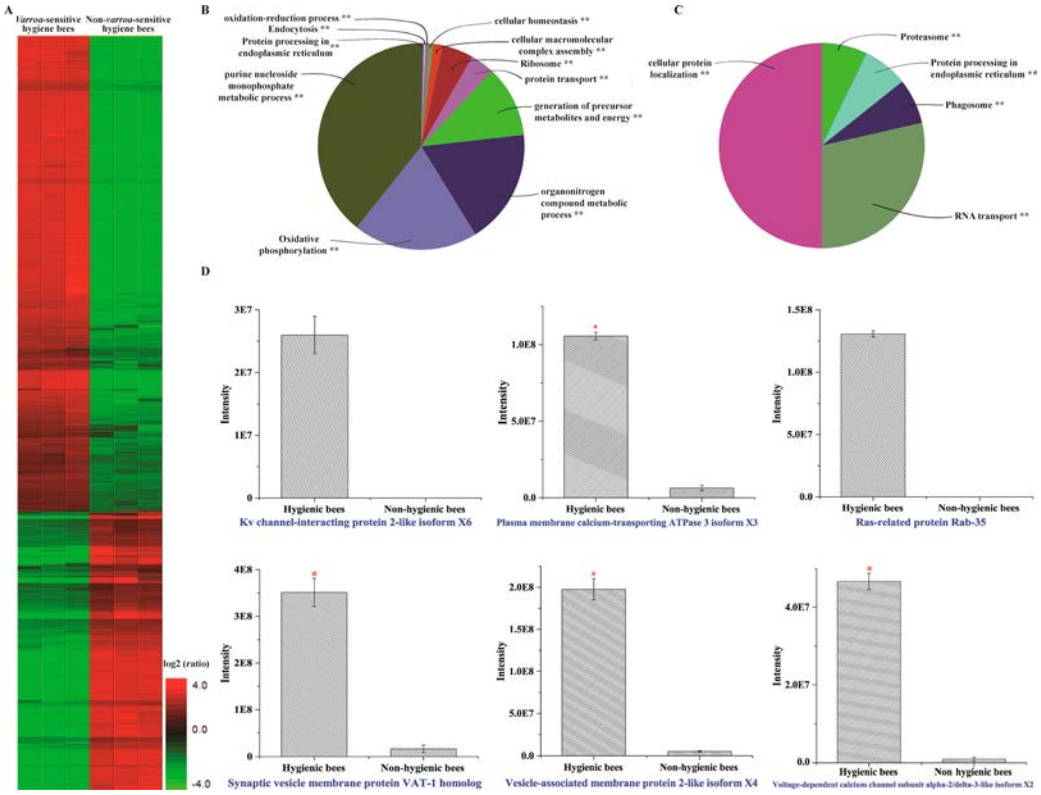

Figure 4. Quantitative comparison of mushroom body proteins between VSH bees and non-VSH bees. (A) The unsupervised hierarchical clustering analysis of the differentially expressed proteins (fold change > 2 and $P < 0.05$); the columns represent the replicates in each of two honeybee lines, and the rows represent the individual proteins. The up- or down-regulated proteins are indicated by red and green, respectively. The color intensity changes with the protein expressional level as noted on the key bar. (B) The significantly enriched functional classes and pathways of up-regulated proteins in VSH bees. For details of the enrichment analysis results, see Figure S4A and Table S11. (C) The significantly enriched functional classes and pathways of up-regulated proteins in non-VSH bees. For details of the enrichment analysis results, see Figure S4B and Table S11. (D) Representative protein expressional levels between VSH bees and non-VSH bees. The two proteins Kv channel-interacting protein 2 and Ras-related protein Rab-35 are specially expressed in VSH bees. *, $P < 0.05$; **, $P < 0.01$.

transport, intracellular protein transport, protein localization, homeostatic process, small molecule metabolic process, oxidation-reduction process, and intracellular transport. Among those 15 protein markers from the hemolymph, they were ribosomal proteins (Rps) RpL13 (7.38-fold) and RpL39 (6.80-fold), heat shock protein beta-1-like isoform X2 (6.31-fold), cytochrome P450 monooxygenas (4.79-fold), Rps11 (4.34-fold), RpL36 (3.00-fold), thioredoxin (2.31-fold), glutathione S-transferase (GST, 2.15-fold), and dynein light chain 2 cytoplasmic (1.58-fold); 6 unique proteins of cytochrome P450 9e2, protocadherin-15 isoform X5, ubiquitin carboxyl-terminal hydrolase 8-like isoform X1, kinesin light chain-like isoform X2, RpL37, and RpL6. In the MBs, 10 proteins related to the synaptic vesicle and calcium channel were voltage-dependent calcium channel subunit alpha-2/delta subunit 3 (Cacna2d3, 73.50-fold), genuine synaptic vesicle protein, associated membrane proteins (VAMP, 57.52-fold), synaptobrevin (38.42-fold), presynaptic membrane proteins syntaxin (22.33-fold), and plasma membrane calcium-transporting ATPase 3 (ATP2B3, 16.56-fold); and 5 unique proteins: NADH dehydrogenase, protein transport protein Sec23A, ras-related protein Rab-35-like, synaptobrevin-like isoform X2 and Kv channel-interacting protein 2-like isoform X6. In the antennae, 5 proteins associated with olfactory and signal transmission were OBP18 (1.74-fold), transmembrane emp24 domain-containing protein (4.94-fold), alphaCop (1.50-fold), gammaCop (2.19-fold), regulator complex protein LAMTOR2 (3.00-fold). Two proteins shared by the 3 tissues were pterin-4-alpha-carbinolamine dehydratase (5.5, 24.20, and 1.62-fold in hemolymph, MBs, and antennae, separately) and uncharacterized protein LOC725661 (2.33-, 3.91-, and 1.53-fold in hemolymph, MBs, and antennae, respectively).

Of the 18 up-regulated proteins in the hemolymph of VSH bees, 11 genes, *CYP9E2*, *HSPB1*, *PCBD1*, *PGM1*, *RpL37*, *CYP314A1*, *GSTS1*, *RpL39*, *RpS11*, *RAB1A*, and *SPARC* were consistent with the tendency of their protein expression. Others did not show a significant consistency between protein expression and their encoding genes (Figure 7). This may be caused by post-translational modification or gene splicing.

4. DISCUSSION

Honeybees are the most important pollinators of agricultural and wild plants, and they are encountering ever-increasing challenges by parasites and pathogens.[17,44,45] To better protect them from these threats, selecting bees resistant to pests and pathogens is of paramount importance and is a never-ending task for the global beekeeping industry. In an effort to gain new insights into honeybee resistance against *Varroa* mites, a high-density proteomic atlas was generated by analyzing three major tissues/organs presumably implicated in regulation of VSH.

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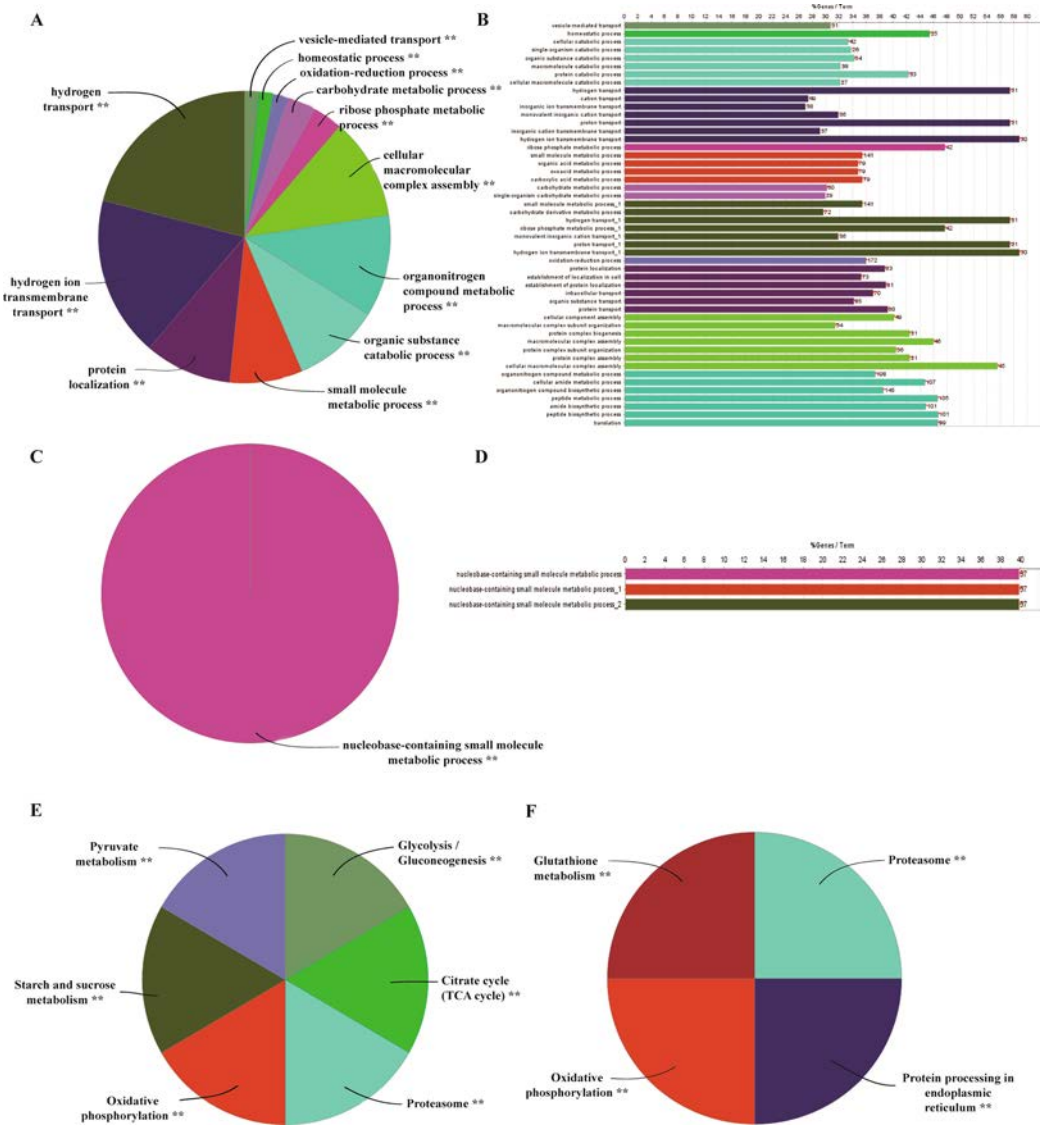

Figure 5. Qualitative comparison of identified antennal proteins between VSH bees and non-VSH bees using ClueGO, a Cytoscape plugin software. The proteins identified in VSH bees and non-VSH bees were analyzed with ClueGO to compare the functional classes and pathways specifically enriched in the two data sets. The detailed parameter settings are available in the methodology section. (A) Pie chart overview of the significantly enriched unique functional classes in VSH bees. (B) The bar chart indicates the specific terms in each functional class corresponding to A in VSH bees. (C) Pie chart overview of the significantly enriched unique functional classes in non-VSH bees. (D) The bar chart indicates the enriched specific terms of each functional class corresponding to C in non-VSH bees. (E) The significantly enriched unique pathways in VSH bees. (F) The significantly enriched unique pathways in non-VSH bees. Note: The name of each functional class is assigned by the lowest *P* value of the term in this class. The same color represents terms belonging to the same functional classes. *, $P < 0.05$; **, $P < 0.01$. See also Table S14.

4.1. Enhanced Energy Metabolism and Protein Biosynthesis in the Pupal Hemolymph Implicated in Boosting the Immunity of VSH Bees

Metamorphosis is a highly energy-demanding process during the pupal stage.[35,38,46−48] This is reflected in our data by the enriched wide spectrum of functional groups and pathways related to metabolizing energy in both lines to support the central roles of the hemolymph of nutrition and energy storage for the bees.[47] In response to about two decades of VSH selection, however, the VSH and non-VSH lines have shaped distinct proteome settings resulting in uniquely enriched divergent functional classes and pathways in each group. The divergent proteome programs are further supported by the uniquely enriched glycolysis/gluconeogenesis and TCA cycle by the VSH pupae, whereas the non-VSH group enriched only pyruvate metabolism. These energy turnover pathways may not only act as an energy source, but also play a defensive role for the VSH bees. The enzymes in the TCA circle are vital for defense against the pathogen *Paenibacillus* larvae,[47] and viral infection hinders the energy metabolism in bee larvae.[49] Again, pathways related to protein synthesis and defense mechanisms still observed quite divergent pathways coverage in the two groups. For instance, the exclusively enriched ribosome pathway and the strongly expressed 32 ribosomal proteins (Rps) were uniquely found in VSH bees. Ribosome plays key roles not only in driving the organogenesis during pupation by providing protein materials,[24,50] but also in larval defense against bacteria and viruses.[47,49] Rps influences the innate immune response by either boosting immune signaling or facilitating pathogen reproduction, depending on the circum-

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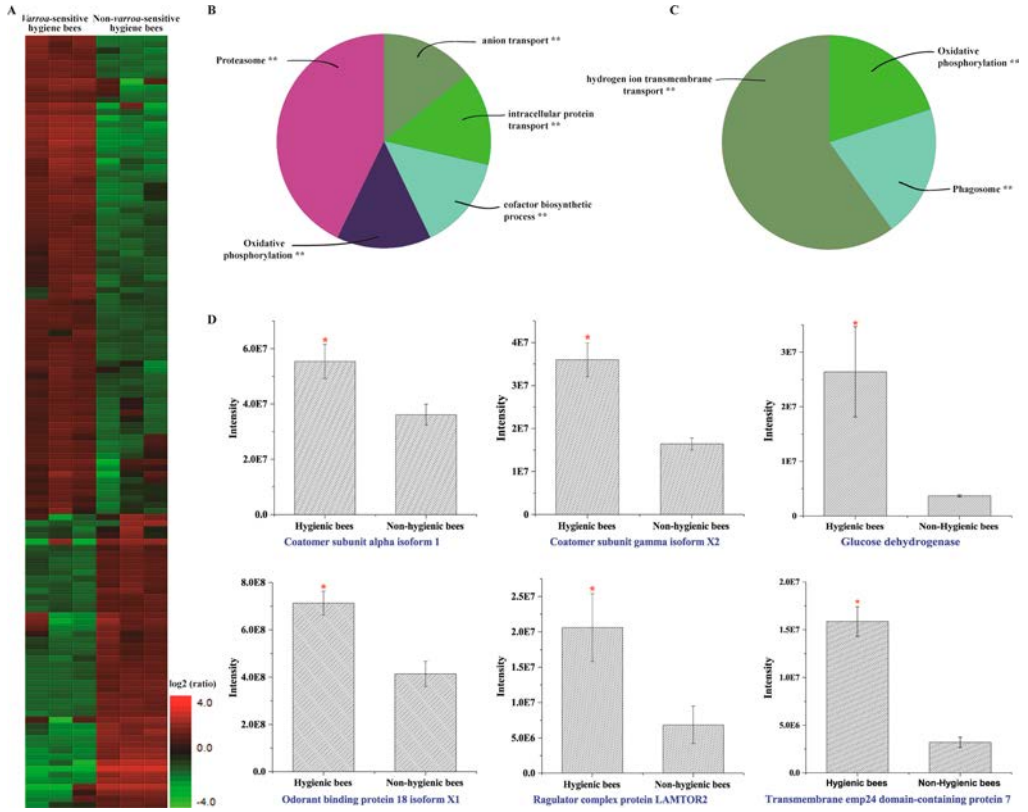

Figure 6. Quantitative comparison of antennal proteins between VSH bees and non-VSH bees. (A) The unsupervised hierarchical clustering analysis of the differentially expressed proteins (fold change > 1.5 and $P < 0.05$); the columns represent the replicates in each of two honeybee lines, and the rows represent the individual proteins. The up- or down-regulated proteins are indicated by red and green, respectively. The color intensity changes with the protein expressional level as noted on the key bar. (B) The significantly enriched functional classes and pathways of up-regulated proteins in VSH bees. For details of the enrichment analysis results, see Figure S6A and Table S16. (C) The significantly enriched functional classes and pathways of up-regulated proteins in non-VSH bees. For details of the enrichment analysis results, see Figure S6B and Table S16. (D) Representative protein expressional level between VSH bees and non-VSH bees. *, $P < 0.05$; **, $P < 0.01$.

stances.[51] Viral invasion counteracts host interference by interrupting regular protein metabolism in cells,[52] which is in concordance with the finding that a large number of Rps are depleted in virus-infected honeybee larvae.[49] Meanwhile, Rpl39 and Rps29 could enhance resistance to insecticides in mosquitoes by changing protein metabolism.[53,54] Again, the uniquely enriched pathways associated with energy and protein metabolism suggest that VSH bees have adapted the strategy by changing their biochemical metabolic route to fit with physiological alteration by selection.

Apart from the above differences, of the 174 up-regulated proteins in the hygienic pupae, a large number of them related to immune response were also found. For example, SPARC plays a vital role in immunity induced by the nuclear polyhydrosis virus, and in densonucleosis infection in *Bombyxmori*[55] and in the larvae of *Heliothisvirescens*.[56] Clcd2 may be associated with HB expression.[22] Thioredoxin and Glutathione S-transferase (GST) play key roles in the defense against viral infections in honeybee larvae.[49] All these observations indicate that selection for VSH has likely led to the increased expression of proteins related to a number of immune functional classes in hygienic bees; albeit, their relation to social immunity is not immediately evident. One possible explanation for this finding is that the breeding line of this work was from the introduction of genetic material of other breeders, which was chosen on the basis of general resistance toward *Varroa* (sometimes based on factors other than VSH). This may have led to the stronger expression of proteins linked to general stress resistance in the VSH selected line. Another hypothesis would be that the pupal immune response is important for the strength of the signal emitted from diseased pupae. This would mean that selection for VSH has led not only to more sensitive reception on the side of the hygienic workers, but also to stronger signals from the side of the diseased brood. Overall, the bees subject to VSH selection have developed a unique biochemical metabolic roadmap to boost the social immunity in concert with the biological demand for executing VSH.

4.2. Strengthened Neuronal Excitability in MBs Associated with Supporting the Initiation of VSH

The MBs are a central part of the honeybee brain that processes the clues received from olfactory sensory neurons, thus implicated in regulating behaviors including VSH.[57] The specificity of the MB proteome is reflected in our data by the significantly enriched four functional categories and one pathway implicated in energy metabolism, signal transduction, transport, and post-translational modification, which is in line with the findings of major protein functions in the brain of honeybees.[26] However, the proteome changes in the MBs of VSH bees are reflected by the wide panel of signal transduction activity. For example, the vesicle-mediated transport process is a cellular transport process of protein and other cargo through the cell by moving in membrane-bounded vesicles,[58] which plays vital roles for neural communication.[57,59]

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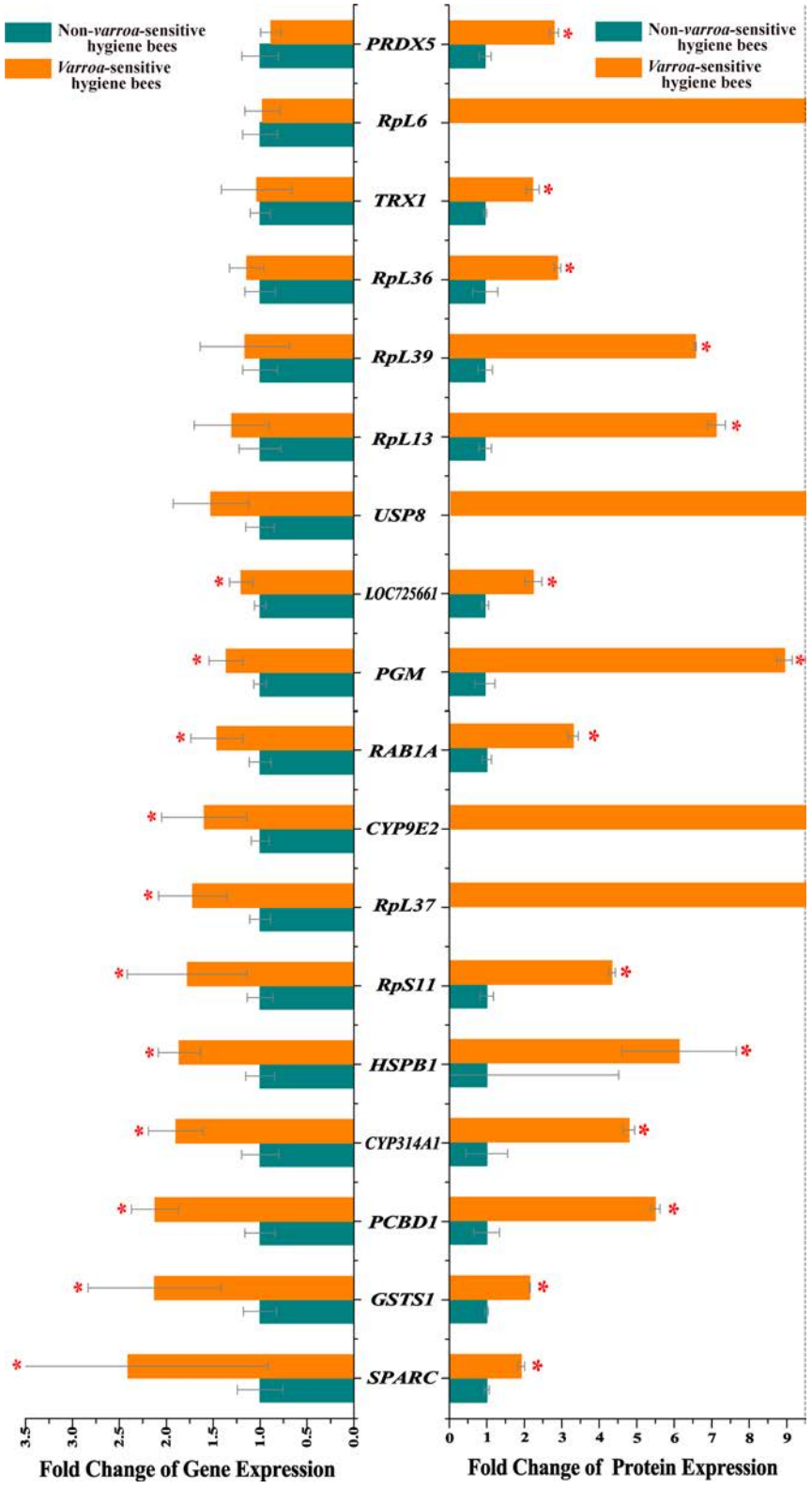

Figure 7. The gene expression level of the differentially expressed proteins in the hemolymph by qPCR analysis. The gene expression level is normalized with the reference gene (*GAPDH*). The primers and corresponding protein names are in Table S1. The error bar is the standard deviation. The protein expression is based on the quantitative analysis of proteome data in Table S5. The missed values of protein expression indicate that they are uniquely found in the hemolymph of VSH bees. *, $P < 0.05$.

In all neurons, communication across the synapse is mediated by neurotransmitters released from synaptic vesicles, and the synaptic vesicle fusion in the presynaptic structure requires a large body of regulators to ensure the spatial and temporal resolution of neurotransmitter release.[59,60] This procedure involves a wide array of proteins because the entire process takes only a fraction of a millisecond (in fast releasing synapses).[59,61] Here, the overexpressed 16, 20, and 15 proteins

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related to synaptic vesicles, synaptic roles, and calcium channels in hygienic bees may be the driving force for fast neurotransmitter release. This is because they are functionally important as organizers and cytoskeletal scaffolding; transporters and channels; sensors and signal transduction; priming, docking, and fusion apparatus; endocytotic and recycling machinery; and linkages between the presynaptic and postsynaptic membranes.[59,62] Of the 20 up-regulated proteins related to synaptic roles, genuine synaptic vesicle proteins, associated membrane proteins (VAMP, 57.52-fold), synaptobrevin (38.42-fold), and vesicle associated membrane proteins (2.58-fold) all act as vesicular soluble NSF attachment protein (SNAP) receptors (v-SNAREs), whereas the presynaptic membrane proteins syntaxin (22.33-fold) and SNAP (5.85-fold) function to target membrane SNAREs (t-SNAREs).[62] The proper targeting and docking of synaptic vesicles are mediated by cognate interactions between v-SNARE and t-SNAREs.[59] SNAREs are controlled by numerous regulators, including Rabs and their direct regulators. Of the 11 enhanced expression of Rabs in hygienic bees, Rab5 (9.22-fold), Rab27 (unique), and Rab11 (64.36-fold) are implicated in regulating vesicle transport, docking, and exocytosis of synaptic vesicles.[59,63,64] These highly expressed synaptic proteins in the MBs of hygienic bees may drive the higher cerebral activity through the rapid transmission of signals, which are likely required by learning, memorization, and communication to perform VSH.[26]

The concentration of intracellular Ca^{2+} regulates the secretory pathway of vesicle-mediated transport processes.[59,63,65,66] This is in line with our finding that several intracellular Ca^{2+} proteins and Ca^{2+}-regulated proteins were up-regulated in VSH bees. For example, Kv channel-interacting protein 2 (KChIP2, unique) can enhance neuronal excitability by inciting a response to changes in intracellular Ca^{2+}.[65] The calcium/calmodulin-dependent protein kinases (CAMKs, 2.28-fold) are activated by increases in the concentration of intracellular Ca^{2+} and transfer phosphates from ATP to defined serine or threonine residues in other proteins.[65,67] Calcium-dependent secretion activator 1 (2.44-fold) is a neural/endocrine-specific cytosolic and peripheral membrane protein required for the Ca^{2+}-regulated exocytosis of secretory vesicles.[65] The homeostasis of intracellular Ca^{2+} is regulated by plasma membrane calcium-transporting ATPase 3 (ATP2B3, 16.56-fold), which removes Ca^{2+} from the cells against very large concentration gradients, and voltage-dependent calcium channel subunit alpha-2/delta subunit 3 (Cacna2d3, 73.50-fold), thereby mediating the influx of Ca^{2+} into the cell upon membrane polarization.[68] The strengthened expression levels of proteins involved in the activities of the Ca^{2+} channel, together with the fact that intracellular Ca^{2+} acts as a secondary messenger in neurotransmitter release from neurons,[69] suggest that the functionality of the vesicle-medicated transport pathway is enhanced, thus promoting the neural excitability of the MBs in VSH bees. This notion is supported by a recent SNP-study,[70] which identified *Apis mellifera* octopamine receptor beta-2R as a candidate gene involved in VSH.

4.3. Increased Sensitivity of Olfaction in Antennae Related to Prime Signal Transport to Brain for VSH

Antennae are the primary sensory organs of the honeybee and are able to sense the presence of either the parasite itself or damaged/diseased pupae.[28] The HB is initiated by a diseased brood detection threshold, which is determined by how quickly a nurse can detect a diseased brood and initiate the removal process.[20,21,28] Therefore, the increased sensitivity of the olfactory system is essential for VSH. Contrasting the only functional group related energy turnover enriched in non-VSH bees, the enriched diverse functional classes and pathways (50 GO terms and 4 pathways) were implicated in olfactory sensitivity in VSH bees, such as small molecule metabolism, transport, vesicle-mediated transport, and ion transport. As is known, the olfactory system detects odorants with a limited set of odorant receptors. To cope with the demand for low odor selectivity, the odorant receptor must address this problem through anion-based signal transduction in which sensory cilia of olfactory receptor neurons accumulate chloride ions at rest and discharge a chloride current upon odor detection. Thereafter, this chloride current amplifies the receptor potential and promotes electrical excitation.[71] These altered proteome signatures between two hygienic phenotypes imply that the VSH bees have developed a distinct olfactory mechanism to drive their behavior by enhancing olfactory sensitivity to promote VSH. Again, it is reflected by our data in that some of the 126 highly expressed proteins in VSH bees support these neuronal and olfactory functions, such as odorant binding proteins (OBPs) and coatomer proteins (Cops). In the antennae, olfactory information is transmitted to the antennal lobe,[72,73] where three types of sensory hairs relay and amplify the olfactory cues. These form synapses with the antennal lobe neurons which, in turn, project axons to higher brain structures in the MB and lateral horn of the protocerebrum.[72] The chemoreception system of insects is mediated by olfactory receptors and soluble OBPs concentrated in the antennae,[27,73−75] which are vital for the normal functionality of the olfactory system.[75−77] The OBPs are the first biochemical step in odor transduction and are involved in carrying lipophilic odorants to the olfactory receptors through hydrophilic surroundings.[72,75,78] Here, the increased expressions of OBP17 precursor and OBP18 in VSH bees support the notion that these two proteins play key roles in responding to outside stimuli related to *Varroa* infestation, and inside regulatory cues. Upon entering the antennae, airborne molecules associated with diseased or dead bees interact with the OBPs.[79] It is known that phenethyl acetate is a key compound associated with *Ascosphaera apis* infection in larvae and is an induction of HB.[80] OBP18 has high affinities to several compounds, including phenethyl acetate and oleic acid, which is released by decaying insect corpses,[27] and it is also a component of pheromones emitted by *Varroa* females.[81] Therefore, the higher expression of OBP18 in VSH bees is indicative of the fact that it is vital for enhancing olfactory sensitivity in VSH bees toward the paralyzed brood or mites.[27]

Based on the anatomy of the synaptic vesicle, coatomer proteins (Cops) are presynaptic proteins. Coatomer protein vesicles, a type of cytosolic protein complex, are principally involved in the transport of cargo between the ER and early Golgi.[59,82] The high expression of intracellular transport protein coatomers (alphaCop, 1.50-fold and gammaCop, 2.19-fold) in VSH bees is involved in vesicle trafficking at synapses and other vesicle sorting pathways, thereby supporting the previously discussed importance of synaptic processes for VSH behavior. The up-regulated proteins related to transport process suggest that their functions are activated to provide strong signal inputs into higher centers of the brain and trigger VSH.[28] In all, the above-mentioned observations indicate that the VSH bees need

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to induce a wide array of biological mechanisms to enable the detection and removal of mite infestation.[27,80]

4.4. Candidate Biomarkers for Selective Breeding of Hygienic Bees

Reliable markers are important to facilitate MAS-based breeding programs. Here, we propose a novel array of protein biomarkers[32] for the prediction of VSH, which cover part of the previously suggested markers.[20,22,27,28] For example, heat shock proteins, ribosomal proteins, and GST in the hemolymph are in line with previous works.[47,49] Also, OBP18, VAMP, and CacyBP, vital presynaptic protein SNAP, and vesicle trafficking and sorting protein gammaCOP in antennae are in agreement with the work of other groups.[27,28] In addition, our suggested markers, such as cytochrome P450 and Dlic2, are in line with findings at the gene level.[20−22] However, some markers suggested by others,[28] such as amphiphsin and helicase 25E, were not found here, which is likely caused by the different bee species, breeding programs, and sampling methods applied in different studies. In addition, the validated tendency between the 11 genes and their protein expressions provides useful evidence in selecting for VSH. To date, breeding for HB is usually done by field assays using a freeze-killed brood.[27,28] However, these assays are not only resource intensive, but also lack sensitivity.[28] Our extended array of protein markers provides additional information for efficient selection of VSH-expressing genotypes by potentially enhancing the sensitivity and accuracy of MAS.

5. CONCLUSIONS

This work is the most comprehensive proteomic study of honeybee VSH to date, and the first to focus on individual bees expressing *Varroa*-specific HB. This approach was made possible by using near-infrared video observations to select real hygienic bees. Using state-of-the-art proteomic technology allowed us to achieve an unprecedented depth in proteome coverage of 3 tissues, representing >55% of the bee proteome, and this made the results more comprehensively useful in deciphering the biology that underlines VSH behavior. The VSH and non-VSH bees have developed very significant differences in proteome settings. Selection for VSH seems to have increased the expression of immune and stress-inducible proteins in the pupal hemolymph, leading to the hypothesis of a genetic bundling of factors related to social immunity. The MBs and antennae of adult workers reveal the great importance of neuronal development and sensory sensitivity for VSH expression. The expanded protein markers associated with VSH are potentially useful for MAS to guide selective breeding for VSH.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.6b00423.

Table S1. Primer sequences used for validatory real-time PCR of targeted genes. Table S2. Identification of proteins expressed in the hemolymph of non-VSH bees. Table S3. Identification of proteins expressed in the hemolymph of VSH bees. Table S4. Comparison of functional GO term enrichment of the hemolymph proteins identified in VSH bees and non-VSH bees. Table S5. Differentially expressed proteins in the hemolymph between VSH bees and non-VSH bees. Table S6. Functional GO term enrichment of proteins up-regulated in the hemolymph of VSH bees. Table S7. Identification of proteins expressed in the mushroom body of non-VSH bees. Table S8. Identification of proteins expressed in the mushroom body of VSH bees. Table S9. Comparison of the functional GO term enrichment of the mushroom body proteins identified in VSH bees and non-VSH bees. Table S10. Differentially expressed proteins in the mushroom body between VSH bees and non-VSH bees. Table S11. Functional GO term enrichment of proteins up-regulated in the mushroom body of VSH bees or non-VSH bees. Table S12. Identification of proteins expressed in the antennae of non-VSH bees. Table S13. Identification of proteins expressed in the antennae of VSH bees. Table S14. Comparison of functional GO term enrichment of the antennal proteins identified at VSH bees and non-VSH bees. Table S15. Differentially expressed proteins in the antennae between VSH bees and non-VSH bees. Table S16. Functional GO term enrichment of the proteins up-regulated in the antennae of VSH bees or non-VSH bees. (XLSX)

Figure S1. Qualitative comparison of proteins between VSH bees and non-VSH bees using ClueGO, a Cytoscape plugin software. Figure S2. The significantly enriched specific terms in each functional class and pathways of hemolymph proteins up-regulated in VSH bees corresponding to the pie chart of Figure 2B. Figure S3. Qualitative comparison of mushroom body proteins between VSH bees and non-VSH bees using ClueGO, a Cytoscape plugin software. Figure S4. The significantly enriched specific terms in each functional class and pathways of mushroom body proteins up-regulated in VSH bees (A) and non-VSH bees (B) corresponding to the pie chart of Figures 4B and C. Figure S5. Qualitative comparison of antennal proteins between VSH bees and non-VSH bees using ClueGO, a Cytoscape plugin software. Figure S6. The significantly enriched specific terms in each functional class and pathway of antennal proteins up-regulated in VSH bees (A) and non-VSH bees (B) corresponding to the pie chart of Figures 6B and C. (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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