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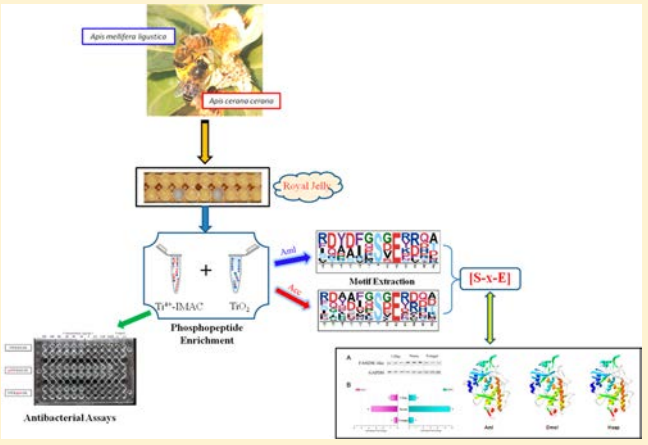
In-Depth Phosphoproteomic Analysis of Royal Jelly Derived from Western and Eastern Honeybee Species

Bin Han, Yu Fang, Mao Feng, Xiaoshan Lu, Xinmei Huo, Lifeng Meng, Bin Wu, and Jianke Li*

Institute of Apicultural Research/Key Laboratory of Pollinating Insect Biology, Ministry of Agriculture, Chinese Academy of Agricultural Science, Beijing 100093, China

Supporting Information

ABSTRACT: The proteins in royal jelly (RJ) play a pivotal role in the nutrition, immune defense, and cast determination of honeybee larvae and have a wide range of pharmacological and health-promoting functions for humans as well. Although the importance of post-translational modifications (PTMs) in protein function is known, investigation of protein phosphorylation of RJ proteins is still very limited. To this end, two complementary phosphopeptide enrichment materials (Ti^{4+}-IMAC and TiO_2) and high-sensitivity mass spectrometry were applied to establish a detailed phosphoproteome map and to qualitatively and quantitatively compare the phosphoproteomes of RJ produced by *Apis mellifera ligustica* (Aml) and *Apis cerana cerana* (Acc). In total, 16 phosphoproteins carrying 67 phosphorylation sites were identified in RJ derived from western bees, and nine proteins phosphorylated on 71 sites were found in RJ produced by eastern honeybees. Of which, eight phosphorylated proteins were common to both RJ samples, and the same motif ([S-x-E]) was extracted, suggesting that the function of major RJ proteins as nutrients and immune agents is evolutionary preserved in both of these honeybee species. All eight overlapping phosphoproteins showed significantly higher abundance in Acc-RJ than in Aml-RJ, and the phosphorylation of Jelleine-II (an antimicrobial peptide, TPFKLSLHL) at S^6 in Acc-RJ had stronger antimicrobial properties than that at T^1 in Aml-RJ even though the overall antimicrobial activity of Jelleine-II was found to decrease after phosphorylation. The differences in phosphosites, peptide abundance, and antimicrobial activity of the phosphorylated RJ proteins indicate that the two major honeybee species employ distinct phosphorylation strategies that align with their different biological characteristics shaped by evolution. The phosphorylation of RJ proteins are potentially driven by the activity of extracellular serine/threonine protein kinase FAM20C-like protein (FAM20C-like) through the [S-x-E] motif, which is supported by evidence that mRNA and protein expression of FAM20C-like protein kinase are both found in the highest level in the hypopharyngeal gland of nurse bees. Our data represent the first comprehensive RJ phosphorylation atlas, recording patterns of phosphorylated RJ protein abundance and antibacterial activity of some RJ proteins in two major managed honeybee species. These data constitute a firm basis for future research to better understand the biological roles of each RJ protein for honeybee biology and human health care.



KEYWORDS: *Royal jelly, phosphoproteome, Ti^{4+}-IMAC, TiO_2, western honeybee, eastern honeybee*

1. INTRODUCTION

Royal jelly (RJ), widely recognized as a healthy and functional food, is a yellowish−white cream secreted by the hypopharyngeal and mandibular glands in the head of young worker honeybees, normally at 5−15 days of age.[1] It is an indispensable diet for the queen bee and is responsible for providing nutrition and acting as a defense mechanism in protecting honeybee larvae within three days of age.[2,3] The biological importance of RJ in honeybee development also lies in its ability to influence honeybee caste determination and act as both an immune agent and nutritional source in the hemolymph of brood to usher their normal development.[4−6] In addition, RJ possesses a wide repertoire of pharmacological activities, such as antibacterial, anticancer, antihypertensive, and antioxidative effects, that can serve therapeutic purposes to promote human health.[7−10] Therefore, RJ is now successfully used in therapeutic products, health foods, and cosmetics.[11] The proteins in RJ are vital in the aforementioned bee biology and health-promoting activities; thus, it is of interest to conduct in-depth investigations into the RJ proteome. Driven by the fast improvement in proteomics and mass spectrometric technology, coverage of the RJ proteome has been largely expanded by identifying novel proteins in it.[12−16] To date, the RJ proteomes of the western (*Apis mellifera ligustica*, Aml) and eastern (*Apis cerana cerana*, Acc) honeybees have been analyzed and compared,[17−19] and major royal jelly protein 5

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(MRJP5) has been identified as a protein freshness marker.[20] Post-translational modifications (PTMs) are crucially important in regulating protein function, and identification of PTM sites is the first initial step toward analysis of protein function.[21] Despite the importance of PTMs for protein functionality, the analysis of PTMs in the RJ proteome still remains to be uncovered. Recently, our group comprehensively identified N-glycosylation modification in RJ.[22] However, mapping the phosphorylation status in RJ proteins still remains in its infancy and has been conducted only in Aml so far.[14,15,23]

Among all PTMs, reversible phosphorylation of proteins at serine, threonine, and tyrosine residues is one of the most important and pleiotropic modifications.[24] Generally, the critical roles of protein phosphorylation involve regulating enzyme activity, subcellular localization, and protein interaction with other molecules as well as enabling further covalent modification.[25−27] In regard to secretory proteins, phosphorylation has also been documented to play pivotal roles in enhancing the biological function of casein in milk,[28] acidic proline-rich protein in human saliva,[29] and melittin in honeybee venom.[30]

Currently, a wide variety of protocols are available for phosphopeptide enrichment, such as immunoprecipitation,[31] chemical modification,[32] and specific adsorbents.[33] Accumulating evidence suggests that applying a combination of different strategies could offer an in-depth identification of the phosphoproteome,[18,34] such as immobilized metal affinity chromatography (IMAC) and titanium dioxide (TiO_2) chromatography. IMAC is a reliable, efficient, and convenient approach for phosphopeptide enrichment. Usually, Fe^{3+}, Ga^{3+}, or Ni^{2+} is used as immobilized metal ions and bound to the stationary phase via iminodiacetic acid (IDA) or nitriloacetic acid (NTA) ligands.[35] Widely used IMAC has demonstrated its power in mapping the phosphoproteome of microorganisms, mammals, and insects.[34,36−38] Moreover, a newly developed IMAC procedure using Ti^{4+} metal ions and phosphate groups as the coordinating ligand to immobilize Ti^{4+} has shown superior specificity and efficiency for phosphopeptide enrichment.[33,39] However, IMAC has a tendency to enrich more acidic and multiply phosphorylated peptides, which restricts its selectivity for single phosphorylated peptides. TiO_2 chromatography is also an affinity-based enrichment approach that selectively isolates phosphopeptides from proteolytic-digested mixtures via bidentate binding at the TiO_2 surface. The major limitation of this method is, however, the retention of both acidic non-phosphopeptides (rich in glutamic and aspartic acid residues) and phosphopeptides.[40] To alleviate this problem, 2,5-dihydroxy benzoic acid (DHB),[32] phthalic acid,[41] and other acidic reagents are added to bind competitively with TiO_2, which significantly enhances the enrichment efficiency of TiO_2 toward phosphopeptides.[42]

Until now, only two major domesticated honeybee species have been widely used for crop pollination and bee-product collection, despite there being nine honeybee species in the genus *Apis*. In the western honeybee (*Apis mellifera*), the Italian bee (Aml) is the most widely distributed species and is used as a honey and RJ producer worldwide. As for the eastern honeybee (*Apis cerana*), the indigenous Chinese honeybee (Acc) comprises the largest population in Asia, with about three million colonies in China, and is mainly used to produce honey.[43] Our previous study showed that the amount of RJ produced by Acc is 10 times lower than that of Aml and that the abundance of most protein components is significantly higher in RJ of Aml than that in Acc.[19] Although recent work of our and other groups has identified several phosphorylation sites in MRJP1, MRJP2, apolipophorin-III-like protein, and icarapin, the phosphoproteome coverage of RJ proteins still remains largely unknown. To further extend our knowledge about RJ, it is of great importance to establish a detailed phosphoproteome map and identify the differences between RJ produced by the two different honeybee species. Therefore, the purpose of this study is to create an in-depth map of the RJ phosphoproteome of the western and eastern honeybee species and to compare differences in the phosphorylation state of these proteomes using two complementary enrichment methods, Ti^{4+}-IMAC and TiO_2 chromatography, as well as high-resolution and high mass accuracy mass spectrometry.

2. MATERIALS AND METHODS

2.1. Chemical Reagents

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Titanium oxide (Titansphere, 5 μm particles) was purchased from GL Science (GL-Sciences Inc., Japan). Ti^{4+}-IMAC material was kindly offered by Prof. H. F. Zhou (Dalian Institute of Chemical Physics, Chinese Academy of Sciences).

2.2. Sample Preparation

RJ was collected 68−72 h after bee larvae were transferred into the queen cells and gathered as a pooled sample from 250 queen cells from each of five colonies (Aml and Acc) at the apiary of the Institute of Apicultural Research, Chinese Academy of Agricultural Science, Beijing. Then, RJ proteins were extracted immediately once the RJ was sampled according to our previously described method with minor modifications. In brief, the fresh RJ was mixed with a lysis buffer (1 mg of RJ/10 μL of buffer) containing 8 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 20 mM tris (hydroxymethyl) aminomethane (tris-base), and 30 mM dithiothreitol (DTT). The mixture was homogenized for 30 min on ice and sonicated 5 s/min during this time; it was then centrifuged at 12 000*g* and 4 °C for 10 min and further centrifuged at 15 000*g* and 4 °C for 10 min. Three volumes of ice-cold acetone were added to the above collected supernatants, and the mixture was kept on ice for 30 min for protein precipitation and desalting. Subsequently, the mixture was centrifuged twice at 15 000*g* and 4 °C for 10 min. The supernatant was discarded, and the pellets were dried at room temperature. A total of 10 mg of dried pellets was dissolved in 40 mM NH_4HCO_3. Proteins were reduced with 10 mM DTT and then alkylated with 50 mM iodoacetamide. Proteins were digested using trypsin in a volume ratio of 1:50 (enzyme/protein) at 37 °C for 14 h. The enzymatic digestion was stopped by adding 1 μL of formic acid to the solution, and the samples were then vacuum-dried using a SpeedVac system (RVC 2-18, Marin Christ, Osterod, Germany).

One-day old bees were obtained by taking frames containing old pupae and placing them into an incubator (34 °C and 80% relative humidity) for their eclosion. The nurse bees and foragers were collected according to standard methods of identification: workers with heads in cells containing small larvae were identified as nurses, and bees flying into the hive with pollen loads were recognized as foragers.[44] More than 50 honeybee nurses and foragers were captured from each of the five colonies; then, the hypopharyngeal glands were dissected, and each kind of sample was pooled together. Three independent batches were collected and stored at −80 °C for later analysis. Next, the

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hypopharyngeal gland proteins were extracted essentially following the same method as that for RJ protein extraction.

2.3. Phosphopeptide Enrichment Using TiO_2

Phosphopeptide enrichment using TiO_2 was performed as previously described[32] with slight modifications. A digested sample was reconstituted in 500 μL of binding solution containing 6.0% trifluoroacetic acid (TFA)/80% acetonitrile (ACN)/0.2 M dihydroxy-benzoic acid (DHB). A TiO_2 slurry (GL Science, Japan) was prepared at a concentration of 10 mg/mL in the binding solution. About 50 μL of the prepared TiO_2 slurry (10 mg/mL in binding solution) was added to the above mixture and incubated at room temperature for 60 min with vigorous shaking. Then, the supernatant was discarded, and the resin was washed sequentially with 200 μL of binding solution, 0.5% TFA/50% ACN solution, and 0.1% TFA/30% ACN solution. The phosphopeptides were eluted from the resin twice with 100 μL of a 0.5 mM K_2HPO_4 solution. Finally, the two fractions were combined and desalted by Zip-Tip C18 columns (Millipore, Billerica, MA, USA). Thereafter, it was dried and stored at −80 °C for further LC−MS/MS analysis.

2.4. Phosphopeptide Enrichment Using Ti^{4+}-IMAC

Ti^{4+}-IMAC material was prepared, and phosphopeptide enrichment was carried out according to our previously described protocol with minor modifications.[33] In brief, the immobilized Ti^{4+} polymer beads (Ti^{4+}-IMAC) were obtained by incubation of 10 mg of polymer beads in 100 mM $Ti(SO_4)_2$ solution at room temperature overnight under gentle stirring. The obtained Ti^{4+}-IMAC beads were centrifuged at 20 000g for 2 min. After the removal of the supernatant, distilled water was used to wash the Ti^{4+}-IMAC beads several times to remove the residual titanium ions. The obtained Ti^{4+}-IMAC beads were dispersed in 30% ACN containing 0.1% TFA before usage. Then, digested samples were reconstituted in 500 μL of binding solution containing 6.0% TFA/80% ACN and incubated with 5 mg of Ti^{4+}-IMAC material at room temperature for 60 min with vigorous shaking. The supernatant was removed after centrifugation, and the beads with captured phosphopeptides were washed sequentially with 200 μL of binding solution, 0.6% TFA/50% ACN/200 mM NaCl solution, and 0.1% TFA/30% ACN solution. The bound phosphopeptides were then eluted twice with 100 μL of 10% ammonia solution with vibration for 10 min. Finally, the two fractions were combined and desalted by Zip-Tip C18 columns, followed by vacuum drying and then storage at −80 °C for further LC−MS/MS analysis.

2.5. Nano-LC−MS/MS Analysis

Phosphopeptide samples were analyzed using a Q-Exactive (Thermo Fisher Scientific, Bremen, Germany) with three replicates. The EASY-nLC 1000 (Thermo Fisher Scientific) nanoliquid chromatography system was coupled with the Q-Exactive via the nanoelectrospray source. Reverse-phase chromatography was performed according to the method described previously with some modifications.[45] The binary buffer system consisting of buffer A (0.5% acetic acid) and buffer B (80% ACN in 0.5% acetic acid) with a flow rate of 350 nL/min was used. The phosphopeptides were separated by the following gradient program: from 3 to 8% buffer B for 5 min, from 8 to 20% buffer B for 55 min, from 20 to 30% buffer B for 10 min, from 30 to 90% buffer B for 5 min, and 90% buffer B for 15 min. The Q-Exactive was operated in data dependent mode with survey scans acquired at a resolution of 70 000 at m/z 400. The top 20 most abundant ions with charge ≥ 2 from the survey scan were selected and fragmented by higher energy collisional dissociation, with normalized collision energies of 25. Xcalibur (version 2.2, Thermo Fisher Scientific) was used to retrieve MS/MS spectra.

2.6. Database Search and Site Localization

MS/MS data extracted in raw format were searched using in-house PEAKS software (version 6.0, Bioinformatics Solutions Inc.) against a constructed database containing protein sequences of *Apis* (downloaded April, 2013) and a common repository of adventitious proteins (cRAP, from The Global Proteome Machine Organization, downloaded April, 2013), totaling 32 515 entries. The following modifications were applied: carbamidomethylation (C)/+57.02 Da was selected fixed modification and oxidation (M)/+15.99 Da and Phospho (S, T, Y)/+79.96 Da were selected as variable modifications. The other parameters used were the following: parent ion mass tolerance, 15.0 ppm; fragment ion mass tolerance, 0.05 Da; enzyme, trypsin; allowing a nonspecific cleavage at neither end of the peptide; maximum missed cleavages per peptide, 2; maximum allowed variable PTM per peptide, 3. The false discovery rate (FDR) was filtered to ≤1.0% (−10 log P ≥ 20.0) with a target-decoy database searching strategy to distinguish positive and negative identifications.[36]

The phosphorylation sites were assigned using Scaffold PTM (version 1.1.3; Proteome Software, Portland, OR, USA) on the basis of the Ascore algorithm.[46] Only a site confidence greater than 95% was considered to be a mapped phosphorylation site in RJ proteins.

2.7. Semiquantitative Analysis

To quantify the abundance of the phosphorylated proteins, a spectral counting-based quantitation approach was employed according to that previously reported.[47] First, the number of MS/MS spectra observed for each phosphopeptide sequence in a single run was counted. The protein spectral count was calculated by summing the number of all peptide spectral counts assigned to the same protein in that run. Then, the spectral counts were converted into a normalized spectral abundance factor (NSAF) value. NSAF is a numerical value based on classical mathematical normalization, and an NSAF approach has proved to be as sensitive and reliable as labeling quantification.[48] Furthermore, the sequence length of a protein was considered when calculating NSAF because the number of spectral counts generated by a protein is correlated with the length of the protein.[49] Therefore, the following formula was applied:

$$(\mathrm{NSAF})_k = \frac{(\mathrm{SpC}/L)_k}{\sum_{i=1}^{n} (\mathrm{SpC}/L)_i}$$

For a protein k, SpC is the total number of MS/MS spectral counts, and L is the length in amino acid residues. So, the NSAF of protein k is the SpC/L of this protein divided by the sum of SpC/L of all n proteins in the experiment. To carry out statistical analysis of the data set, the NSAF values from three replicates were then natural log-transformed and statistically compared using an independent two-sample t-test. To avoid division by zero, zero spectral count values were replaced with an empirically determined fractional value of 0.5.[50] $p < 0.05$ was used to identify any significant differentially expressed phosphoproteins.

2.8. Verification of Phosphorylation Sites

To confirm the assigned phosphorylation sites on RJ proteins, four phosphorylation peptides were commercially synthesized using a solid-phase peptide synthesis process (ChinaPeptides

Ltd. Co., Shanghai, China). The MS/MS spectra were compared between the digested phosphorylation peptides from RJ proteins and the synthetic phosphorylation peptides. The phosphorylation sites were validated when a very similar peak profile was observed and the b-ions and y-ions between the digested peptides from RJ and synthetic peptides were almost identical, especially the ions next to the phosphorylation sites.

2.9. Bioinformatics Analysis

Specific motifs were extracted from the data set using the Motif-X algorithm (http://motif-x.med.harvard.edu/index.html),[51] and the *Drosophila* Proteome was chosen as the background. All single phosphorylation sites a with phosphorylation localization probability of >95% were used for motif extraction. The prealigned mode was used, so all candidate sequences had been aligned to 13 amino acids with a phosphorylated residue in the center before submission. The minimal reported number of occurrences for a given motif was set at 10. For our data set, the minimal number of motif occurrences was set to 10 for phosphorylated Ser and 5 for phosphorylated Thr and phosphorylated Tyr, and the threshold for significance was set to $p < 10^{-5}$.

The Group-based Prediction System (GPS, version 2.0) was adopted to predict kinase-specific phosphorylation sites. GPS software predicts kinase-specific phosphorylation sites in a query sequence based on the sites' similarity to known sites of kinases.[52] The thresholds for both serine/threonine kinases and tyrosine kinases were set to high, which means FDRs are 2 and 4%, respectively. FASTA format of all phosphoproteins was submitted, and after prediction, only unambiguous phosphorylation sites (phosphorylation localization probability 100%) were considered. If one phosphorylation site was predicted as the recognition site of multiple kinases, then the kinase with the highest value of score/cut-off was retained as the most likely candidate.

Protein sequences were aligned by Molecular Evolutionary Genetics Analysis (MEGA) software (version 6.0).[53] The Phyre2 online server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) was used to predict the three-dimensional structure of the interested proteins.[54] Then, the three-dimensional structure and the phosphorylated site were displayed by SPDBV software (version 4.1).[55]

2.10. Antibacterial Assays

Native Jelleine-II (TPFKLSLHL) and phosphorylated Jelleine-II (pTPFKLSLHL and TPFKLpSLHL) were commercially synthesized using a solid-phase peptide synthesis process (ChinaPeptides Ltd. Co., Shanghai, China) and then submitted to antimicrobial assays. Antibacterial activity of the peptides was examined according to a previously described method with some modifications. Briefly, bacteria were grown at 37 °C in Luria−Bertani medium (the remaining bacterial species), harvested in exponential phase (OD_{600nm}: 0.6−0.8), and resuspended in Muller−Hinton broth at a concentration of approximately 10^6 colony-forming units/mL. A double-diluted series of synthesized peptides was produced by physiological solution dilution. In sterile 96-well plates, 80 μL of a bacterial suspension was mixed with 20 μL of peptide solution series, making the final concentration gradient from 0.625 to 320 μg/mL. Bacterial growth inhibition was determined by measuring the absorbance at 492 nm following incubation for 18−24 h at 37 °C. Antibacterial activity was expressed as the minimal inhibitory concentration (MIC), the concentration at which 100% growth inhibition was observed after 18−24 h of incubation. The bacteria used were *Staphylococcus aureus* (ATCC 6538), *Bacilus subtilis* (ATCC 6051), *Escherichia coli* (CCT 1371), *Pseudomonas aeruginosa* (ATCC 13883), and *Paenibacillus larvae* (ATCC 13537); the yeast *Candida albicans* (ATCC 10231) was also used.

2.11. Real-Time Quantitative PCR (qPRC)

To validate whether the expression of FAM20C-like protein changes with RJ secretion at the transcription level, total RNA was extracted from the hypopharyngeal glands of newly emerged bees (1 day old), nurse and forager bees of Aml, using TRIzol reagent (Takara Bio, Kyoto, Japan). Each sample was analyzed individually and processed in triplicate. The mRNA level of FAM20C-like protein was examined by qPCR, and the primer sequences were based on the sequences in the honeybee cDNA library (GeneID: 410517); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (GeneID: 410122) was used as the reference gene. The following PCR primers were used: FAM20C primers, forward: 5′-TGGCGGAGGAGTTATCACAAGAG-3′, reserve: 5′-CCATGTCCATTAGATCCAGCAACC-3′; GAPDH primers, forward: 5′-GACTACTGTTCATGCTGTTACTG-3′, reserve: 5′-GTACTGGTACACGGAAGGC-3′. Reverse transcription was performed using a RNA PCR Kit (Takara Bio, Kyoto, Japan), according to the manufacturer's instructions. Real-time qPCR amplification was conducted on iQ5Multicolor real-time PCR detection system (Bio-Rad, Hercules, CA, USA) as previously described.[45] Gene expression data was normalized by GAPDH. After verifying amplification efficiency of the selected genes and GAPDH in approximately equal levels, the differences in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method.[56] The statistical analysis of gene expression was performed by one-way ANOVA (SPSS version 16.0, SPSS, Inc. Chicago, IL, USA) using Duncan's multiple-range test. An error probability $p < 0.05$ was considered statistically significant.

2.12. Western Blot Analysis

To further verify whether the expression tendency of extracellular serine/threonine protein kinase FAM20C-like protein was consistent with RJ secretion, western blot analysis was performed using a method described previously with some modifications.[45] Briefly, equal amounts of a protein sample (12 μg/lane) were separated by stacking (4%) and separating (12%) SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gels and then transferred to a nitrocellulose transfer membrane (0.2 μm pore size) (Invitrogen, Eugene, OR, USA) using an iBlot apparatus (Invitrogen). After blocking, the membranes were incubated for 2 h at room temperature with primary rabbit polyclonal antibodies recognizing FAM20C protein (Abcam, Cambridge, MA, USA) at a dilution of 1:500. Following three washes, the membranes were further incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary athentibody at a dilution of 1:2000 for 1.5 h. Immunoreactive protein bands were detected using the ECL western blotting substrate (Pierce, Rockford, IL, USA) and quantified by densitometry using Quantity-One image analysis system (Bio-Rad). GAPDH was detected simultaneously as a loading control of the analysis.

3. RESULTS

3.1. Extended Phosphoproteome of Aml-RJ

To achieve a more comprehensive map of the Aml-RJ phosphoproteome, two phosphopeptide enrichment strategies

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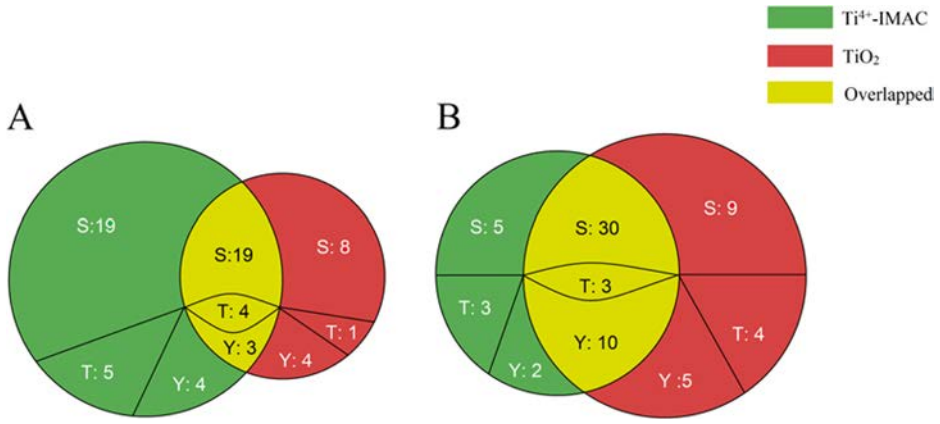

Figure 1. Comparison of the nonredundant phosphorylation sites identified by Ti^{4+}-IMAC and TiO_2. Only phosphorylatioin sites with a confidence score greater than 95% were included. S represents phosphorylated serine residues, T represents phosphorylated threonine residues, and Y represents phosphorylated phosphotyrosine residues. (A) Phosphorylation sites identified in royal jelly of *Apis mellifera ligustica*. (B) Phosphorylation sites identified in royal jelly of *Apis cerana cerana*.

with complementary efficiency, Ti^{4+}-IMAC and TiO_2 chromatography, were employed. In total, 67 phosphorylation sites with a >95% confidence assignment in 93 unique phosphopeptides, corresponding to 16 phosphoproteins, were identified in Aml-RJ with a FDR < 1.0% at the peptide and protein levels. Specifically, 54 phosphorylation sites resided on 59 unique phosphopeptides derived from 13 phosphoproteins, as determined by Ti^{4+}-IMAC. Using TiO_2 chromatography, 39 phosphorylation sites harbored on 52 unique phosphopeptides from 14 phosphoproteins were identified. All of the identified phosphorylated peptides and corresponding spectra are provided in Supporting Information Table 1 and Supporting Information Figure 1.

At the protein level, 11 phosphoproteins were identified by both approaches, including MRJP1−5, MRJP7, icarapin, defensin, apolipophorin-III-like protein, hypothetical protein LOC408608, and protein DDB_G0271606-like. Meanwhile, two proteins, extracellular serine/threonine protein kinase FAM20C-like and uncharacterized protein LOC100577669 isoform X1, were uniquely identified by the Ti^{4+}-IMAC approach. Three proteins, f-box only protein 7-like, activating molecule in BECN1-regulated autophagy protein 1-like isoform X1, and protein CREG1, were identified only by TiO_2 chromatography. Among the 67 unambiguously mapped phosphorylation sites, 46 were phosphoserine (pS), 10 were phosphothreonine (pT), and 11 were phosphotyrosine (pY), in which 26 sites were identified by both strategies, and 28 and 13 phosphorylation sites were exclusively identified by Ti^{4+}-IMAC and TiO_2 chromatography, respectively (Figure 1A). Of all of the identified phosphorylation sites, only five have been identified before, and the other 62 phosphorylation sites were newly assigned.

3.2. Novel Phosphoproteome of Acc-RJ

The same approaches applied to Aml-RJ were also applied to analyze the Acc-RJ phosphoproteome. In total, 71 phosphorylation sites were mapped onto 113 unique phosphopeptides that correspond to nine phosphoproteins. Among these, 53 phosphorylation sites localized in 66 unique phosphopeptides corresponding to eight phosphoproteins were identified by Ti^{4+}-IMAC. Similarly, 61 phosphorylation sites assigned to 78 unique phosphopeptides corresponding to nine phosphoproteins were identified by TiO_2 chromatography (Supporting Information Table 2 and Supporting Information Figure 1). All of these phosphorylation sites and phophoproteins were reported for the first time here.

Of nine phosphoproteins in Acc-RJ, eight phosphoproteins, MRJP1−5, MRJP7, icarapin, and defensin, were identified by both enrichment methods, whereas hymenoptaecin was exclusively identified by TiO_2 enrichment. Of all 71 unambiguous phosphorylation sites, 44 were pS, 10 were pT, and 17 were pY, of which 43 sites were identified by both strategies, whereas 10 and 18 phosphorylation sites were specifically identified by Ti^{4+}-IMAC and TiO_2 chromatography, respectively (Figure 1B).

3.3. Qualitative and Quantitative Comparisons between the Phosphoproteomes of Aml-RJ and Acc-RJ

In order to reveal differences in the phosphorylation status between Aml-RJ and Acc-RJ, all identified nonredundant phosphoproteins were compared qualitatively and quantitatively. Eight proteins were found to be phosphorylated in both samples, including six major royal jelly proteins (MRJP1−5 and MRJP7), icarapin, and defensin. However, the number of phosphorylation sites and site locations on the same protein were different. Specifically, in Aml-RJ, the number of identified phosphorylation sites in MRJP1−5,MRJP7, icarapin, and defensin was 10 (S: 6, T: 1, Y: 3), 10 (S: 4, T: 3, Y: 3), 10 (S: 8, T: 1, Y: 1), 4 (S: 1, T: 1, Y: 2), 6 (S: 3, T: 1, Y: 2), 4 (S: 3, T: 1), 4 (S: 3, T: 1), and 1 (T:1), respectively. Likewise, in Acc-R,J the number of assigned phosphorylation sites in MRJP1−5, MRJP7, icarapin, and defensin was 20 (S: 11, T: 1, Y: 8), 10 (S: 5, T: 3, Y: 2), 9 (S: 6, Y: 3), 11 (S: 6, T: 4, Y: 1), 10 (S: 7, T: 1, Y: 2), 4 (S: 3, Y: 1), 4 (S:4), and 2 (S:1, T:1), respectively. Apart from the eight shared phosphoproteins above, eight other phosphoproteins (apolipophorin-III-like protein, hypothetical protein LOC408608, putative uncharacterized protein DDB_G0271606-like, extracellular serine/threonine protein kinase FAM20C-like, uncharacterized protein LOC100577669 isoform X1, f-box only protein 7-like, activating molecule in BECN1-regulated autophagy protein 1-like isoform X1, and protein CREG1) were identified only in Aml-RJ, whereas hymenoptaecin was exclusively identified in Acc-RJ (Figure 2).

To gain a better understanding of the different phosphorylation levels of the two RJ samples, a spectral counting-based strategy was used for a semiquantitative comparison of all identified phosphoproteins. Quantitation of the abundance of phosphoproteins was preformed separately in terms of the two phosphopeptide enrichment protocols. Noticeably, the abun-

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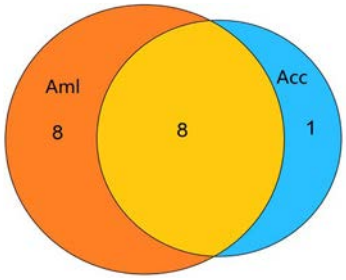

Figure 2. Comparison of the number of nonredundant phosphorylated proteins identified in royal jelly of *Apis mellifera ligustica* and *Apis cerana cerana*. The Venn diagram shows the taxonomical distribution of 17 nonredundant phosphorylated proteins. The pie chart on the right represents phosphorylation proteins identified in royal jelly of *Apis cerana cerana*, and the pie chart on the left represents proteins identified in royal jelly of *Apis mellifera ligustica*.

dance level of all eight shared phosphoproteins was significantly higher in Acc-RJ than in Aml-RJ ($p < 0.05$). As for the phosphoproteins identified only in one sample, their respective abundance was also statistically significant (Figure 3 and Supporting Information Table 3).

3.4. Verification of Phosphorylation Sites

To validate the phosphorylation sites, four peptides, CENPNNDNTPFKIsIHL, VtCDLLSFK, GYGVsVYEGVARIsGEDPSDNPTLGELKK, and KNVDtVLVLPsIER, which are from MRJP1, defensin, extracellular serine/threonine protein kinase FAM20C-like protein, and icarapin, respectively, were commercially synthesized and identified by MS, and then their MS/MS spectra were compared with the digested peptides from RJ proteins. Of the four synthetic peptides, CENPNNDNTPFKIsIHL belongs to Acc, VtCDLLSFK was identified in both Aml-RJ and Acc-RJ, and the other two phosphorylated peptides were from Aml proteins. The results showed a very similar peak profile of b-ions and y-ions between the digested peptides from RJ and artificially synthesized peptides. This confirms that phosphorylation modifications occur at S^{430} in MRJP1, at S^{135} and S^{144} in FAM20C-like protein, and at T^{37} and S^{43} in icarapin. Meanwhile, in defensin, phosphorylation occurs at T^{20} in Aml and at T^{45} in Acc (Figure 4).

3.5. Motif Analysis and Kinase Prediction

To uncover the relationships between phosphorylation sites in RJ proteins and protein kinases, all phosphorylated sites having >95% probability were used for motif analysis. In this study, due to the simplicity of the RJ protein constitution, [S-x-E] was the only extracted motif from both samples (Figure 5 and Supporting Information Table 4); neither a threonine nor tyrosine motif was extracted.

Possible kinase families were predicted by submitting FASTA sequences of all phosphoproteins to GPS 2.0. After filtering by phosphorylation localization probability (>95% probability) and score/cut-off value (the highest), 45 and 55 pairs of predicted kinase−substrate relationships were obtained from Aml-RJ and Acc-RJ, which belonged to 23 and 30 kinase groups, respectively. The top three serine/threonine families represented in Aml-RJ were CAMK, STE, and TKL. Likewise, STE, AGC, and CAMK/TKL were the top three serine/threonine groups in Acc-RJ. The

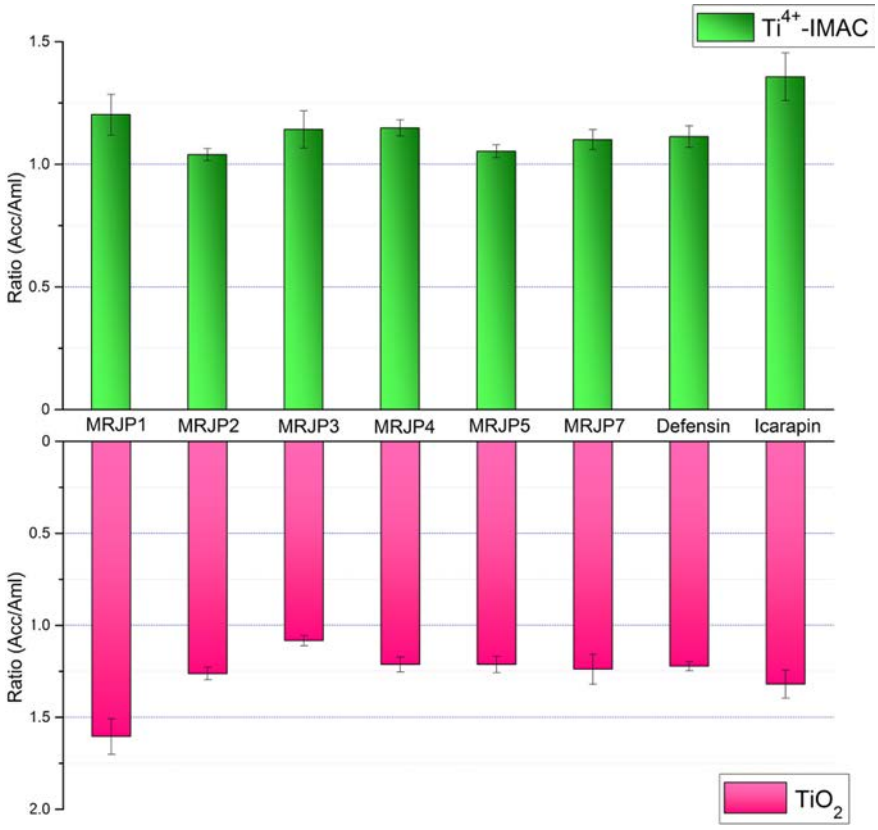

Figure 3. Quantitative comparison of phosphoproteins identified by Ti^{4+}-IMAC and TiO_2 in royal jelly of *Apis cerana cerana* and *Apis mellifera ligustica*. The ratio of the protein abundance is shown as *Apis cerana cerana* to *Apis mellifera ligustica*. All compared phosphoproteins are significantly higher in royal jelly of *Apis cerana cerana* compared to that in *Apis mellifera ligustica*.

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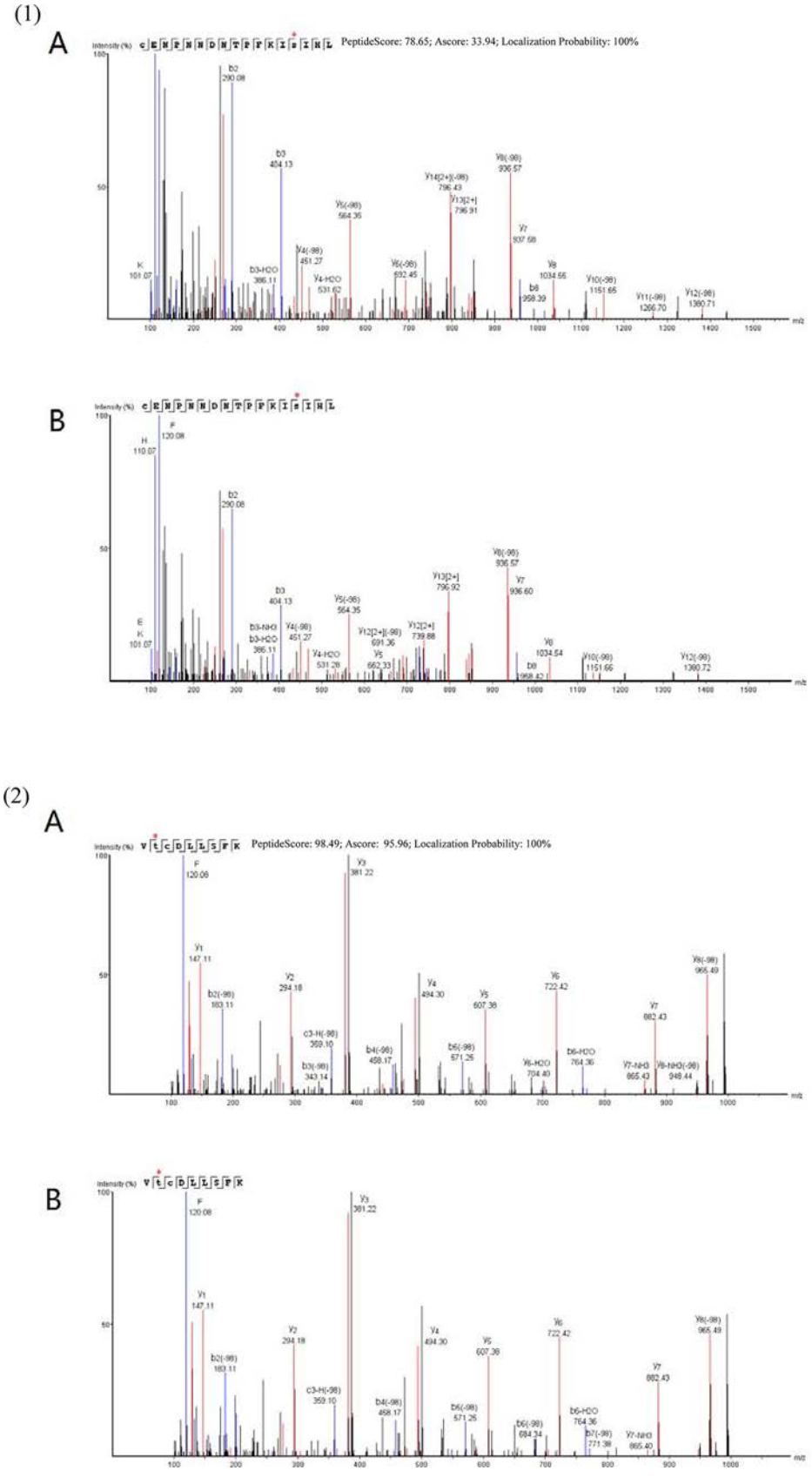

Figure 4. continued

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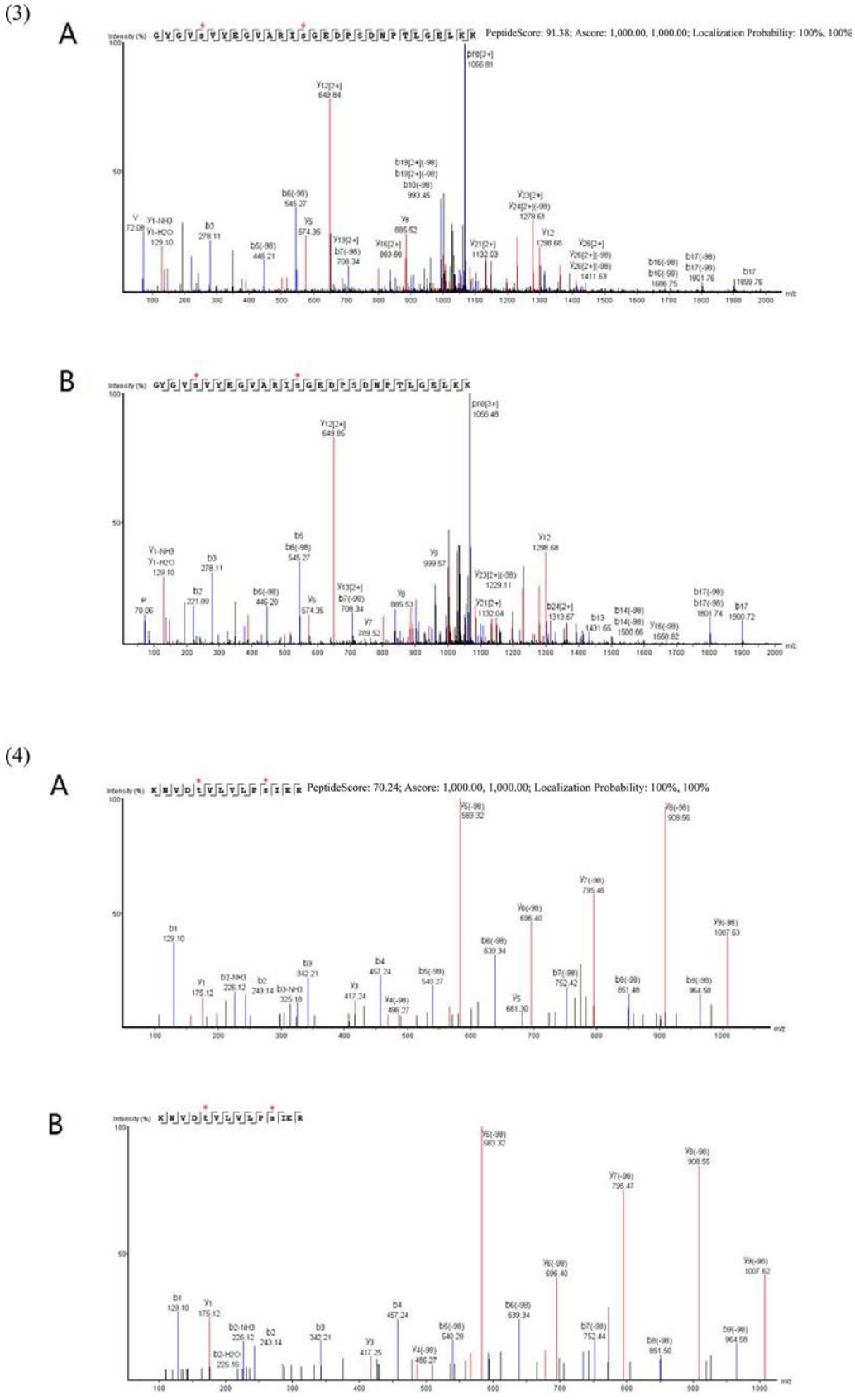

Figure 4. Tandem mass spectra comparison between the digested phosphorylation peptides from RJ proteins (A) and the synthetic phosphorylation peptides (B). (1) CENPNNDNTPFKIsIHL from MRJP1. (2) VtCDLLSFK from defensin. (3) GYGVsVYEGVARIsGEDPSDNPTLGELKK from extracellular serine/threonine protein kinase FAM20C-like protein. (4) KNVDtVLVLPsIER from icarapin.

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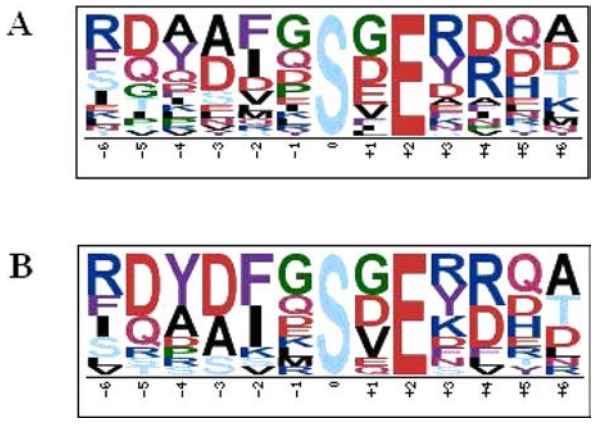

Figure 5. Phosphorylation motifs extracted from the data set using the Motif-X algorithm. Only phosphorylatioin sites with a confidence score greater than 95% were considered. (A) Motif extracted from royal jelly proteins of *Apis mellifera ligustica*. (B) Motif extracted from royal jelly proteins of *Apis cerana cerana*.

VEGFR was the most frequently observed tyrosine kinase group in both samples (Figure 6 and Supporting Information Table 5).

3.6. Antibacterial Assays

With the aim of determining the influence of protein phosphorylation on the antibacterial activity of the Jelleines, native Jelleine-II (TPFKLSLHL) and two forms of phosphorylated Jelleine-II, Jelleine-II (pT) and Jelleine-II (pS), were synthesized for antibacterial assays against five species of bacteria and one species of yeast. The results (Table 1) showed that native Jelleine-II was active against all five bacteria species and the yeast, whereas the Jelleine-II (pS) presented a reduced spectrum of antibiosis that had no inhibitory effect on *S. aureus* and *P. aeruginosa*; Jelleine-II (pT) inhibited only *E. coli* and had no antibacterial activity against the other microorganisms, even at the concentration of 320 μg/mL.

Table 1. Antimicrobial Spectrum of Jelleine-II, Jelleine-II (pT), and Jelleine-II (pS) Indicated by the Values of Minimal Inhibitory Concentration (MIC) for Each Sensitive Microorganism

	MIC (μg/mL)		
microorganisms	TPFKLSLHL	pTPFKLSLHL[a]	TPFKLpSLHL[a]
Gram-Positive Bacteria			
B. subtilis (ATCC 6051)	40	R	40
S. aureus (ATCC 6538)	80	R	R
P. larvae (ATCC 13537)	40	R	40
Gram-Negative Bacteria			
E. coli (CCT 1371)	10	160	40
P. aeruginosa (ATCC 13883)	80	R	R
Yeast			
C. albicans	10	R	80

[a]R: microorganism resistant to the peptide.

3.7. Test of FAM20C-Like Protein Expression in the Hypopharyngeal Gland by RT-qPCR and Western Blot Analysis

RJ is mainly produced by the hypopharyngeal gland of nurse honeybees, and newly emerged bees and foragers cannot produce it.[57] To validate the hypothesis that FAM20C-like protein is responsible for phosphorylating RJ proteins through [S-x-E] motifs,[58] total RNA and protein were extracted from the hypopharyngeal gland of newly emerged bees (day one after emergence), nurse bees, and foragers of Aml for qRT-PCR and western blot analysis. The expression levels of both mRNA and protein of FAM20C-like were significantly higher in nurse bees than that in newly emerged and forager bees. Specifically, the mRNA and protein expression of FAM20C-like in nurse bees were significantly higher than that in newly emerged bees and foragers (Figure 7).

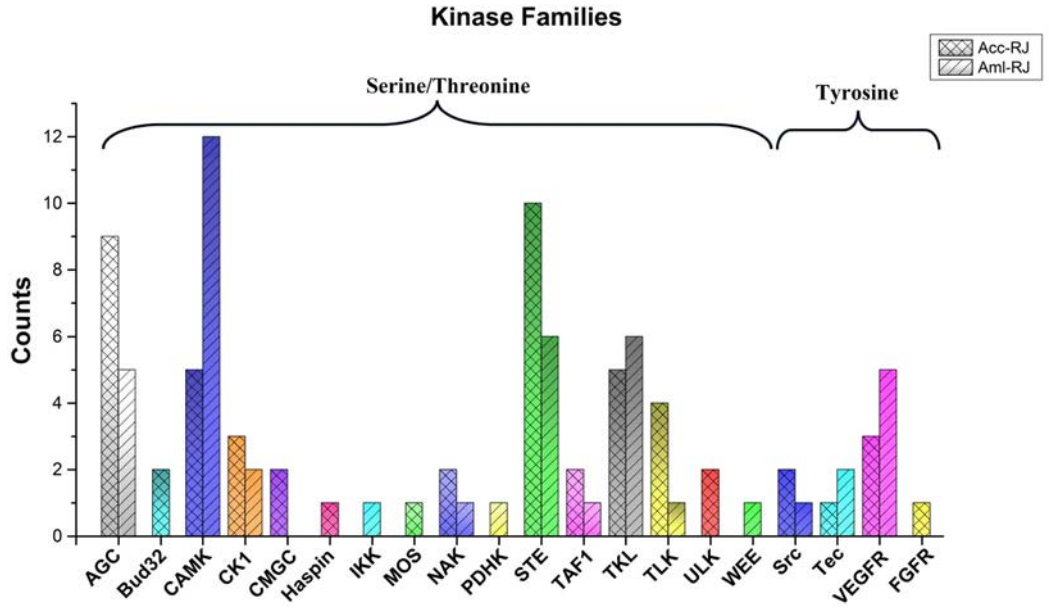

Figure 6. Predicted kinase families against phosphorylation sites in the royal jelly proteins of *Apis cerana cerana* and *Apis mellifera ligustica* using GPS 2.0. Only the phosphorylated sites with a confidence score greater than 95% and the highest score/cut-off value were used. The *y* axis is the number of kinase-substrates under each kinase family and is represented by the bars.

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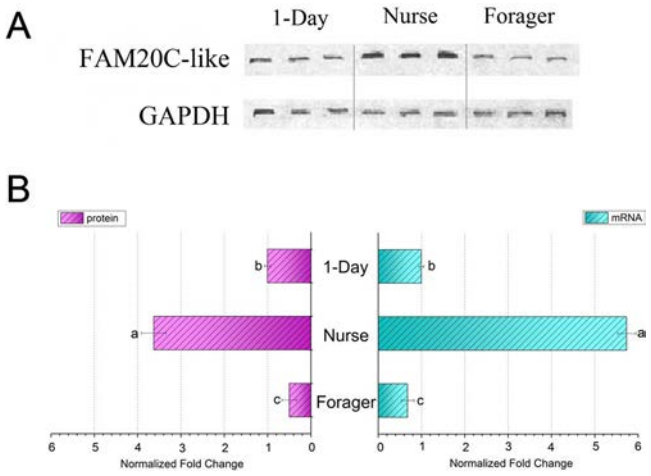

Figure 7. Western blot and RT-qPCR analysis of extracellular serine/threonine protein kinase FAM20C-like protein (FAM20C-like). Total protein and RNA were extracted from the hypopharingeal gland of newly emerged bees (day one), nurse bees, and foragers of *Apis mellifera ligustica* for western blot and qRT-PCR analysis. The protein samples from the hypopharingeal gland were subjected to SDS-PAGE followed by western blot analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference control. (A) Western blot bands of FAM20C-like and GAPDH. (B) Normalized fold change of FAM20C-like in the three different aged honey bee hypopharingeal glands (normalized by GAPDH) (a is significantly higher than b and c, and b is significantly higher than c). Error bars represent the standard deviation.

4. DISSCUSSION

In-depth, large-scale mapping of phosphorylation sites is of vital importance to gain insight into the biological functions of RJ proteins. Here, we employed a complementary enrichment strategy (Ti^{4+}-IMAC and TiO_2) coupled with high-resolution and high mass accuracy mass spectrometry to identify and compare the phosphoproteome of RJ derived from Aml and Acc. In total, 16 phosphoproteins carrying 67 phosphorylation sites were identified in Aml-RJ, and nine phosphoproteins with 71 phosphorylation sites were found in Acc-RJ. These results covered most of the MRJPs, accounting for >90% of RJ proteins,[59] and other RJ proteins. Due to the biological nature of RJ, there are not many biological samples for determining the absolute protein species that comprise it; therefore, our proteomic survey ranks among the largest reported in RJ, and our phosphorylation survey is among the largest accrued. Furthermore, all data have been collected and analyzed using the same state-of-the-art techniques, ensuring results of a consistently high quality. Our study identified phosphorylated sites in six MRJP members in both RJ samples, significantly expanding the phosphoproteome of Aml-RJ and comprehensively characterizing the phosphoproteome of Acc-RJ for the first time. Specifically, eight proteins phosphorylated in both RJ samples were mainly MRJPs, and the same motif ([S-x-E]) was extracted. This data, combined with the similar kinase families that were predicted from the two RJ samples, indicates that the principal functions of RJ proteins as nutrients and immune agents have been evolutionarily preserved in both the western and eastern honeybee (Figures 3 and 4). On the other hand, although these two bee species are of the same *Apis* genus, their native ecological ranges vary dramatically; western bees colonize a diverse native range over almost all the world, whereas eastern bees are confined to the Asian continent.[45] To better survive in the current ecological landscape, both species have experienced strong physiological changes through selection. The different phosphorylation sites, abundances of phosphorylated RJ proteins, and changes in the antibacterial activity of some antimicrobial peptides observed between these two honeybee species may serve the purpose of helping them adapt to their current ecology through employing different protein phosphorylation strategies in nutrient and defense mechanisms. It must be emphasized that an important protein kinase, FAM20C-like protein, which recognizes the [S-x-E] motif,[58] was novelly found in Aml-RJ. Furthermore, RT-qPCR and western blot evidence showed that the mRNA and protein expression of FAM20C-like protein reached the highest level in the hypopharyngeal gland of nurse bees, which is in-line with RJ secretion, suggesting that FAM20C-like protein in the hypopharyngeal gland of honeybees may be the driving force for phosphorylation of RJ proteins by recognizing the [S-x-E] motif. To our knowledge, this is the first comprehensive report of phosphorylation sites in proteins of Acc-RJ and thus significantly expands our knowledge of the phosphorylation sites in proteins of Aml-RJ.

4.1. Phosphorylation of MRJPs: Modulating Biological Functions

Although efforts have been made to identify protein phosphorylation in MRJPs,[15] only potential phosphorylated sites for MRJP2 and MRJP7 in Aml-RJ have been reported but without site assignment.[14] Our previous work has identified only seven phosphorylation sites on MRJPs of Aml-RJ due to the poor MS resolution and sensitivity of the applied method.[23] In this study, a total of 44 and 64 phosphorylation sites were assigned to MRJPs of Aml-RJ and Acc-RJ, respectively.

MRJPs are well-known for their nutritional roles as a protein source in boosting the speed of development of honeybee larvae and the fecundity of the honeybee queen, due to their high level of essential amino acids and their nitrogen storage function (concentrated with nitrogen-rich amino acids).[60,61] In human and bovine milk, phosphorylated caseins can stabilize amorphous calcium phosphate to support the early development of the newborn.[62,63] Therefore, the phosphorylated MRJPs may serve the same purpose as that of mammalian milk in the enhancement of nutritional efficiency via binding calcium ions to satisfy the nutritional demands of honeybee larvae and queens.

In addition to serving as a nutrient supply, MRJPs have been experimentally confirmed as having diverse biological functions. MRJP1 functions as a growth factor that increases the growth and proliferation of rat hepatocytes,[64] human lymphoid cells,[65] and myeloid cells.[66] Recent evidence has suggested that MRJPs are important defense agents in hemolymph for young larvae, in which glycosylated MRJP2 can effectively inhibit American foulbrood (*P. larvae*).[6,53,67] Moreover, MRJP1−3 are reported to modulate immune responses in mice.[68,69] Although they exist mainly in RJ, MRJPs have been also found in honeybee venom[70] and several honeybee tissues, such as the brain and ovaries;[71−73] their expression in these tissues is largely influenced by age, division of labor, and bacterial challenges, suggesting that MRJPs play context-dependent roles in honeybee development and colony organization.[74] The predicted kinase groups of CAMK, STE, AGC, TKL, and VEGFR suggest likely roles in driving the phosphorylation of RJ proteins (Figure 6). CAMK is a family of calcium/calmodulin regulated kinases comprising MLCK, DAPK, MAPKAPK, and PHK,[75] of which MLCK has been demonstrated to phosphorylate the myosin II regulatory light chain (RLC) to stimulate muscle contraction.[76] RLC is a well-known eukaryotic motor protein that interacts with filamentous

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actin. Intriguingly, MRJP1 has also been found to be associated with proteins of filamentous structures in the honeybee brain;[72] hence, phosphorylation of MRJP1 may facilitate its interaction with filamentous actin. The STE group is part of the mitogen-activated protein kinase (MAPK) cascade kinases,[77] involved in transducing environmental and developmental signals into adaptive intracellular responses, which are employed for activating immune responses against microorganisms that invade both insects and plants and eliminating abiotic-induced oxidative stresses.[78−80] Importantly, MRJP1 has been reported to activate the MAPK pathway in the fat body to increase cell size and reduce developmental time in *Drosophila*.[4] It is thus believed that MRJP1 is potentially phosphorylated by MAPK to regulate this developmental process in insects. Likewise, the AGC group of cyclic nucleotide-dependent kinases mainly comprises the PKA, PKG, and PKC families.[81] In honeybees, PKA is implicated in managing chemosensory stimulation in the antennal lobe,[82] and both PKA and PKC are associated with olfactory learning and memory formation.[83,84] Because MRJPs are predicted to be substrates of these protein kinases, this suggests that MRJPs may be functionally important in these signaling pathways. Given the significant contribution of phosphorylation modifications in modulating protein function, it is likely that phosphorylated MRJPs perform a wide array of biological functions for the good of the species.

Due to the high similarity of the MRJPs amino acid sequences between these two honeybee species, some family members, such as MRJP2 and MRJP3 (identities of 91 and 88%), were quite similar in their number and location of phosphorylation sites. On the contrary, for proteins that shared relatively low identities, such as MRJP4 (83%), MRJP5 (79%), and MRJP7 (86%), there were larger differences in the number and/or position of phosphorylation sites. Since the specificity of kinase−substrate interactions is largely determined by sequence motifs in the substrates, the residues around the phosphorylation site are crucially important.[85] Also, changes in the amino acid sequence among these proteins partially influenced their spatial structures, which was clearly shown in the predicted 3D structures of the proteins (Supporting Information Figure 2). Accordingly, differences in the motifs and spatial structures in these proteins may lead to variation in their phosphorylation sites. These differences may be functionally important for different bee species to achieve their biological functions, which is reflected in our data whereby the phosphorylation of Jellssin-II at S^6 of Acc-RJ has stronger antibacterial activity than that at T^1 in Aml-RJ (Table 1).

Noticeably, in spite of the high sequence similarity in MRJP1 between these two species, the number of phosphorylation sites in Acc-MRJP1 is twice that of Aml-MRJP1. This is generally in accordance with the higher degree of polymorphism found in MRJP1 of Acc-RJ than that in Aml-RJ observed via a 2-DE gel.[19] Although our previous work has showed a higher abundance of MRJPs in Aml-RJ than in Acc-RJ at the total protein level,[19] the abundance of phosphorylated MRJPs in Acc-RJ was higher than that in Aml-RJ. As the native ecological ranges of Aml and Acc are quite different,[45] they have evolved large differences in physiology and behavior to better survive their present ecological ranges.[86] For instance, Acc has unique biological characteristics for resisting both wasps and the ectoparasitic mite, *Varroa destructor*, whereas most Aml are susceptible to both of these.[87,88] Acc workers have smaller body sizes and start working earlier in the day than Aml workers, and they can survive extreme fluctuations in ambient temperature and long periods of rainfall.[89] Thus, it is believed that their ecology has shaped these two bee species by applying different protein phosphorylation strategies to modify their own MRJPs to meet their respective biological needs. This is also supported by the evidence that phosphorylated Jelleine-II contained in MRJP 1 of Acc-RJ showed stronger a propensity to resist *P. larvae*, *B. subtilis*, *E. coli*, and *C. albicans* than that in Aml-RJ (Table 1).

4.2. Phosphorylation of Antimicrobial Peptide: Altering Antibiotic Activity

Antimicrobial peptides are natural antibiotic substances that display broad-spectrum activity against a wide variety of microorganisms and play important roles in innate immunity in the plant, insect, and animal kingdoms.[90,91] However, in contrast to that for mammals, insects do not have a lymphocyte-based immune system, so antimicrobial peptides are their most effective weapon to fight against invading pathogens.[92] In honeybees, four potent antimicrobial peptides are released into the hemolymph once they are infected by a pathogen, i.e., apidaecins, abaecin, hymenoptaecin, and defensin.[92] Notably, in this experiment, defensin was phosphorylated at one site, T^{20}, in Aml-RJ and at two sites, T^{45} and S^{85}, in Acc-RJ. Meanwhile, hymenoptaecin was phosphorylated at S^{52} in Acc but not in Aml. In general, both defensin and hymenoptaecin are cationic peptides with a characteristic six cysteine/three disulfide bridge pattern.[93] It has been reported that honeybee defensin in RJ executes antibacterial activity against the growth of some Gram-positive bacteria and fungi, particularly showing inhibitory effects toward *P. larvae*.[94] By comparison, hymenoptaecin has a wider antibacterial spectrum against both Gram-negative and -positive bacteria that challenge the honeybee, which even includes several human pathogens.[95] Recent work has shown that phosphorylation changes the toxicity of melittin in bee venom, an antimicrobial peptide with hemolytic activity.[39] Accordingly, the different phosphorylation status of defensin and hymenoptaecin indicates that different honeybee species may employ distinct strategies to boost their defense system. It has also been reported that more defensin and hymenoptaecin cDNA and peptide are generated by Acc than by Aml, specifically hymenoptaecin.[96] Here, we present new evidence that the variation in the phosphorylation of these antimicrobial peptides between the western and eastern honeybee may also be related to their different biological traits.

The Jelleines represent another family of antimicrobial peptides identified in RJ, containing four members with 8−9 amino acids (Jelleine-I, PFKLSLHL; Jelleine-II, TPFKLSLHL; Jelleine-III, EPFKLSLHL; Jelleine-IV, TPFKLSLH). Functionally, Jelleine-I to III can be effective against yeast and Gram-negative and -positive bacteria.[86] Since Jelleines are identical to the C-terminal region of the MRJP1, existing work suggests they are produced by serial tryptic digestion and the exoproteinase action of MRJP1.[86] In this scenario, considering the phosphorylated MRJP1 at T^{424} in Aml-RJ and at S^{430} in Acc-RJ, there is a high possibility that phosphorylated Jelleine-II (pTPFKLSLHL) and Jelleine-IV (pTPFKLSLH) exist in Aml-RJ and that phosphorylated Jelleine-I (PFKLpSLHL), Jelleine-II (TPFKLpSLHL), and Jelleine-IV (TPFKLpSLH) exist in Acc-RJ. Therefore, to analyze the phosphorylation−activity relationships of Jelleines, we synthesized native Jelleine-II and phosphorylated Jelleine-II at two different sites, Jelleine-II (pT) and Jelleine-II (pS), for use in antibacterial assays. The results showed an apparent reduction in the antibacterial activity and antibiotic spectrum of Jelleine-II after phosphorylation

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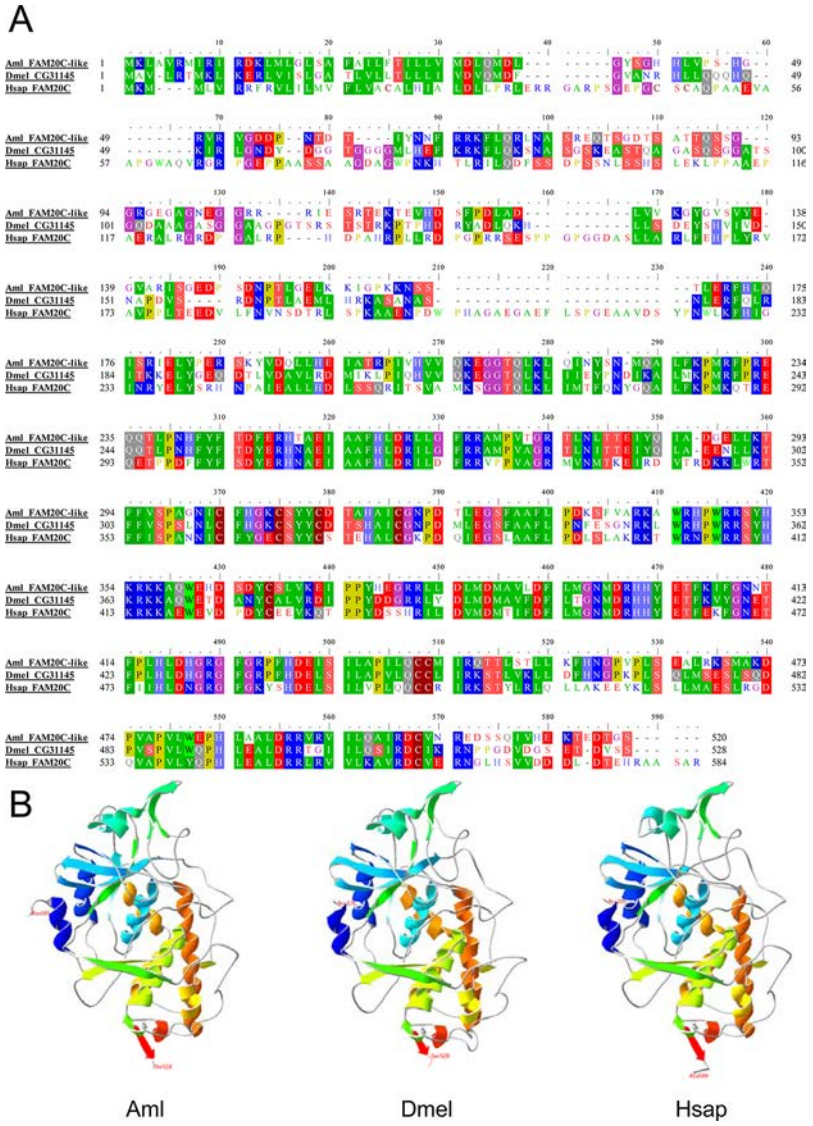

Figure 8. Protein sequence alignment and three-dimensional structure comparison of FAM20C proteins. FAM20C protein of *Apis mellifera ligustica* (Aml FAM20C-like), *Drosophila melanogaster* (Dmel CG31145, isoform C), and *Homo sapiens* (Hsap FAM20C) were aligned by Molecular Evolutionary Genetics Analysis (MEGA) software (version 6.0). Three-dimensional structures were predicted by the Phyre2 online server and displayed using SPDBV software (version 4.1). (A) Protein sequence alignment. (B) Three-dimensional structure comparison.

(Table 1). The Thr residue in the N-terminus of Jelleines does not seem to be very important for antibiotic activity because native Jelleine-I and -II exhibit similar antibiotic properties.[86] However, the antibacterial activity of Jelleine-II was significantly decreased after the Thr residue was phosphorylated. Because phosphorylation provides a negative charge to the peptide, the net charge of the Jelleines seems to play a crucial role in their antibacterial function. Intriguingly, although they were both phosphorylated at only one site, Jelleine-II (pS) was observed to be stronger at resisting *B. subtilis*, *P. larvae*, *E. coli*, and *C. albicans* growth than Jelleine-II (pT). Circular dichroism spectra indicate that Jelleine-II adopts a 3_{10} helix conformation. Different from the position of the Thr residue at the N-terminus, the Ser residue is in the middle of the peptide, so phosphorylation at the Ser residue might be buried inside the helix.[97] Therefore, phosphorylated Thr has a more negative effect on the interaction between Jelleine-II and the membrane bilayer of microorganisms, which influences its antibacterial property. Although further study is required to reveal the mechanism of Jelleines against yeast and bacteria, our results indicate that the reduced antibacterial activity of Jelleine-II is likely caused by phosphorylation due to alteration of the charge status on the surface of the peptide structure. Nevertheless, it is worth mentioning that Acc does not forage propolis (a plant resin) but Aml does. Propolis is a valuable antibiotic substance that shields the colony from pathogen invasion.[98] Despite the phosphorylated Jelleines being of low abundance in RJ and less effective than the nonmodified peptide, the stronger antibacterial effect of Jelleine-II (pS) in Acc-RJ than Jelleine-II (pT) in Aml-RJ indicates that Acc evolved protein modification as a strategy to boost its immune defense.

4.3. Phosphorylation of Immunity-Related Proteins: Strengthening Immune Function

Icarapin (or venom protein 2) has been identified as a phosphoprotein in both RJ and the venom of Aml.[23,39] In RJ, phosphorylation of icarapin has been found at S^{43}, S^{202}, and S^{205},[99] whereas in venom, S^{43} and S^{205} are phosphorylated.[70,100] In the present study, a new phosphorylation site was found at T^{37} in icarapin of Aml, whereas in Acc, icarapin was phosphorylated

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at S^{43}, S^{107}, S^{201}, and S^{204}. Apolipophorin-III protein is an exchangeable hemolymph protein, also identified in Aml-RJ. Here, phosphorylated apolipophorin-III-like protein was identified in Aml-RJ with five serine phosphorylation sites, including two sites identified before (S^{117}, S^{118}). In the honeybee colony, the high population density puts the colony at risk for disease propagation, making the larvae with a congenitally weak immune system more susceptible to infection.[101] However, because they are surrounded and fed by RJ, the larvae are shielded with a natural protective barrier because RJ contains immunity-related proteins. Functionally, both icarapin and apolipophorin-III-like protein are involved in promoting the innate immunity of insects.[23,102] Moreover, protein phosphorylation, especially tyrosine phosphorylation, is a key mechanism for modulating the immune response through direct protein interaction or cell−cell communication.[103] Hence, it is suggested that phosphorylation of these two proteins in RJ may function to modulate the strength of the immunity of the larvae.

4.4. FAM20C-Like Protein: The Potential Kinase Responsible for Phosphorylating RJ Proteins with a [S-x-E] Motif

Of the identified phosphoproteins, phosphorylated FAM20C-like protein in Aml-RJ caught our attention. FAM20C is a Golgi-resident protein kinase engaged in the phosphorylation of several secreted proteins.[104] In mammals, FAM20C phosphorylates secreted proteins related to biomineralization, and these phosphoproteins have important roles in modulating odontoblast differentiation and bone formation.[58,105] Recently, FAM20C has been defined as the Golgi casein kinase in humans and flies, the existence of which, although described in the Golgi apparatus of lactating rat mammary glands over a hundred years ago, has not been molecularly identified before.[106] FAM20C has also been reported as being secreted from cells and undergoing an autophosphorylation reaction;[106] therefore, identification of FAM20C adds a new member to the RJ protein family. The 3D structural comparison of FAM20C among honeybees, flies, and humans showed high similarity in tertiary structure (Figure 8). Given that FAM20C has a preference for the phosphorylation of peptides with a [S-x-E] motif,[58] which is in line with the extracted motif from RJ proteins, it is assumed that honeybee FAM20C-like protein expressed in the hypopharyngeal gland of nurse bees plays an important role in the phosphorylation of RJ proteins. Furthermore, RT-qPCR and western blot results proved that the transcription and translation levels of FAM20C-like protein were both significantly higher in the hypopharyngeal gland of the nurse bees than that in newly emerged and forager bees (Figure 7). This is consistent with the fact that nurse bees are the most active in secreting RJ, whereas newly emerged bees (one day) and foragers do not secrete RJ.[57] Therefore, the variation of FAM20C-like protein expression, tuned to the fluctuation of RJ secretion, indicates that FAM20C-like protein is likely responsible for the process of phosphorylating RJ proteins. Although our results provide indirect evidence that FAM20C-like protein is a potential kinase catalyzing the phosphorylation process of RJ proteins with a [S-x-E] motif, further research involving the expression and purification of FAM20C-like protein to perform kinase assays with RJ proteins as substratesis is still required .

5. CONCLUSIONS

Through our complementary phosphopeptide enrichment platform, 16 phosphoproteins with 67 phosphorylation sites and nine phosphoproteins with 71 phosphorylation sites have been identified in Aml-RJ and Acc-RJ, respectively. Of which, eight phosphoproteins are shared in both RJ samples. These findings not only greatly increase the phosphoproteome coverage of Aml-RJ but also depict the phosphoproteome of Acc-RJ for the first time. Antibacterial assays of antibacterial peptides in RJ reveal that antimicrobial activities are largely influenced by phosphorylation and that, even though the phosphorylation occurred at different sites on the same peptide, they have distinct effects on antimicrobial activity and antibiotic spectrum. This provides a new perspective for the directional selection of antimicrobial peptides for medical applications. Importantly, the phosphorylation of RJ proteins with a [S-x-E] motif is likely driven by FAM20C-like protein in the hypopharyngeal gland of nurse bees. Overall, the identified phosphorylated RJ proteins in this study warrant further validation in additional mechanistic studies of honeybee biology as well as of their human health promoting activity. Further knowledge gained through such studies has the potential to lead to major advances in these areas.

■ ASSOCIATED CONTENT

Supporting Information

Annotated spectra and ion tables of all identified phosphorylation peptides; protein sequences alignment and three-dimensional structure comparison of major royal jelly proteins (MRJPs); identified phosphopeptides from royal jelly proteins of *Apis mellifera ligustica* by Ti^{4+}-IMAC and TiO_2; identified phosphopeptides from royal jelly proteins of *Apis cerana cerana* by Ti^{4+}-IMAC and TiO_2; quantitative comparison of phosphoproteins identified by Ti^{4+}-IMAC and TiO_2 in royal jelly of *Apis mellifera ligustica* and *Apis cerana cerana*; motif extraction from the data set using the Motif-X algorithm; and kinase-specific phosphorylation site prediction. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel/Fax: +86 10 6259 1449. E-mail: apislijk@163.com.

Notes

The authors declare no competing financial interest.

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