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ORIGINAL PAPER

Differential protein expression analysis following olfactory learning in *Apis cerana*

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Abstract Studies of olfactory learning in honeybees have helped to elucidate the neurobiological basis of learning and memory. In this study, protein expression changes following olfactory learning in *Apis cerana* were investigated using isobaric tags for relative and absolute quantification (iTRAQ) technology. A total of 2406 proteins were identified from the trained and untrained groups. Among these proteins, 147 were differentially expressed, with 87 up-regulated and 60 down-regulated in the trained group compared with the untrained group. These results suggest that the differentially expressed proteins may be involved in the regulation of olfactory learning and memory in *A. cerana*. The iTRAQ data can provide information on the global protein expression patterns associated with olfactory learning, which will facilitate our understanding of the molecular mechanisms of learning and memory of honeybees.

Keywords *Apis cerana* · Proboscis extension response · Learning and memory · Isobaric tags for relative and absolute quantification (iTRAQ)

Abbreviations

A. cerana	*Apis cerana*
A. mellifera	*Apis mellifera*
AKT	RAC Serine/threonine-protein kinase
CAMK	Calcium/calmodulin-dependent protein kinase
iTRAQ	Isobaric tags for relative and absolute quantification
MAP2	Microtubule-associated protein 2
NCDN	Neurochondrin
NMDAR1	Glutamate [NMDA] receptor-associated protein 1
PER	The proboscis extension reflex
RGN	Regucalcin
SGMS1	Phosphatidylcholineceramide cholinephosphotransferase 1
SLC6A15	Orphan sodium- and chloride-dependent neurotransmitter transporter NTT73
SNAP25	Synaptosomal-associated protein 25
STX1	Syntaxin-1A
VAChT	Vesicular acetylcholine transporter

Introduction

Honeybees, an important model organism for neuroethological studies, exhibit high behavioral plasticity and a remarkable ability to learn. Previous studies revealed that honeybees not only accurately remember the odor (Menzel et al. 1996), color (Frisch 1914) and shape (Srinivasan 1994) of a target but also learn the characteristics and sequences of landmarks to ensure a safe return to the hive (Collett et al. 2003). Moreover, they can generate associative memory to facilitate the search for a food source (Srinivasan et al. 1998). More recent study has also shown that honeybees exhibit cross-modal interaction between visual and olfactory learning (Zhang et al. 2014).

Honeybees exhibit strong olfactory abilities to ensure intra-specific communication and search for food (Sandoz

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et al. 2007; Wright et al. 2002). At present, the olfactory learning ability of honeybees is assessed in the laboratory using the proboscis extension reflex (PER) (Bitterman et al. 1983; Smith et al. 1992; Giurfa and Sandoz 2012). This behavioral response was initially used in gustative physiology studies of honeybees (Frings 1944). Specifically, it was first developed by Takeda (1961) as a research method of olfactory learning based on associating a sucrose reward with odorant.

In the last two decades, the molecular mechanisms of olfactory learning and memory in honeybees have been investigated substantially. The reduced expression of Protein Kinase A (PKA) and *N*-methyl-D-aspartate (NMDA) receptors were found to impair long-term memory during the olfactory learning of honeybees (Fiala et al. 1999; Si et al. 2004). In addition, an acetylcholine receptor (AChRs) (Dacher and Gauthier 2008), a metabotropic glutamate receptor (AmGluRA) (Kucharski et al. 2007), calcium/calmodulin-dependent kinase II (CaMKII) (Matsumoto et al. 2014), octopamine receptors (Farooqui et al. 2003) and an AmDOP1 receptor (Blenau et al. 1998) have also been demonstrated to be involved in the learning and memory processes of honeybees.

High-throughput sequencing and microarray technology are important methods to comprehensively unravel the underpinnings of olfactory learning in honeybees. Using a tag-based digital gene expression (DGE) and microarray transcriptome analysis, Wang et al. (2013a) and Cristino et al. (2014), respectively, demonstrated a general down-regulation of protein-coding genes after associative olfactory learning in *Apis mellifera*. Qin et al. (2014) found that 88.40 % of differentially expressed mRNAs are down-regulated after maze learning, as evidenced by DGE. The above-mentioned studies focused on RNAs and found many more down-regulated than up-regulated coding RNAs. However, very few studies have examined which proteins are involved in olfactory learning and memory in honeybees.

Isobaric tags for relative and absolute quantification (iTRAQ) is the latest, highly sensitive and accurate technique for the quantitative examination of proteomics. The technology, in combination with multidimensional liquid chromatography and tandem mass spectrometry, can simultaneously relatively or absolutely quantify up to eight protein samples. Moreover, iTRAQ can separate and identify a variety of proteins, including membrane proteins, proteins of high molecular weight, insoluble proteins, acidic proteins and alkaline proteins. This technology has been widely applied in various life science fields such as analyses of orange leaf proteomics (Song et al. 2012), molecular mechanisms underlying the regulation of the plant flowering phase (Ai et al. 2012), the formation mechanism of mollusk shells (Zhang et al. 2012), the protein expression spectrum of cancer cells (Wang et al. 2013b) and mammalian organelle assessment (Hakimov et al. 2009).

Apis cerana, an Asian honeybee, is found in China, Japan and Pakistan. Compared with *A. mellifera*, *A. cerana* has actually been shown to learn better in a controlled laboratory setting (Chen 2001; Qin et al. 2012). Wang and Tan (2014) showed that *A. cerana* is as amenable as *A. mellifera* to the study of olfactory learning using the PER assay. However, little is known about the molecular mechanisms of learning and memory in *A. cerana*. In this study, the iTRAQ approach was used to identify the protein expression associated with the olfactory learning of *A. cerana*.

Materials and methods

Insect

A honeybee (*Apis cerana*) colony was maintained at the Honeybee Research Institute of Jiangxi Agricultural University in Nanchang, China (28.46 N, 115.49 E). The colony consisted of 4 frames and approximately 6000 bees. Frames with hundreds of 11-day-old pupae were packaged in a nylon net in the evening.

The next morning, newly emerged bees were removed from the nylon net and placed into a rectangular box containing 1 M sucrose and bee-bread (pollen and sugar solution). After 1 week of incubation in the box, honeybees were collected from the box for the experiment.

PER experiment

The honeybees were collected from the rectangular box in the morning of the eighth day for the PER experiment. The PER experimental procedure was based on the reports of Letzkus et al. (2006) and Wang et al. (2013a). The honeybees were briefly immobilized on ice for 3–5 min. Subsequently, each honeybee was mounted in a thin-walled copper tube (6 mm inner diameter) using a thin strip of GAFFA tape to immobilize the whole body, leaving the head and prolegs exposed. The fixed honeybees were randomly divided into two groups, the trained group and untrained (control) group. They were then allowed to recover in an incubator at a constant temperature of 34 °C and a constant humidity of 90 %.

In the afternoon, the honeybees were conditioned to both a rewarded odorant (CS+) and a punished odorant (CS−). The rewarded odorant contained a lemon essence plus 1 M sucrose solution and the aversive odorant contained a vanilla essence plus saturated NaCl solution, constituting a punishment. The odorant used in the present study are natural flavoring essences used for food (Queen Fine Foods Pty Ltd., Australia). The stimuli were presented as drops emerging from a 2.5 ml needle. A suction fan (20 cm × 23 cm) attached to a pipe was placed behind the honeybees to ensure both a

continual stream of scented air during stimulus presentation and the quick removal of the residual odorant traces before the next bee was trained. On the first trial, the rewarded stimulus was presented approximately 1–2 cm away from the antennae of each honeybee for 5 s until the honeybee extended its proboscis and consumed a small amount of the stimulus. If the honeybee did not extend its proboscis within 5 s, the antennae were briefly touched with the stimulus to ensure that the scent was associated with the sucrose reward. Subsequently, the same procedure was performed with the unrewarded stimulus. During training, each honeybee was allowed to consume some of stimulus drop so they could learn to distinguish between lemon and vanilla odorants. This training trial for each bee was repeated three times with intervals of 5 min. The rewarded stimulus was always presented prior to the unrewarded stimulus. After training, the honeybees were fed using a 1 M sucrose solution and then returned to the incubator (34 °C, humidity of 90 %) overnight. To reduce death, the honeybees were fed twice; at 23:00 h on the eighth day and at 6:00 h on the ninth day.

Retention tests were performed in the afternoon of the ninth day (24 h after training). The order of the presentation stimuli was reversed with respect to the training sessions—the unrewarded stimulus was offered first, followed by the rewarded stimulus. The drops of unrewarded stimulus and rewarded stimulus were presented at a distance of 1–2 cm in front of the antennae and for 5 s without touching the antennae. The test trial was repeated three times at an interval of 5 min for each bee. When all testing was done, only trained bees that showed a correct proboscis extension response (extended their proboscis when the rewarded stimulus was presented) in all three retention tests and control bees that were active and extended their proboscis in response to sucrose water were flash frozen in liquid nitrogen and stored at −80 °C.

Protein extraction and iTRAQ labeling with iTRAQ reagents

Approximately 150 samples were collected from each group after the PER experiment. Pools of 50 brain tissues served as a biological replicate for protein extraction, and three biological replicates were employed for each group.

Whole brain tissue was manually dissected to obtain protein lysates. The protein was extracted according to the methods reported by Chen et al. (2012). The protein concentration of each sample was determined using Bovine serum albumin (BSA) as a protein standard based on the Bradford method, and the quality of proteins was analyzed by SDS-PAGE (120 V, 120 min). The extracted proteins were then digested using Trypsin Gold (Promega, Madison, WI, USA) at 37 °C for 16 h at a protein:trypsin ratio of 30:1.

After trypsin digestion, the peptides were dried and resuspended in 0.5 M TEAB (Applied Biosystems, Milan, Italy). The peptides were then labeled using 8-plex iTRAQ reagent according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). The labeling reaction was incubated at room temperature for 2 h. The samples were then labeled as follows: 114 trained group; 116 untrained group; 117 trained group; 118 untrained group; 119 trained group; 121 untrained group. Subsequently, the labeled peptides were pooled, dried by vacuum centrifugation and stored at −80 °C for mass spectrometry (MS) analyses.

SCX fractionation of peptides

The iTRAQ-labeled peptide mixtures were fractionated with an Ultremex SCX column (Phenomenex) according to the description by Kuss et al. (2012). A total of 20 fractions were collected, desalted with an Ultremex SCX column (Phenomenex) and vacuum-dried for LC–MS/MS analysis.

LC–ESI–MS/MS analysis based on Q EXACTIVE

The iTRAQ-labeled peptides were analyzed using a LTQ Orbitrap velos instrument (Thermo Fisher Scientific, San Jose, CA) coupled with a LC-20AD nanoHPLC (Shimadzu, Kyoto, Japan). Each fraction was resuspended, then separated with a 2 cm C18 trap column and finally packed with a 10 cm analytical C18 column. The peptides were eluted with an acetonitrile gradient from 5 to 80 % for 44 min at a velocity 300 nL/min. The peptides eluted from the column directly entered the ESI–MS/MS at a resolution of 17,500. For MS scans, the *m/z* scan range was 100–2000, and the electrospray voltage was 1.6 kV. High-energy collision dissociation (HCD) operating mode and automatic gain control (AGC) were used to select peptides and optimize the spectra from the orbitrap, respectively.

Mass spectrometric data analysis

The raw data files obtained from the orbitrap were converted into MGF files using proteome and the proteins were identified using the Mascot search engine (Matrix Science, London, UK; version 2.3.02) against a database containing *Apis_cerana* (5594 sequences) (http://www.ncbi.nlm.nih.gov/protein?term=txid7461[Organism]). The following search parameters were employed: peptide mass tolerance at 10 ppm; fragment mass tolerance at 0.05 Da; trypsin as the enzyme with allowance for one missed cleavage; Carbamidomethyl (C), iTRAQ8plex (N-term), and iTRAQ8plex (K) as fixed modifications; Gln->pyro-Glu (N-term Q), Oxidation (M), and Deamidated (NQ) as the potential variable modifications; and a peptide charge of

Table 1 Summary statistics for iTRAQ analyses of brain proteins of trained and untrained *Apis cerana*

Group name	Total spectra	Spectra	Unique spectra	Peptide	Unique Peptide	Protein
Apis cerana	307,252	65,256	60,662	14,500	13,995	2406

2+ or 3+. To reduce the probability of false peptide identification, the peptides were filtered with significance scores (≥20) at the 99 % confidence interval and involved at least one unique peptide.

For protein quantification, a protein was required to contain at least two unique peptides. The quantitative protein ratios were weighted and normalized by the median ratio in Mascot.

Identification of differentially expressed proteins

The differentially expressed proteins were selected according to the following cut-off criteria: the protein ratio in at least one biological replicate meets the fold change (≥1.2 or ≤0.833 at $p < 0.05$), and the tendency of protein expression (the fold change ≥1.0 or ≤1.0) is consistent with the three biological replicates.

A GO annotation analysis can determine the main biological function of the differentially expressed proteins by searching for significantly enriched GO terms in differentially expressed proteins compared with the enrichment in all identified proteins. Specifically, a GO enrichment analysis applies a hypergeometric test to map all differentially expressed proteins to terms (molecular function, cellular component and biological process) in the GO database (http://www.geneontology.org/). The test employs the following formula:

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

where N is the number of all proteins with GO annotation; n is the number of differentially expressed proteins in N; M is the number of all proteins that are annotated to the certain GO terms; and m is the number of differentially expressed proteins that are annotated to certain GO terms.

The KEGG pathway enrichment analysis (http://www.genome.jp/kegg/) was utilized to identify significantly enriched biochemical pathways or signal transduction pathways in differentially expressed proteins compared with all identified proteins. The formula used for the pathway analysis is the same as that used for the GO analysis.

Results

iTRAQ analysis of protein identification

Using a PER assay, *A. cerana* was trained to associate one odorant with a sugar reward and another with a salt water punishment. After the third training trial, the bees showed a good level of learning for the odorant as nearly 75 % of bees responded correctly (Supplemental Figure S1a). Approximately 14 % of the trained bees also showed correct proboscis extension responses in all three retention trials the following day (Supplemental Figure S1b). Consequently, these bees were considered for long-term memory training and further sampled for iTRAQ analysis.

The iTRAQ technique was performed to obtain a global view of the proteome differences between the trained and untrained groups of *A. cerana*. In the mass spectrum experiment, a total of 307,252 MS spectra were obtained. A total of 65,256 spectra were successfully matched to peptide fragments, and 60,662 spectra were matched to unique peptide fragments with a Mascot analysis (Table 1). Among these spectra, 1.5 % showed multiple matches, and 78.76 % did not match the peptide. Moreover, 2406 proteins were identified from 13,995 unique peptide sequences deduced from 60,662 spectra based on the *A. cerana* (5594 sequences) database (Table 1). The identified proteins were used to further analyze the differential expression of proteins.

A statistical analysis showed that 45.14 % of the identified proteins had a coverage greater than 20, and 14 % of proteins had a coverage below 5 % (Supplemental Figure S2). In addition, approximately 1806 (75.47 %) of all identified proteins were identified with at least two peptides per protein, and 2049 (85.16 %) were identified within ten peptides (Supplemental Figure S3).

Differentially expressed proteins

All identified proteins were filtered based on a ratio ≥1.2-fold or ≤0.833-fold at $p < 0.05$ in at least one biological replicate and the consistency of protein expression in the three biological replicates. Moreover, the three biological replicates of each sample exhibited a mean CV (coefficient of variation) of 0.10 (Supplemental Figure S4), suggesting the high reliability of the results. A total of 147 proteins were differentially expressed between the trained and untrained groups, 87 of which (59.18 %) were up-regulated and 60 of which (40.82 %) were down-regulated in the trained group compared with the untrained group, based on the above criteria (Fig. 1, Supplemental file 1).

Function of the differentially expressed proteins

The biological functions of these differentially expressed proteins were investigated based on the Gene Ontology

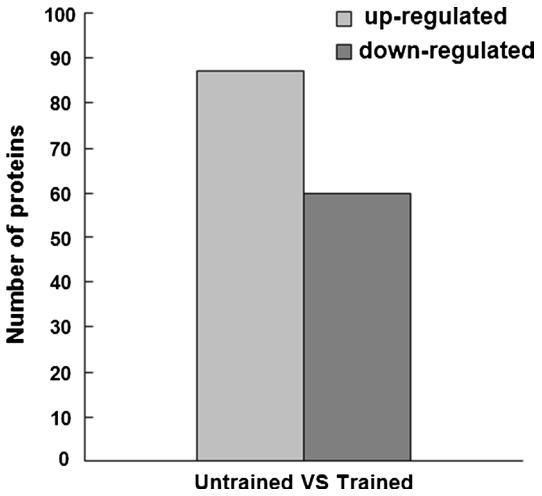

Fig. 1 Differential expression analysis of proteins in the trained and untrained groups. A total of 147 differentially expressed proteins were detected, with 87 up-regulated proteins and 60 down-regulated proteins in the trained group compared with the untrained group

database. Of the 147 differentially expressed proteins, 98 proteins were successfully mapped to one or more GO terms, whereas 49 proteins were not classified. Among the 98 proteins mapped to the GO terms, 77 (78.57 %), 63 (64.28 %), and 83 (84.69 %) are involved in biological processes, cellular components and molecular functions, respectively (Fig. 2). Of the 77 proteins involved in biological processes, 57, 45, and 41 are implicated in the cellular processes, metabolic processes and single-organism processes, respectively (Fig. 2a). Similarly, 39, 39, and 29 of the proteins involved in cellular components are related to cells, cell parts, and membranes of the cellular components, respectively (Fig. 2b). A total of 53 proteins exhibit catalytic activity, and 46 proteins function in molecular binding (Fig. 2c). Moreover, the GO significant enrichment analysis indicated that 37, 19 and 6 terms were significantly enriched (Q value <0.05) from the biological process ontology, the molecular function ontology and the cellular components ontology, respectively, compared with all identified proteins (Supplementary file 2).

The biochemical pathways of the differentially expressed proteins were investigated based on the KEGG database. Of the 147 differentially expressed proteins, 114 proteins were associated with a KO ID and involved in 132 pathways (Supplementary file 3). Compared with the background of all identified proteins, three pathways

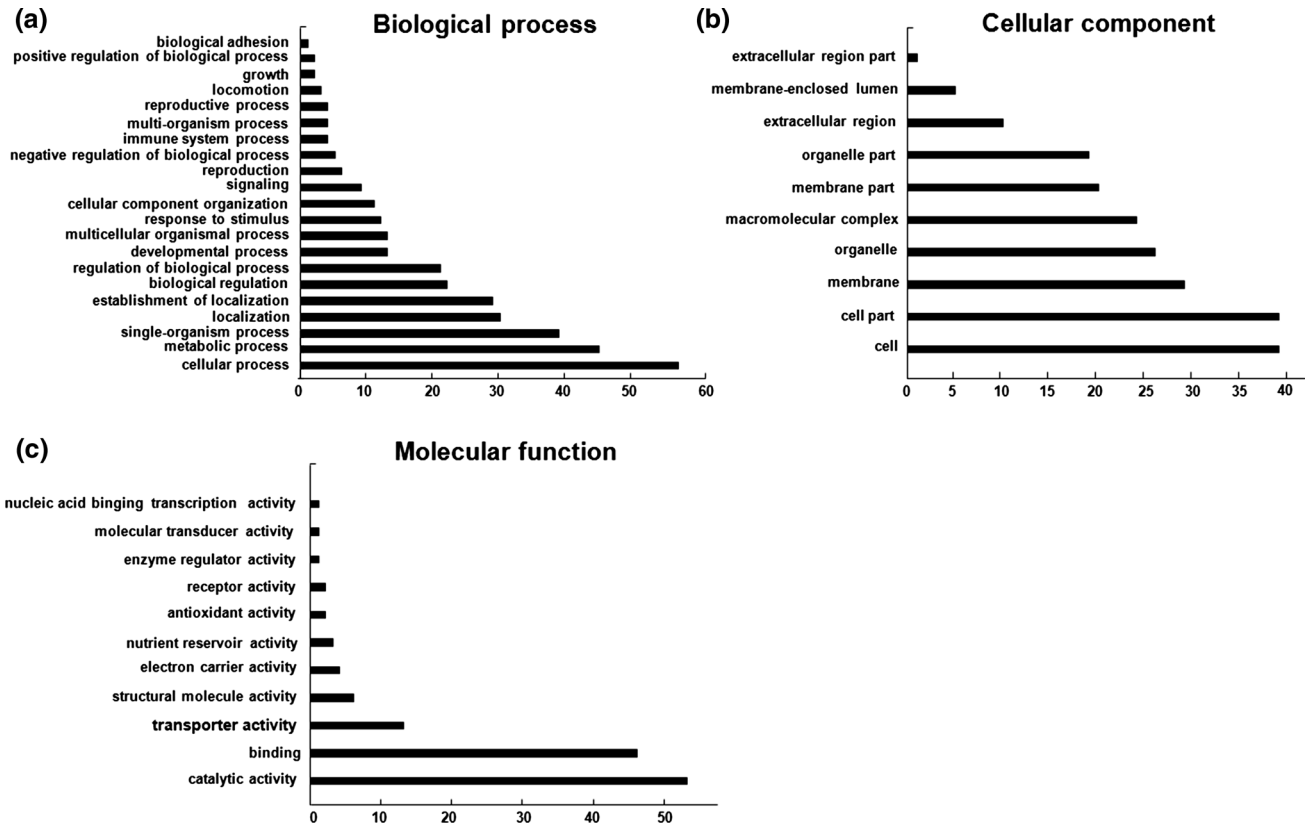

Fig. 2 Functional categorization of the differentially expressed proteins between the trained and untrained groups. The proteins were arranged in terms of GO classification, and the number of proteins in each category is displayed based on **a** biological process, **b** cellular component, and **c** molecular function

Table 2 List of part differentially expressed proteins implicated the learning and memory between trained and untrained group by iTRAQ analysis

Accession ID	Protein name	Fold change			No. of peptides
		116/114	118/117	121/119	
Up-regulated					
gi|373198053|	Phosphatidylcholine:ceramide cholinephosphotransferase 1	1.29*	1.34	1.197	2
gi|373195337|	Vesicular acetylcholine transporter	1.183	1.221*	1.173	4
gi|373217289|	RAC serine/threonine-protein kinase	1.216*	1.305*	1.183*	4
gi|373208367|	Regucalcin	1.38*	1.595*	1.6	5
gi|373204511|	Neurochondrin	1.285*	1.404*	1.007	1
Down-regulated					2
gi|373197014|	Synaptosomal-associated protein 25	0.82	0.776*	0.94	3
gi|373198326|	Microtubule-associated protein 2	0.926	0.811*	0.952	19
gi|373194043|	Calcium/calmodulin-dependent protein kinase	0.792*	0.928	0.942*	1
gi|373212420|	Syntaxin-1A	0.876	0.707	0.778*	11
gi|373205317|	Glutamate [NMDA] receptor-associated protein 1	0.899*	0.549*	0.918	1
gi|373212984|	Orphan sodium- and chloride-dependent neurotransmitter transporter NTT73	0.833*	0.777*	0.937	1

ID represents accession numbers

* Indicates significance of differential expression; fold change represents the ratios of trained/untrained group; no. of peptides represents number of peptides identified for each protein

(peroxisome, vitamin digestion and absorption, riboflavin metabolism) were significantly enriched (Q value <0.05).

Discussion

The results of the iTRAQ analysis quality assessment, including the distribution of protein sequence coverage (Supplementary Figure S2), peptide number distribution (Supplementary Figure S3) and reproducibility analysis (Supplementary Figure S4) showed that our iTRAQ data were accurate and reliable. Although the gene and protein databases of *A. cerana* were incomplete, 147 proteins were found to be differentially expressed between the trained and untrained groups, and some of these proteins are reportedly related to learning and memory, such as neurotransmitter transporter proteins, synaptic proteins, neurotransmitter receptor proteins and neurochondrin.

Intriguingly, although the expression levels of two proteins (gi\|151368183\| and gi\|373194840\|) were in line with the results reported by Wang et al. (2013a), 87 (59.15 %) differentially expressed proteins were up-regulated after learning in *A. cerana*. This observation was not consistent with the reported down-regulation of coding genes after learning in *A. mellifera* by Wang et al. (2013a) and Cristino et al. (2014). The present experimental procedure was similar to that utilized by Wang et al. (2013a), but different results were obtained. This difference may be due to significant changes in the mRNA expression levels after the test that are not necessarily accompanied by corresponding changes in protein expression during the same period because of delays in protein translation related to mRNA transcription. In addition, the protein expression reported herein also differs from the findings reported by Cristino et al. (2014), and these differences may be attributed to differences in the experimental procedures, including the odorant presentation, feeding appetitive and aversive stimuli and treatment of the control group. Recent studies have indicated that changes in gene expression are not frequently reflected at the protein level (Guo et al. 2008; Vogel and Marcotte 2012). This phenomenon may also be responsible for the differences between the reported mRNA and protein expression levels in response to bee learning.

Among these differentially expressed proteins, two neurotransmitter transporter proteins; orphan sodium- and chloride-dependent neurotransmitter transporter NTT73 (SLC6A15, gi\|373212984\|) and vesicular acetylcholine transporter (VAChT, gi\|373195337\|) were found to show significant expression differences after memory formation (Table 2). Acetylcholine (ACh) is major neurotransmitter in the central and peripheral nervous system, whereas VAChT mediates the storage and release of Ach by synaptic vesicles (de Castro et al. 2009). Furthermore, the elimination of the VAChT gene from the forebrain impairs synaptic plasticity and causes deficits that interfere with learning and memory (Martyn et al. 2012; de Castro et al. 2009). SLC6A15 mRNA is widely expressed in neurons of the olfactory bulb, cerebral cortex and hippocampus (Inoue et al. 1996). SLC6A15-null mice are less likely to avoid an

aversive olfactory stimulus in initial experiments, but these data have not been reproducible (Drgonova et al. 2007). In addition, two synaptic proteins; synaptosomal-associated protein 25 (SNAP25, gi|373201736|) and syntaxin-1A (STX1, gi|373212420|) were found to be significantly down-regulated after learning (Table 2). HPC-1/syntaxin-1 is involved in the synaptic plasticity of the hippocampus in vivo. Furthermore, this protein participates in the consolidation of conditioned fear memory as a member of the syntaxin super-family (Fujiwara et al. 2006). Previous studies have also suggested that SNAP-25 is involved in cognitive dysfunction, verbal memory and memory consolidation (Spellmann et al. 2008; Golimbet et al. 2010; Hou et al. 2004). Moreover, STX1 interacts with SNAP-25 protein, which plays an essential role in the regulation of neurotransmitter release (Lin and Scheller 2000). Thus, SLC6A15, VAChT, STX1 and SNAP-25 may be required during the olfactory learning process of *A. cerana*.

In our study, glutamate [NMDA] receptor-associated protein 1 (NMDAR1, gi|373205317|), calcium/calmodulin-dependent protein kinase (CAMK, gi|373194043|) and microtubule-associated protein 2 (MAP2, gi|373198326|) were found to be significantly down-regulated after memory formation. Conversely, phosphatidylcholine: ceramide cholinephosphotransferase 1 (SGMS1, gi|373198053|), regucalcin (RGN, gi|373208367|), RAC serine/threonine-protein kinase(AKT, gi|373217289|)and neurochondrin (NCDN, gi|373204511|) were significantly up-regulated after memory formation (Table 2). Previous studies also demonstrated that these proteins are directly or indirectly involved in learning and memory (Si et al. 2004; Matsumoto et al. 2014; Woolf et al. 1999; Lim and Suzuki 2008; Yamaguchi 2000; Yao et al. 2011; Wang et al. 2009). NMDAR1 is expressed throughout the brain, in neurons and in glial cells (Zannat et al. 2006), and plays a critical role in olfactory long-term memory formation in Drosophila (Xia et al. 2005) as well as *A. mellifera* (Si et al. 2004). CaMKII has also been reported to play an essential role in memory formation and storage (Cammarota et al. 1998; Coultrap and Bayer 2012). Recently, a pharmacological experiment showed that CaMKII was also required for the formation of LTM during the olfactory conditioning of honeybees (Matsumoto et al. 2014). The changes in the MAP-2 levels may reflect dendritic remodeling related to contextual memory storage (Woolf et al. 1999). The reduced expression of MAP-2 and synaptophysin in the hippocampus of rats is thought to contribute to cognitive impairment (Hai et al. 2010). SGMS1 is required to convert sphingomyelin and diacylglycerol to phosphatidylcholine and ceramide, and phosphatidylcholine can improve the maze-learning performance of adult mice (Lim and Suzuki 2008). Regucalcin, a calcium-binding protein, has been demonstrated to play an important role in Ca^{2+} signaling and is implicated in the long-term potentiation of neuronal plasticity (Brocher et al. 1992; Yamaguchi 2000). Furthermore, regucalcin may be involved in learning and memory. In addition, AKT is involved in the PI3K/Akt signaling pathway, which is implicated in learning and memory (Musumeci et al. 2009; Yao et al. 2011). Furthermore, a deficiency in NCDN, which is predominantly expressed in the nervous system, has been shown to cause serious spatial learning defects in neurons (Shinozaki et al. 1997; Dateki et al. 2005) and impaired synaptic plasticity (Wang et al. 2009).

The results of the KEGG pathway enrichment analysis indicate that some metabolic or signaling pathways may be involved in olfactory learning in *A. cerana*, including SNARE interactions in vesicular transport, the neurotrophin signaling pathway, the MAPK signaling pathway and the dopaminergic synapse signaling pathway (Supplementary file 3). The differentially expressed proteins, such as SNAP25, STX1, AKT, AKT, MAP-2, and AKT, participate in these signaling pathways. These proteins have been documented to be related to learning and memory, as described above.

In conclusion, the iTRAQ technology was used to identify a total of 147 proteins that were differentially expressed in *A. cerana* in response to olfactory learning. Some of these proteins are thought to be implicated in learning and memory from previous studies. The present study provides the first report of olfactory learning- and memory-related proteins in *A. cerana*, and these proteins will help to elucidate the detailed molecular mechanisms underlying learning and memory in honeybees in future studies.

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Compliance with ethical standards

Conflict of interest None.

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