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A N I N T E R N A T I O N A L J O U R N A L

Expression and Localization of Olfactory Receptor AcerOr1 from Chinese Honey Bee, Apis cerana cerana (Hymenoptera, Apidae)

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ABSTRACT

Olfactory receptors are essential for recognition and detection of odor in honeybees. Although we have cloned and characterized the sequence of olfactory receptor AcerOr1 before, the tissue distribution and location of this gene in the nurse and the forager worker Apis cerana cerana were not very clear. To further investigate this information of AcerOr1 gene, we analyzed its expression and localization. The results showed that AcerOr1 mRNA was predominantly expressed in the antennae of nurse and forager bees, while the AcerOr1 protein was predominant in thorax, and its expression in antennae was higher in forager than in nurse. IHC revealed that AcerOr1 mainly localized in the olfactory neurons of the antennae. In addition, the staining intensity of AcerOr1 protein by IHC was consistent with the results of qRT- PCR and western blotting. The expression of AcerOr1 in non-olfactory tissues implied that, in addition to olfaction, it may involve in other physiological processes. The localization of AcerOr1 in antennae further suggests that it is involved in the olfactory system. Our research suggest that AcerOr1 may participated in perceive pheromone and odours of floral in nurse and forager bees, and also critical for the behavior of collecting, defending, cleaning between the nurse and the forager worker bees.

Key words: Apis cerana cerana, olfactory receptor, AcerOr1, expression, localization, in vivo.

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INTRODUCTION

The olfactory system plays an essential role in the survival and reproduction of insects. Insects rely on their olfactory system to detect and recognize environmental and chemosensory signals to explore food sources, locate mates, avoid toxic substances, withdraw from predators and other environmental threats, and select oviposition sites. Insects sense the odorants through specialized olfactory receptors (ORs) on the dendrites of olfactory receptor neurons (ORN) present in the antennae. The ORs are involved in the transduction of chemical signals present in the environment. Insect olfactory receptors (ORs) are heteromeric, ligand-gated, seven-transmembrane ion channels that consist of a highly conserved co-receptor subunit (Orco) and an odorant-specific binding subunit (OR). They are mainly expressed on the dendrites of olfactory receptor neurons (ORN) in olfactory and gustatory organs [1-3], and participate in the detection of volatile odors and the conversion of these chemical cues into electrical signals [4, 5].

To understand the molecular basis of odor responses in insects, the identification of insect ORs is needed.

There are 119 ORs in Apis cerana [6], and more than 177 in Apis mellifera [7, 8] based on honeybee genome analysis, but only a few studies have shown that the honeybee olfactory receptor Or151 is expressed at higher levels in worker bees [9]. AcerOr2, which is orthologous to the co-receptor, was expressed in all development stages of worker antennae in Apis cerana cerana [10], and a similar expression profile was found for the Apis mellifera Orco gene AmelOr2 [11]. AcerOr1 is expressed in both worker and drone antennae at different developmental stages [12]. These observations suggest that AcerOr2 and AcerOr1 are involved in sensory processes and may be caste- or task-dependent.

In the present study, we used qRT-PCR and western blot techniques to analyze the expression patterns of AcerOr1, in antennae (the main olfactory organ) and non-olfactory tissues of nurse and forager worker bees. Immunohistochemistry (IHC) was also performed to determine the localization of AcerOr1. Understanding the detailed expression and localization of AcerOr1 would be highly valuable, not only to gain a better overview of the biology of Apis cerana cerana but also provide insight into future research directions for further functional studies of AcerOr in non-olfactory tissues.

MATERIALS AND METHODS

Samples

The honeybees (Apis cerana cerana) were obtained from an apiary at the Shanxi Agricultural University Experimental Station of Animal Science and Veterinary Medicine (Shanxi Taigu, China). The capped worker combs were incubated at 32 ± 2°C and 70% relative humidity in an incubator (XT5107 Humidity Incubators, Ningbo, China). Some of the newly emerged workers were painted with non-toxic and tasteless enamel paint at the back of the thorax and were returned to the colony. The nurse worker bees were collected at the age of 6 to 18 days and the forager worker bees carrying pollen or nectar were collected on their return to the hive entrance after 18 days. Antenna, head (after removal of antennae), thorax, abdomen, and legs from the samples were dissected and immediately frozen in liquid nitrogen for expression analysis.

Quantitative real time PCR

Total RNA was isolated using Tirol reagent (Takara, Dalian, China) according to the manufacturer's protocol. The cDNA was synthesized from total RNA using PrimeScript® RT Reagent Kit (TaKaRa) according to the manufacturer's instructions. The amplifications were carried out in a real-time PCR system (7500 Real Time PCR System, ABI, USA) using SYBR®Green Master Mix (ABI). The primers (F, 5′-AGGATTCGCCGATTTACGAG-3′, R, 5′-CGCAGC AGTGCATGGTTATAG-3′) were designed based on the AcerOr1 full-length sequence (GenBank accession number JN544932). The constitutively expressed β-actin gene of Apis cerana cerana (GenBank accession number JX899419.1) was used as an internal control and was amplified using its specific primers (F, 5′-GTGACGACGAAGTAGCAGC-3′ and R, 5′-TGACCCATACCGACCAT-3′). All the samples were analyzed in triplicate using the following conditions, 95°C for 30 s, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The analysis was performed by the $2^{-\Delta\Delta CT}$ method.

Antibody preparation

In order to acquire polyclonal antigens (pAb_AcerOr1) of high titer and specificity against olfactory receptor AcerOr1, according to the primary amino acid sequence and protein secondary structure of information about AcerOr1 in NCBI GenBank, A polypeptide antigen were raised against the antigenic peptides AcerOr1 ENTTNYRNIHYKSD (14 aa) was designed based on the AcerOr1 cDNA sequence of Apis cerana cerana by using bioinformatics software contained Blastn, Blastx and Expasy, DNAstar and ANTHEPROT (The bioinformatic software were used to analyze the amino acid sequence features of AcerOr1 proteins including their hydrophilicity, flexibility, surface probability and antigenicity and their secondary structures). And antibody was synthesized by AbMax (AbMax Biotechnology Co., Ltd. China). 0.5 mg antigen was injected into a New Zealand rabbit subcutaneously as well as intramuscularly. The injections were administered four times at 10-day intervals. The antibody titer reached the standard serum titer ≥ 1:50,000 and the antiserum against AcerOr1 were collected on day 42 after immunization. Purification of the Apis cerana cerana anti-rabbit AcerOr1 polyclonal antibody was performed by HisTrap rProtein A FF Affinity Purification Kit (Amersham Pharmacia Biotech) according to the manufacturer's instruction. The rabbits for this study were housed in a large cage at a constant temperature. All the operations were performed in accordance with the code of ethics for minimizing pain and discomfort of animals.

Antiserum and purified antibody titer detection

The titers of AcerOr1 antiserum and the purified antibody were determined using indirect ELISA. The wells of the ELISA plate were coated with the polypeptide. The polypeptide was coupled with BSA, and 100 ng was added to each well. The antibody titers detected in the rabbit antiserum were in the range 1:100,000–1:1,000 and those in the purified antibody were in the range 1–0.0005 μg/mL. The wells in the negative control were coated with 5% milk prepared in phosphate-buffered saline. The absorbance was determined at 490 nm.

Western blotting

Total protein was isolated from the samples using a tissue protein extraction kit (Boster, Wuhan, China). Before isolation, a protease inhibitor, phenylmethanesulfonyl fluoride (Boster), was added and the samples were incubated at 4°C for 30 min. The

soluble protein was obtained from the supernatant after centrifugation at 12,000 × g for 20 min at 4°C. After centrifugation, the supernatant was collected and stored at -20°C for further use. The total protein quantity was determined by BCA Protein Assay Kit (Boster), according to the manufacturer's instructions; BSA was used as a standard. The extracted proteins (100 μg per sample) were separated by 12% SDS-PAGE, and then transferred onto nitrocellulose filter membrane (Boster). The membranes were blocked for 1.5 h at room temperature in 5% skimmed milk (Boster), washed with tris-buffered saline, with Tween-20 (TBST, pH 8.0), and then incubated overnight at 4°C with rabbit polyclonal anti-AcerOr1 (1:2,000 [v/v]) and mouse anti-β-actin (1:500 [v/v]) (Boster) antibodies. Thereafter, the membranes were washed with TBST and incubated with the secondary antibodies, namely horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:5,000 [v/v]) (Boster) and goat anti-mouse (1:2,000 [v/v]) IgG (Boster), respectively, for 2 h at room temperature. Finally, the membranes were washed with TBST three times. The bands were detected using the Super ECL Plus detection reagent (Boster) and analyzed using Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA) and Image J 1.49 software.

Immunohistochemistry

The antennae were collected from Apis cerana cerana nurse in the hive and forager workers returning to hive with pollen loads, respectively. They were embedded in optimum cutting temperature (OCT) compound at temperatures below -20°C and sagittal sections (5 μm thick) were cut using a freezing microtome (Leica CM1950, Germany). The sections were fixed in 4% paraformaldehyde solution and kept at room temperature for 30 min. After fixation, the slides were treated with 3% H2O2 for 10 min at 37°C. Antigen retrieval was performed by heating in a water bath for 30 min; this was followed by blocking with 5% BSA (Boster) for 40–60 min at room temperature. Subsequently, the sections were incubated with rabbit anti-AcerOr1 polyclonal antibody (1:1000) overnight at 4°C and with Horseradish Peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:200) for 1 h at 37°C. For negative control, sections were subjected to the same manipulation, using pre-immune serum in place of the primary antibody. The stained sections were visualized under an Olympus microscope (Olympus BX53, Japan) and images were captured using the Olympus Fluoview program. The optical density of the staining was analyzed using Image J 1.49 software.

Statistical analysis

Data were evaluated by SPSS17.0 software and expressed as mean ± SD. The datasets were analyzed by t-test and ANOVA. In all the cases, statistical significance was accepted at $P<0.05$.

RESULTS

mRNA expression of AcerOr1 in nurse and forager worker bees

We investigated the expression patterns of AcerOr1 in different tissues of nurse and forager worker bees by qRT-PCR. The results obtained were as follows (Figure. 1): Highest expression levels of AcerOr1 were detected in the antenna of both the nurse and forager workers. The expression of AcerOr1 in the antenna was significantly higher than in the other four tissues, with the difference being significant at $P<0.01$. The expression levels of AcerOr1 in the head (without antennae), thorax, abdomen,

and leg were not significantly different (P>0.05). In summary, the expression levels of AcerOr1 were higher in different tissues of forager bees than in those of nurse bees.

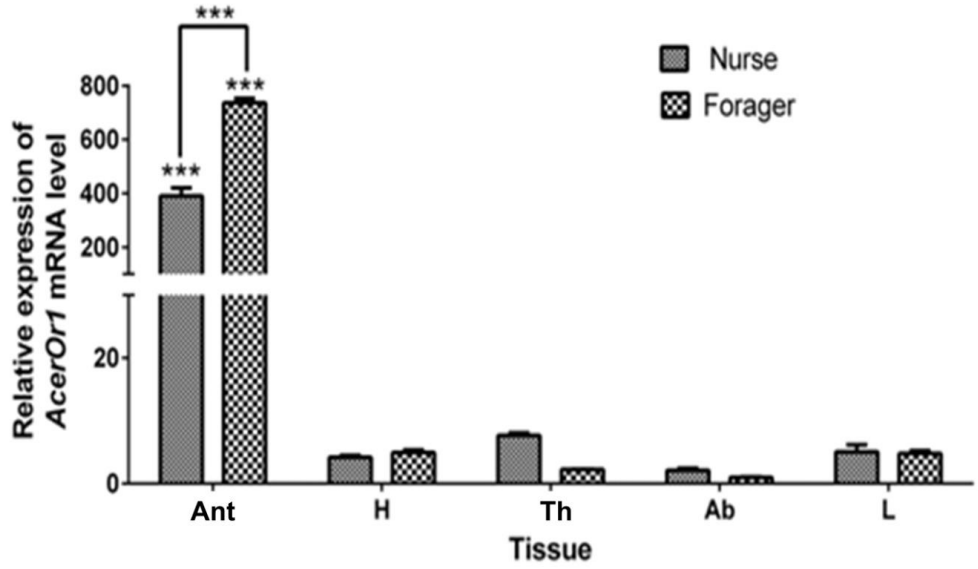

Figure. 1 Relative expression of AcerOr1 mRNA in different tissues of nurse and forager bees
The transcription levels of AcerOr1 were normalized with that of the internal housekeeping gene β-actin. Each sample had triplicates. P values were generated by one-way analysis of variance (ANOVA). *** P＜0.01.

Determination of antiserum titer

High serum titers (1:5×107) were achieved in the immunized rabbit. The ELISA results revealed that the antibodies were specific for the Apis cerana cerana AcerOr1 protein (Table 1 and Figure. 2); with the decrease in antibody concentrations, the color gradually became weak, consequently decreasing the absorbance.

Table 1 Results of ELISA performed using the rabbit antiserum

Dilution	1:1,000	1:5,000	1:10,000	1:50,000	1:100,000	NC
coating	Peptide					
OD	3.672	2.437	1.588	0.367	0.233	0.049
coating	Peptide-BSA					
OD	3.722	3.554	3.024	2.59	1.53	0.047

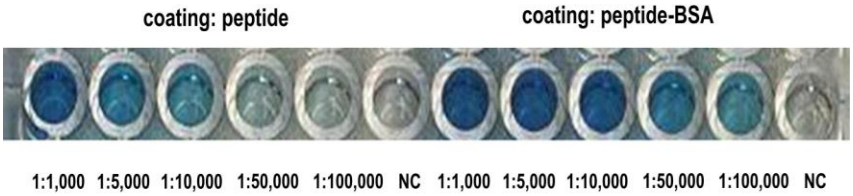

Figure. 2 Results of ELISA performed using the rabbit antiserum
Note: the absorbance readings at 490 nm in the ELISA are the average readings for two replicate samples; the absorbance of the healthy control was considered to be a positive result.

We used the rabbit polyclonal antiserum with high titer for affinity purification. Indirect ELISA results showed that after affinity purification of the serum, purified antibody against AcerOr1 was obtained with a titer reaching 0.001 ng/mL. Moreover, no cross-reactivity with BSA, the coupling agent, was observed (Table 2 and Figure. 2).

Then the antibody specificity and sensitivity was determined by Western blot (Figure. 3). There has a clear and purity band with a molecular weight of 50 kDa on the negative result compared with the negative result. The result indicated that the antibody is the target protein and have a good specificity.

Table 2 Results of ELISA performed using affinity purified antibody

Coating primary antibody	Dilution					
Serum	1:1,000	1:5,000	1:10,000	1:50,000	1:100,000	NC
Purified antibody 1.03 mg/ml	1	0.5	0.05	0.005	0.0005	NC
	0.1	0.01	0.001	0.0001	0.00001	NC
OD value	3.892	3.136	2.411	0.58	0.349	0.053
	1.914	0.735	0.112	0.039	0.033	0.036
	3.465	0.563	0.043	-0.007	-0.016	-0.003

Note: NC is negative control.

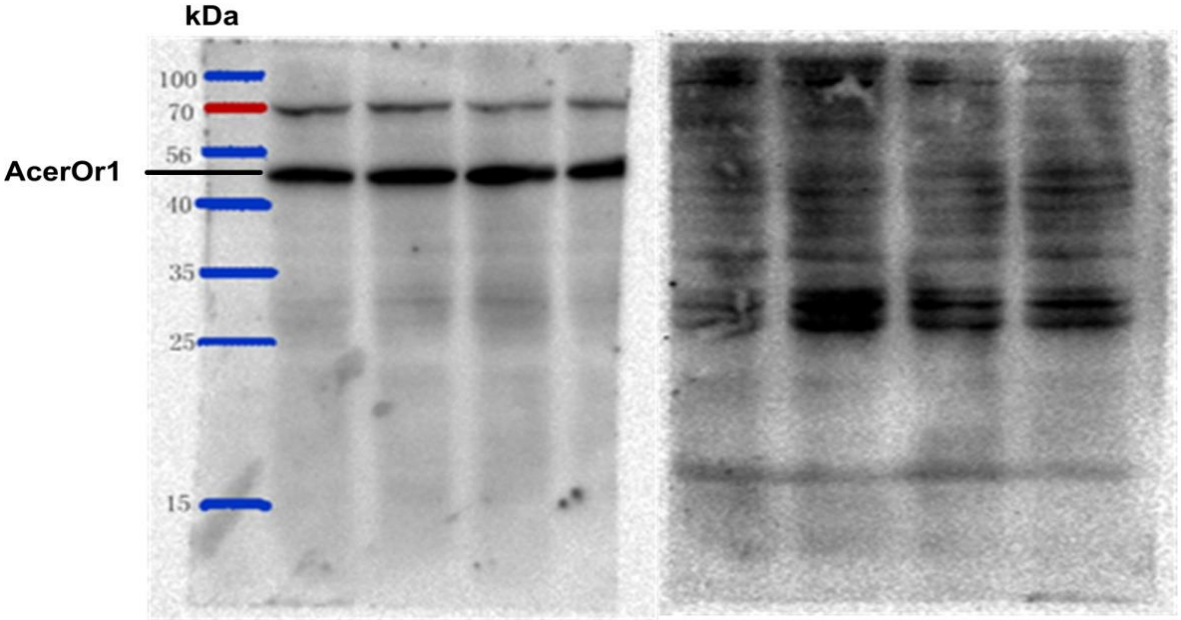

Figure. 3 The positive and negative serum results of AcerOr1 polyclonal antibody test
Note: The picture on the left is the positive result (the molecular weights of AcerOr1 about 50 kDa), the picture on the right is the negative result.

Protein expression of AcerOr1 in nurse and forager worker bees

The expression of AcerOr1 (50 kDa) was assessed by western blotting using the specific polyclonal antibodies. The protein was found to be expressed in the antenna, head, thorax, abdomen, and legs of nurse and forager worker bees, with the expression in thorax being the highest (Figure. 4A). The average band intensity was used for quantitative analysis (Figure. 4B). The analysis of the band average intensity revealed that the expression of AcerOr1 in the antenna, abdomen, and legs of forager bees was significantly higher than that in the nurse bees ($P<0.01$), In the forager bees' head the expression of AcerOr1 was higher than in the nurse bees ($P<0.05$). The expression levels of AcerOr1 in the thorax were significantly higher than other four tissues both in the nurse and the forager ($P<0.01$) and not significantly different between the nurse and the forager ($P>0.05$). Some differences in the mRNA and protein expression were observed owing to the differences between the transcription and translation process.

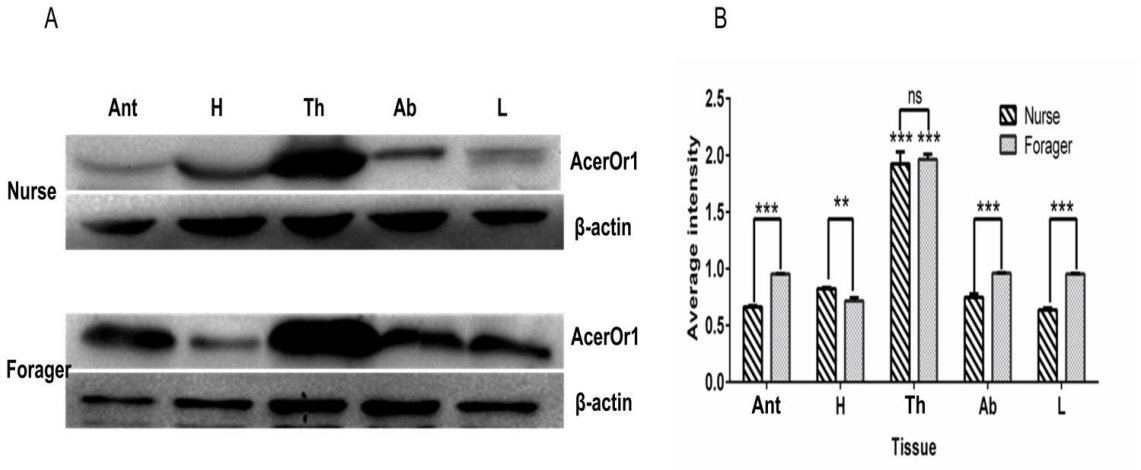

Figure. 4 Expression of AcerOr1 in different tissues of nurse and forager bees
(A) Western blot analysis of AcerOr1 and β-actin proteins in different tissues of nurse and forager bees. β-actin (100 μg/lane) was used as a control protein for normalization (the molecular weights of AcerOr1 and β-actin are 50 and 43 kDa, respectively). (B) Histograms showing the average band intensities. The values are presented as means ± SD. P values were generated by one-way analysis of variance (ANOVA). *** $P<0.01$; ** $P<0.05$.

Localization of AcerOr1 in the antennae of worker bees

There are different types of sensilla in the antennae of honeybee, the placodea sensilla and trichoidea sensilla are associated with olfaction (Ljungberg 1993). The localization of AcerOr1 was assessed in the frozen sections of the antennae of worker bees using immunohistochemistry. In our study, the expression pattern of AcerOr1 was observed in the antennae of the workers. The positive signals were distributed in the most basal region of the antennal cuticle olfactory sensory neurons (OSNs) of placodea sensilla (Figure. 5A). This suggested that AcerOr1 is linked to olfactory recognition and odor reception (Figure. 5A). The analysis of the average color intensity revealed that the expression of AcerOr1 in forager bees was significantly higher than that in the nurse bees (Figure. 5B, $P<0.01$). These results were consistent with the expression patterns of AcerOr1 in nurse and forager worker bees, as assessed by qRT-PCR and western blotting. No labeled spots were detected in the negative control section.

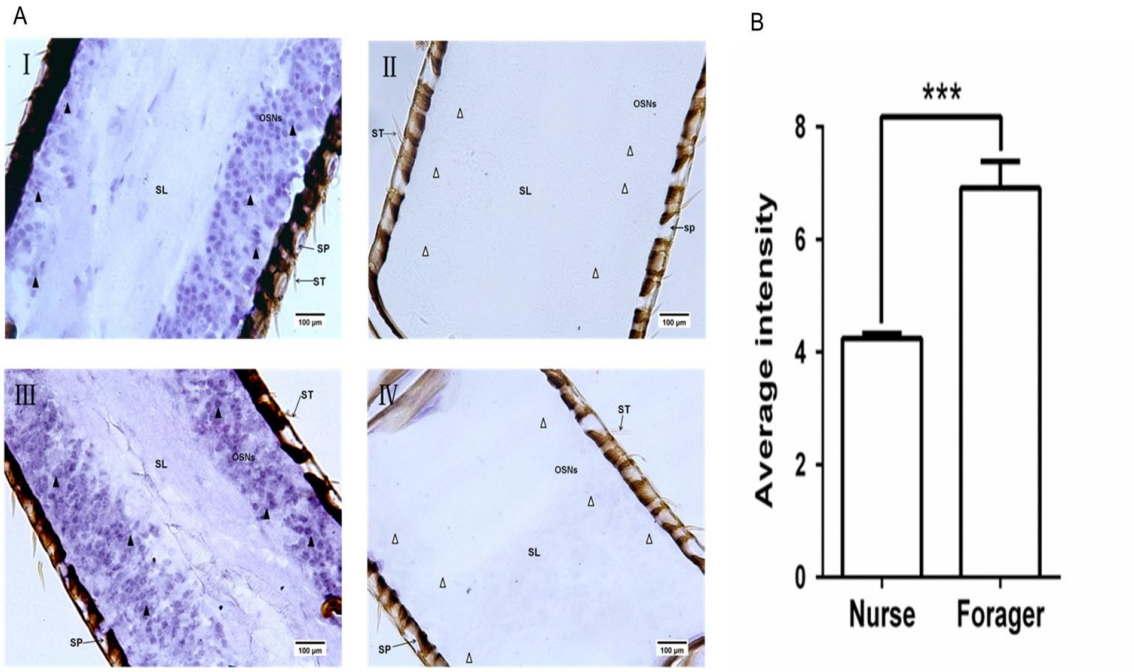

Figure. 5 Immunohistochemical analysis of AcerOr1 in the antennae of nurse and forager bees
(A) Panels I and III show positive staining results of AcerOr1 in the antenna of nurse and forager bees; panels II and VI show negative controls with no AcerOr1 staining in the antenna of nurse and forager bees. Scale bars, 100 μm. (B) The intensity of DAB staining in the antenna of nurse and forager bees. P values were generated by one-way analysis of variance (ANOVA). *** $P < 0.01$.

DISCUSSION

The developmental and tissue-specific expression profiles of conventional odorant receptors, are highly distinct and species-specific; for example, AgOR1 is expressed specifically in female Anopheles gambiae [13] and AgOR4 is highly expressed in the antennae [14]. AalOR7, AalOR10, and AalOR88 were detected significantly and specifically in female antennae [15]. Among the 57 odorant receptor genes in Drosophila, 32 are specifically expressed in the antenna, with seven being exclusively expressed in the maxillary palp, and one in both the antenna and maxillary palp. Seventeen have neither been detected in embryonic, larval, or adult olfactory organs nor in the adult head [16]. The mRNAs of the odorant receptors LmigOR1, LmigOR3, and LmigOR4 of migratory locust have been detected exclusively in antennae, whereas the LmigOR2 transcripts were abundantly detected in the mouthparts [17]. In the cotton bollworm, Helicoverpa armigera, the expression of HarmOR51 was limited to the adult male antenna, implying its role in mating. HarmOR1, 53, 54, and 58 were found to be specifically expressed in the antennae of larvae [18]. In our previous studies we find that AcerOr1 expressed in both worker and drone antennae at different developmental stages [12]. However, in the present study, we found that AcerOr1 is expressed not only in antennae but also in the non-olfactory organs, such as head, thorax, abdomen, and legs, both in the nurse and the forager worker bees, where numerous gustation-related chemosensillae (chaetic sensillae) are located.

The main organs associated with odorant sensitivity in honey bees are the antennae, and the localization of AcerOr1 in antennae therefore indicates its involvement in olfactory functions. However, AcerOr1 expression has also been seen in non-olfactory organs like the legs, thorax and abdomen in the Apis cerana cerana. This not only indicates a dual role of AcerOr1 in non-olfactory functions like gustation, but also that

non-olfactory organs whose main functions are movement (legs and thorax) and reproduction (abdomen) may also have secondary olfactory functions. For instance, AcerOr1 in the legs of Apis cerana cerana may play a critical role in contact chemosensory pathway when the bees land on the anthers to collect and carry pollen. Honey bees communicate with their nest-mates through a variety of signals. A collection pheromone secreted by the gland of Nasanov which is located near the tip of the abdomen is used to attract nest-mates, and to mark the source of nectar or water. Expression of AcerOr1 in abdomen may therefore be associated with both olfaction and gustation. However, whether AcerOr1 functions as a chemosensor in the non-olfactory tissues needs to be validated by further studies.

AcerOr1 may also have different functions in the antennae of the nurse and forager worker bees: recognition of nest pheromone in the former, and perception of volatile floral scents in the latter. Taken together, AcerOr1 likely participates in behaviors like pollination, collection, defense and cleaning in the nurse and forager worker bees.

In insect, the ORs are extensively expressed in the membranes of OSNs, which can detect volatile chemicals and are housed in sensilla of the antenna, such as basiconica sensillae, trichodea sensillae, coeloconica sensillae [19]. In Drosophila, 12 members of 60 Or (Odor receptor) genes have been demonstrated to be expressed in trichoid sensilla [19]. The pheromone receptors Or65a, Or88a, Or47b, and Or67d are expressed in the trichoid sensilla [20] and are thought to be critical for sociosexual communications between males and females [21]. Or83c is expressed in intermediate sensilla neurons [22]. The expression of transgenic Ors in Drosophila OSNs is either in ab3 or T1 antennal sensilla [23]. Or43b is expressed in a subset of olfactory neurons localized to basiconica sensilla in the region of the antenna [24].

The immunohistochemical analysis in the present study showed that AcerOr1 is abundantly expressed in the neurons in trichodea sensilla and placodea sensilla of the antennae of Apis cerana cerana. These sensillae are specialized for pheromone detection in many insects. The localization of AcerOr1 in antennae further suggests that it is involved in the olfactory system because antennae are the main organs associated with odorant sensitivity.

However, the molecular function, and cellular signal transduction pathways of AcerOr1 have not yet been reported. Therefore, further investigation of AcerOr1 is needed. Moreover, Ors play important roles in olfaction, and future comparative analysis should help in clarifying these roles of Ors in insects. We hope that the results obtained in the present study would provide the research community with information that would encourage further studies on these genes in Apis cerana cerana.

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