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Study of the *obp5* gene in *Apis mellifera ligustica* and *Apis cerana cerana*

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ABSTRACT. *Apis mellifera ligustica* and *A. cerana cerana* exhibit differences in olfactory sensitivity to odors from nectariferous plants and diseased broods. It is presumed that the differences in odorant-binding proteins (OBPs) between these 2 species contribute to their olfactory sensitivity. We compared the sequences, temporal expression pattern, and binding properties of the 2 OBP-encoding genes. We cloned the *Amobp5* and *Acobp5* genes. Among the ligands tested, phenethyl acetate was the most variable, with AcOBP5 showing high affinity and AmOBP5 having no apparent affinity for this ligand. While AmOBP5 had high affinity to both benzyl alcohol and 2-phenylethanol, the binding affinity of AcOBP5 to these compounds was moderate. However, the fluorescence intensity of these compounds was not decreased below 50%; thus, the dissociation

constants could not be calculated. The *Amobp5* gene showed significantly higher expression in 10- and 15-day-old workers than in other stages, while the *Acobp5* gene had the highest expression in 30-day-old workers. Both the *Amobp5* and *Acobp5* genes had the lowest expression level in 1-day-old workers. These results suggest that the binding properties and temporal expression patterns of the *obp5* genes in *A. mellifera* and *A. cerana* play a critical role in the olfactory sensitivity of workers.

Key words: *Apis cerana cerana*; *Apis mellifera ligustica*; Olfaction; Odorant-binding proteins (OBPs); Gene expression

INTRODUCTION

Olfactory sense plays a critical role in the life of honeybees. As social insects, honeybees rely on their complicated olfactory system to manage their complex behaviors such as regulating the bee colony (Swanson et al., 2009a) through the division of labor (Dani et al., 2005; Iovinella et al., 2011), searching for food sources like nectar and pollen (Slessor et al., 2005), and recognizing nestmates *vs* alien conspecifics.

Antennae are the main olfactory sensory organs in honeybees that detect odor molecules. The first step in the detection of molecules such as volatiles is the transportation of hydrophobic signaling molecules by odorant-binding proteins (OBPs) to receptor neurons through the sensillum lymph. Honeybee OBPs are small, water-soluble, and extracellular proteins that participate in receptor events of odor-pheromone detection by carrying, deactivating, and selecting odor stimuli. Typically, OBPs show an affinity to hydrophobic compounds and exhibit a globular, alpha-helical structure. The honeybee genome contains 21 genes encoding OBPs (Forêt and Maleszka, 2006). In the antennae of foragers, OBP1, 2, 4, 8, 11, 16, 19, 20, and 21 are present in high concentrations, and OBP5, 14, 15, 17, and 18 occur at lower concentrations (Dani et al., 2010). The *obp5* gene is located on chromosome 9 and belongs to the N-minus OBPs. The mRNA expression levels of the *Apis mellifera ligustica obp5* (*Amobp5*) gene in queen and worker antennae are similar.

Apis cerana cerana Fabricius, an Asian honey bee, is an economically important species that is bred in China. This honeybee has many unique biological characteristics such as the presence of a highly sensitive olfactory system that detects small odor molecules from dispersed nectariferous plants, high resistance to ectoparasites such as *Varroa destructor* and chalkbrood disease because of a specific grooming behavior, and tolerance to low environmental temperatures such as 4°C (Radloff et al., 2010). On the other hand, the widely cultured *A. mellifera* is a species that is cultured economically to produce honey, pollen, propolis, and royal jelly. However, *A. mellifera* is susceptible to infection by *V. destructor* and chalkbrood disease most likely because of its low olfactory sensitivity. However, the olfactory systems of these 2 species have not been directly compared. Such a comparison could reveal insights into the mechanisms that offer disease resistance to *A. cerana* and may allow manipulation of *A. mellifera* to become more tolerant to diseases and parasites. In this study, we cloned the *obp5* gene from *A. mellifera* and *A. cerana* worker antennae and compared the gene sequences, binding characteristics, and expression profiles.

MATERIAL AND METHODS

Insects and sample collection

Colonies of *A. mellifera* and *A. cerana* were reared in an experimental apiary at the College of Bee Science in Fujian Agriculture and Forestry University during the spring of 2011-2012. Broods from healthy colonies were incubated at 34°C and 80% relative humidity (Kucharski and Maleszka, 2002). Honeybees that emerged within 24 h were collected, marked with enamel paint on the back of the thorax, and returned to the colony.

Total RNA extraction and cDNA synthesis

Approximately 1000 antennae were dissected from workers of each species for RNA isolation. Total RNA was extracted by Trizol reagent according to the manufacturer protocol (Invitrogen, USA). Subsequently, cDNA was synthesized using the Promega RT-PCR System following the manufacturer manual (USA).

Cloning and expression of the recombinant *obp5* gene

The *obp5* gene was amplified from both *A. mellifera* and *A. cerana* antennae by polymerase chain reaction (PCR) using primers *Aobp5* (Table 1), which were designed according to the *A. mellifera obp5* sequence. The volume of all PCRs was 50 μL and contained 1X ExTaq polymerase buffer, 0.2 mM dNTPs, 1 μM each of the *Aobp5* primers, and 2.5 U ExTaq polymerase (Promega). PCRs were performed on a Mastercycler Gradient PCR machine (Eppendorf, USA) with the following cycling conditions: a denaturation step at 95°C for 5 min; 35 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. PCRs were separated on a 1.2% agarose gel. Products in the estimated size range were excised, purified with a Gel Extraction Kit (Sangon Shanghai, China), and ligated into the pGEM-T vector. The ligation mixtures were transformed into *Escherichia coli* DH5α, and the positive colonies were selected by restriction endonuclease digestion of plasmids isolated from them.

The fragments that were excised with *Eco*RI and *Xho*I from the pGEM-*Amobp5* and pGEM-*Acobp5* plasmids were purified with the Gel Extraction Kit (Sangon Shanghai), cloned into the pET-28a(+) vector, and digested with the same restriction endonucleases to construct the recombinant plasmids pET-28a-*Amobp5* and pET-28a-*Acobp5*. Subsequently, the plasmids were transformed into *E. coli* Rosseta competent cells. Single colonies were grown overnight in 10 mL Luria-Bertani broth including 50 μg/mL kanamycin and 34 μg/mL chloramphenicol. The culture was diluted 1:100 with fresh medium and grown at 37°C until the optical density at 600 nm was 0.5, at which point 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce expression of the target proteins and cultured overnight.

Bacterial cultures were centrifuged at 4000 rpm for 10 min at 4°C. The expression of recombinant proteins in *E. coli* leads to the formation of inclusion bodies. Therefore, the pellets were subjected to ultrasonication, and the proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with copper staining solution for 5 min with agitation and rinsed in Milli-Q water; the target bands were cut from the gels. The target protein band that was identified by SDS-PAGE was dialyzed

and used to assess binding characteristics using N-phenyl-1-naphthylamine (1-NPN) as the fluorescent ligand.

Table 1. Oligonucleotide primers used for isolation and expression analysis of odorant-binding proteins from *Apis mellifera ligustica* and *Apis cerana cerana*.

Primer	Sequence (5'-3')
Aobp5	F: GAATTCATGCACGTAAAAGTCGGTAT
	R: CTCGAGTCACGGGAAGAAAAACTTTTC
Bobp5	F: GATCACGATCTTCAATGCTACAC
	R: TCCACCAGTATCCTTTCCTTTAG
β-actin	F: TGCCAACACTGTCCTTTCTG
	R: AGAATTGACCCACCAATCCA

Binding affinity measurements

To investigate the binding affinities of the 2 target OBPs, 3 potential ligands, benzyl alcohol (Sigma, USA), 2-phenylethanol (Fluka, USA), and phenethyl acetate (Aldrich, USA), which are present in the volatile compounds of chalkbrood disease-infected honeybee larvae, were used.

Emission fluorescence spectra were recorded on an RF-5301PC instrument at 25°C with a 1-cm light path quartz cuvette and 10 nm slits for both excitation and emission. To measure the binding constants of the 1-NPN fluorescent probe with each OBP, a 2-mM solution of each protein in 50 mM Tris-HCl, pH 7.4, was titrated with 100-mM aliquots of each ligand dissolved in methanol to final concentrations that ranged from 2 to 16 mM. The 1-NPN probe was excited at 337 nm, and emission spectra were recorded between 300 and 550 nm. The affinity of OBPs to the test ligands was measured in competitive binding assays using 2 mM 1-NPN and 2-16 mM competitor.

To determine the binding constants of 1-NPN, intensity values corresponding to the maximum fluorescence emission were plotted against ligand concentrations. Bound ligand was then evaluated using the fluorescence intensity values, assuming that the protein was active, with a 1:1 stoichiometric ratio of protein:ligand. The curves were linearized using Scatchard plots. Dissociation constants of bound ligand were calculated from the corresponding half maximal inhibitory concentration (IC_{50}) values, using the equation: $KD = [IC_{50}] / (1 + [1 - NPN] / K_{1\text{-}NPN})$, where 1-NPN is the free concentration of 1-NPN and $K_{1\text{-}NPN}$ is the dissociation constant of the Protein/1-NPN complex.

Measurement of temporal gene expression patterns

Antennae from 100 worker bees aged 1, 4, 10, 15, 20, 25, and 30 days were collected from *A. cerana* and *A. mellifera*. Total RNA was isolated using Trizol, and cDNA was synthesized using the Promega RT-PCR system.

Each sample was analyzed in triplicate in 20-µL reactions, containing 100 nM of each primer, 10 µL SYBR Green, 0.2 µL CXR, and 2 µL diluted cDNA (1:10). Quantitative reverse transcription PCRs were performed on the Applied Biosystems Step One Plus Real-Time PCR System with the SYBR Green dye (Promega) on 96-well plates (ABI, USA). The following PCR program was used: 95°C for 3 min and 40 cycles of 95°C for 15 s and 61°C for 1 min.

The specificity of the amplification was checked with a heat dissociation curve (60-95°C) following the final PCR cycle. The relative quantification analysis was performed according to the threshold values generated from the Promega GoTaq 2-Step RT-qPCR system (Promega). A standard curve was prepared using the purified PCR products of the *obp5* and β-actin genes. For each experiment, the β-actin gene was analyzed as the endogenous control (Li et al., 2012), and a no-template negative control and a water only blank control were also included. The relative quantification analysis was performed using the comparative relative standard method (Schefe et al., 2006).

Statistical analysis

All quantitative data are reported as means ± standard deviation. One-way analysis of variance (SPSS 17.0 Statistical software) was used to determine the significance of differences in gene expression between the different ages.

RESULTS

Molecular cloning and sequence analysis of the *Amobp5* and *Acobp5* genes

To characterize the *Amobp5* and *Acobp5* genes from *A. mellifera* and *A. cerana*, respectively, a 432-bp open reading frame (ORF) was first amplified from the antennae of 10-day-old adult workers, cloned, and sequenced. The *Acobp5* ORF encodes 144 amino acids and contains a putative hydrophobic signal peptide with 23 amino acids at the N-terminus, which was predicted by Signal P 4.0 (Petersen et al., 2011). The predicted molecular weight of OBPs is 16 kDa, and the theoretical isoelectric point is 6.81 (http://web.expasy.org/cgi-bin/protparam/protparam). The deduced amino acid sequences of AmOBP5 and AcOBP5 were analyzed and aligned using the DNAMAN software (Figure 1). AmOBP5 and AcOBP5 share 95.8% identity, and the different amino acids are marked by circles and all 6 conserved cysteines are shaded in gray (Figure 1).

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AcOBP5-P.seq   MHVKSVLLLITIVTFVVLKPVKSMSADQVEKLAKNMRKSCLQKIAITEELVDRMRRGEFP
AmOBP5-P.seq   MHVKSVLLLITIVTFVALKPVKSMSADQVEKLAKNMRKSCLQKIAITEELVGGMRRGEFP
AcOBP5-P.seq   DDHDLQCYTTCIMKLLRTFKNGNFDFDMIVKQLEITIPPEEVVIGKEIVAVCRNEEYTGD
AmOBP5-P.seq   DDHDLQCYTTCIMKLLRTFKNGNFDFDMIVKQLEITMPPEEVVVGKEIVAVCRNEEYTGD
AcOBP5-P.seq   DCQKTYQYVQCHYKQNPEKFFFPZ
AmOBP5-P.seq   DCQKTYQYVQCHYKQNPEKFFFPZ
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Figure 1. Alignment of the deduced AcOBP5 and AmOBP5 protein sequence. Conserved cysteine residues are shaded in gray, and amino acids that are different in the two sequences are indicated with stars.

Using the TMHMM 2.0 posterior probabilities, the sequences outside the transmembrane domain of AmOBP5 and AcOBP5 were found to be extracellular. According to the Kyte and Doolittle method, AmOBP5 and AcOBP5 were predicted to be hydrophilic, although these proteins have aliphatic amino acids with an aliphatic index of 83.12. OBP5 also contains 3 regions of lipophilicity (Figure 2) (http://web.expasy.org/protparam/).

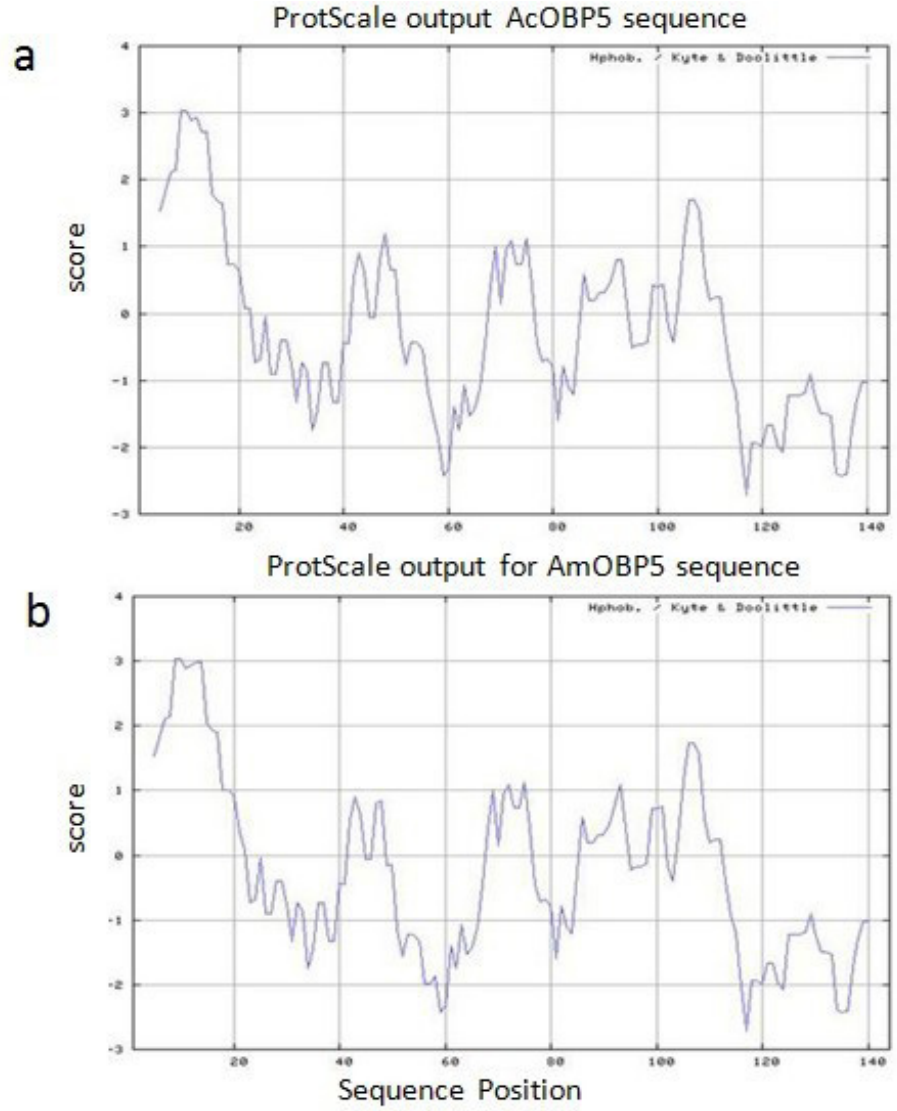

Figure 2. AcOBP5 (**a**) and AmOBP5 (**b**) are hydrophilic (Kyte and Doolittle method) and contain 3 lipophilic regions.

Binding properties of the AmOBP5 and AcOBP5 proteins

Both the *Amobp5* and *Acobp5* genes were heterologously expressed in *E. coli*. About 8 h after induction at 20°C, the inclusion bodies with the AmOBP5 and AcOBP5 recombinant proteins were precipitated and resuspended (1 mg/mL) in phosphate-buffered saline, pH 7.4. Samples from the expression medium before and after inducing protein expression with IPTG were also analyzed by SDS-PAGE. Induction resulted in an increase in the intensity of a band at 16 kDa in the cultures with the recombinant *Amobp5* and *Acobp5* genes (Figure 3).

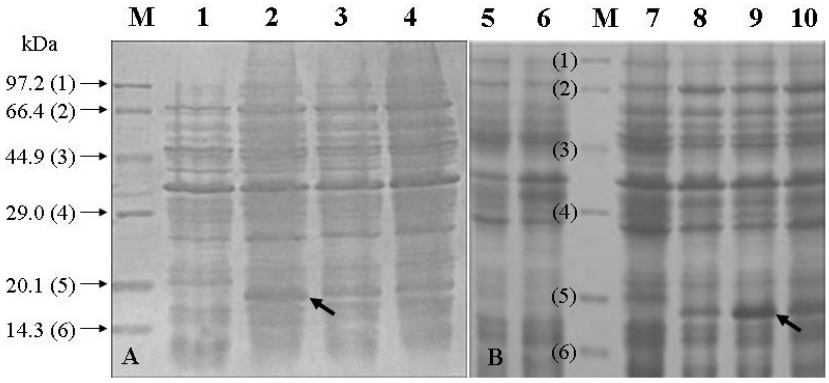

Figure 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of recombinant AcOBP5 (**A**) and AmOBP5 (**B**). **A.** *Lane M*: protein molecular weight marker; *lane 1*: culture medium without isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce expression of pET-28a-*Acobp5*; *lanes 2-4*: induction of pET-28a-*Acobp5* expression; **B.** *Lane 5*: culture from *Escherichia coli* Rosseta competent cells without plasmids; *lane 6*: *E. coli* Rosseta competent cells with pET-28a; *lane M*: protein molecular weight marker; *lane 7*: culture medium without IPTG to induce expression of pET-28a-*Amobp5*; and *lanes 8* and *9*: induction of pET-28a-*Amobp5* expression. Arrows show the OBP5 proteins.

Binding assays were also performed by measuring the affinity of AmOBP5 and AcOBP5 to a fluorescent reporter (1-NPN). An increase in the fluorescence intensity at 431 nm was observed when increasing amounts of 1-NPN were added to the AcOBP5 and AmOBP5 (Figure 4).

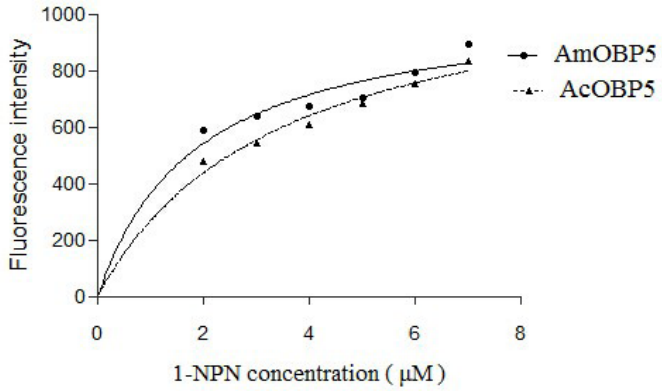

Figure 4. Binding curve of N-phenyl-1-naphthylamine (1-NPN) to recombinant AmOBP5 and AcOBP5.

The dissociation constants of the AmOBP5/1-NPN and AcOBP5/1-NPN complexes were 1.872 ± 0.69 and 3.441 ± 0.8712 μM, respectively. In the competitive binding assays, differences were observed between AmOBP5 and AcOBP5. Among the ligands that were tested, phenethyl acetate was the most variable, with AcOBP5 showing high affinity and AmOBP5 having no apparent affinity for the ligand. While AmOBP5 had high affinity to both benzyl alcohol and 2-phenylethanol, the binding affinity of AcOBP5 to these compounds was moderate (Figure 5a and b). However, when benzyl alcohol, 2-phenylethanol, and phenethyl acetate

were added at a final concentration of 16 μM, the fluorescence intensity did not decrease below 50%; thus, the dissociation constants could not be calculated.

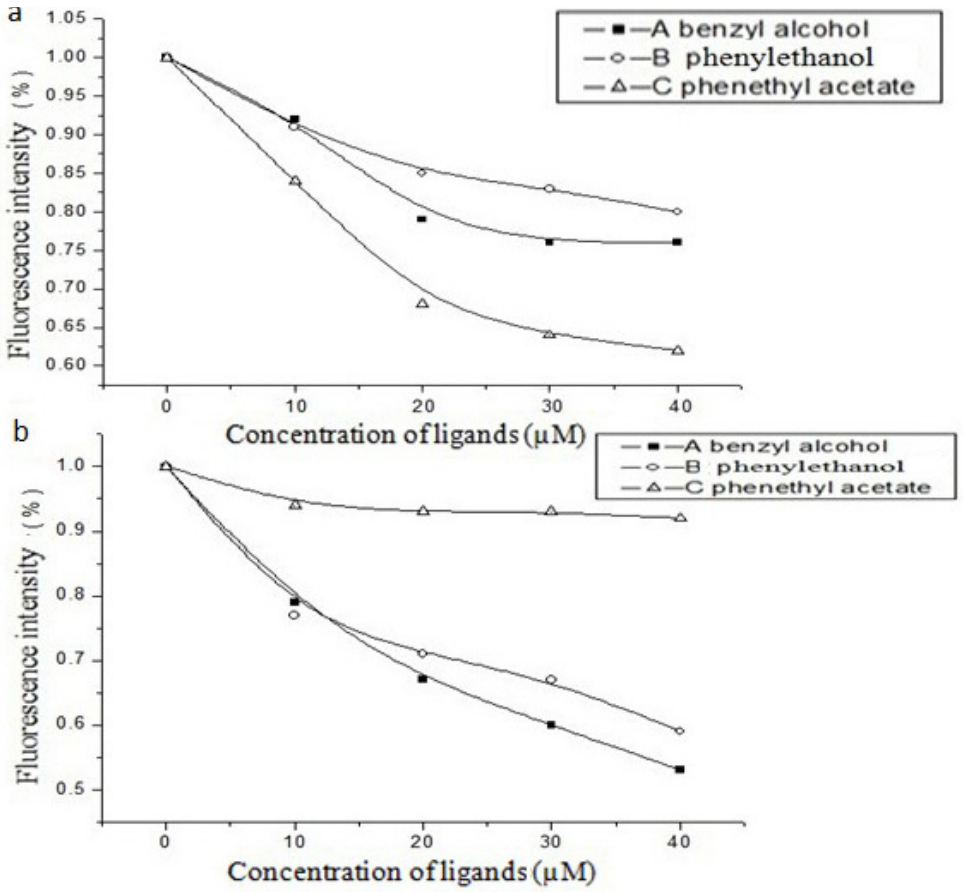

Figure 5. Competitive binding curves of selected ligands to the protein AcOBP5 (**a**) and AmOBP5 (**b**).

Temporal expression pattern of the *Amobp5* and *Acobp5* genes

The standard curve based on a broad range (10^5-fold) of concentrations showed a 96.39% amplification efficiency (slope = -3.412) for the β-actin gene and 85.57% for the *obp5* gene (slope = -3.724). These indicate that the relative standard curve method (Livak and Schmittgen, 2001) for real-time PCR analysis with SYBR Green dye is suitable. The transcript abundance was calculated according to the differences in the Ct values between the *obp5* gene transcripts and the β-actin gene transcripts.

The expression of the *Amobp5* gene was the highest in 10-day-old workers and was followed by 15-day-old workers, which also had relatively high expression. The lowest expression was observed in 1-day-old workers (Figure 6a).

On the other hand, the expression of the *Acobp5* gene was the highest in 30-day-old workers and was also relatively high in 25-day-old workers. The lowest expression was in

1-day-old workers, which is similar to the *Amobp5* gene, and the expression varied between 1 and 25 days (Figure 6b).

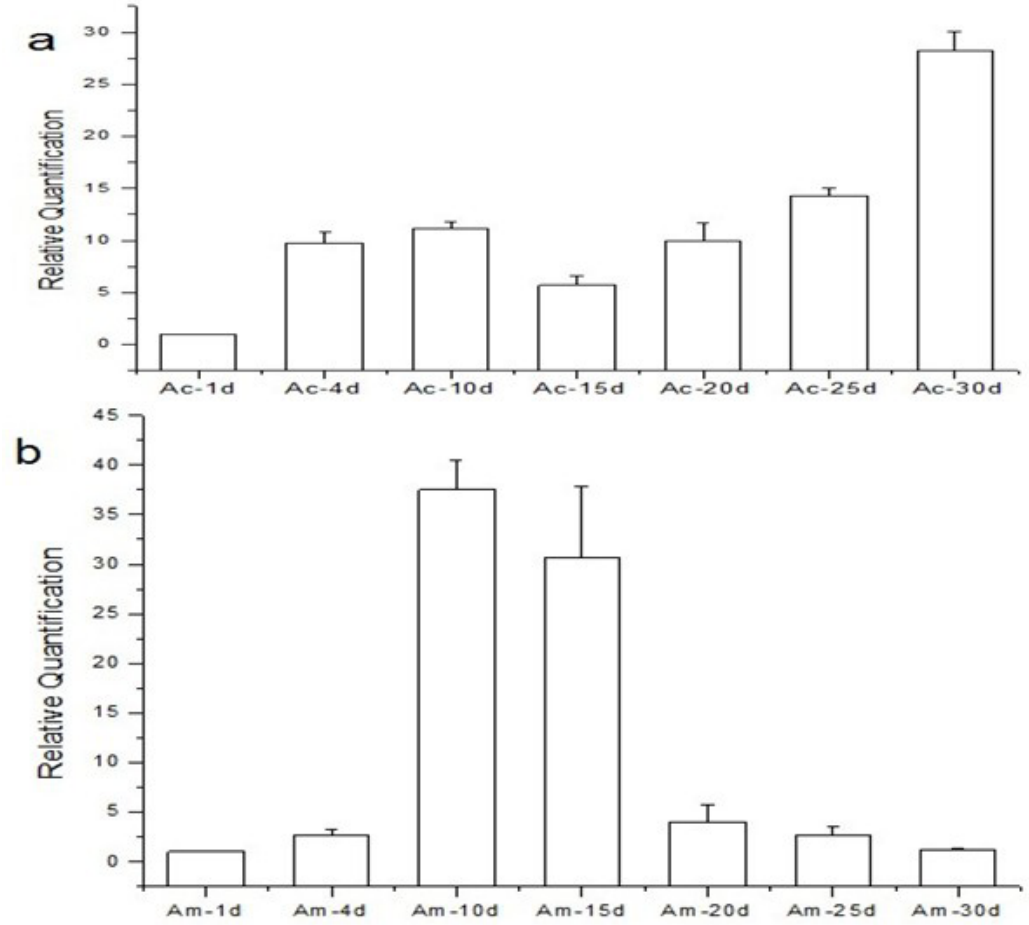

Figure 6. Expression patterns of *Acobp5* (**a**) and *Amobp5* (**b**). The relative expression levels of *obp5* at different time points were normalized against the threshold cycle (Ct) values for β-actin. All samples were tested in triplicate. The mean ± SD was used to analyze the relative transcript levels for each time point using the relative standard curve method. *Amobp5* expression was significantly higher in 10- and 15-day-old *Apis mellifera* workers than in other ages. *Acobp5* expression was significantly higher in 30-day-old workers than in other ages of *A. cerana* workers. Asterisks represent significance at $P < 0.01$.

DISCUSSION

Olfaction plays an important role in almost every biological behavior of honeybees, not only providing the colony with a sensory network that maintains the internal cohesion of the hive but also recognizing the various kinds of airborne molecules that aid in the identification of foods and partners. OBPs mediate the recognition and discrimination of a huge variety of odor compounds. Honeybees have the smallest known set of OBPs, and only 21 OBP-like

proteins were identified in the honeybee genome. In comparison, insects such as *Anopheles gambiae* and *Drosophila melanogaster* contain 70 and 51 OBPs, respectively. However, OBP families in honeybees show a high level of sequence diversity (Forêt and Maleszka, 2006). Because OBPs could act as selective filters in odor recognition (Kim et al., 1998), each OBP could perform a different function and task (Spinelli et al., 2012). Consistent with this, OBP5 is mainly expressed in the antennae of adult workers and is only weakly present in the head and legs (Forêt and Maleszka, 2006). During the experiment, we detected the *Amobp5* and *Acobp5* genes in different tissues of *A. mellifera* and *A. cerana*, including head, leg, and wing. The expression of *obp5* gene in the antennae of 1-day-old workers was 10- to 100-fold higher than the expression in other tissues; yet, 1-day-old workers had the weakest expression of *obp5* in the antennae. Thus, the *Amobp5* and *Acobp5* genes were mainly expressed in the worker antennae (Zhao HX and Luo YX, unpublished results).

In this study, we successfully cloned the *Amobp5* and *Acobp5* genes from *A. mellifera* and *A. cerana* antennae, respectively. Using the fusion expression vector pET-28a, the expression of the *Amobp5* and *Acobp5* genes was induced, and the recombinant proteins were analyzed by SDS-PAGE. The observed molecular weight of the protein was consistent with the predicted molecular mass of the native honeybee protein. These proteins share typical characteristics of honeybee OBPs that include 6 conserved cysteine residues and a signal peptide. We observed that the few differences between AmOBP5 and AcOBP5 were not in the conserved cysteine residues (Figure 1). Similar to previous reports (Forêt and Maleszka, 2006) that OBPs have an affinity to hydrophobic compounds, AmOBP5 and AcOBP5 are hydrophilic molecules with 3 lipophilic regions that bind lipid-soluble compounds. According to these features of AmOBP5 and AcOBP5, 3 lipid-soluble compounds were utilized to determine the binding affinities of AmOBP5 and AcOBP5.

In fluorescent competitive binding assays, AcOBP5 had an apparent affinity for phenethyl acetate. In a previous report, phenethyl acetate induced honeybee hygiene behavior when infected with *Ascosphaera apis* (Swanson et al., 2009b). Thus, we proposed that AcOBP5 binds phenethyl acetate to transfer a message to neurons, and *A. cerana* insects promptly clean diseased larvae to prevent outbreaks of this disease. Further experiments are needed to assess the function of AcOBP5. Although benzyl alcohol and 2-phenylethanol had high competitive binding affinities to AmOBP5, phenethyl acetate had a weak competitive binding affinity to AmOBP5. These 3 volatile compounds, which are detected in larvae that are infected with fungal pathogens (Swanson et al., 2009a,b), were not found in healthy broods. Olfaction mediates the hygienic behavior of these bees (Masterman et al., 2000; Gramacho and Spivak, 2003). According to the crystal structure of *A. mellifera* OBP5, *N*-butyl-benzenesulfonamide was an ideal competitive ligand. Therefore, it should be used in further analysis of ligand binding and release.

In a previous study, a laser interferometer was used to detect the movements released through the capping of brood cells containing live, dead, or *V. destructor*-infested pupae. The results showed that honeybees did not use acoustic signals to detect disease- or parasite-infected broods. In addition, an infrared thermographic system revealed no differences in temperatures between live, dead, and diseased broods. Therefore, honeybees might not use temperature or acoustic signals to detect a brood cell that is infested with *V. destructor* or diseased larvae (Gramacho et al., 1997). This powerful behavioral model indicates that, in addition to

olfaction, foragers could play an important role in the search for nectar and pollen and that honeybees could require a visual sense for flight orientation.

In our study, the *obp5* gene from *A. mellifera* and *A. cerana* exhibited differential expression in the antennae of workers. This result confirmed the association between the gene expression patterns of the bee species and the colony (Whitfield et al., 2006). The expression of the *Amobp5* gene in 10- and 15-day-old workers was significantly higher than the expression at other ages. According to bee biology, 10- to 15-day-old workers are hive bees that nurse and clean in the dark hive according to olfactory signals for tasks. It has been reported that both 11- and 15-day-old workers performed hygienic activities (Invernizzi Castillo and Corbella, 1999; Arathi et al., 2000). Moreover, Palacio et al. (2010) demonstrated that 15-day-old workers rapidly initiated uncapping behaviors depending on a stimulus from a dead brood. Workers display a dynamic age-related division of labor and natural behavioral plasticity (Ben-Shahar, 2005; Le Conte and Hefetz, 2008). The change from cell cleaning to nurse and other in-hive tasks (Winston, 1991) are performed by 21-day-old workers. From a molecular perspective, the shift to foraging nectar and pollen could be regulated by various gene expression patterns (Whitfield et al., 2003; Behura and Whitfield, 2010). In our experiment, 1-day-old workers had weak expression levels of the *Amobp5* and *Acobp5* genes; this result is consistent with reports that the maturation of the olfactory neuron function begins 2 days before emergence (Masson and Arnold, 1984). When bees emerge, 1-week-old workers assume cleaning behaviors in their colony (Ben-Shahar et al., 2004; Leoncini et al., 2004; Whitfield et al., 2006). After about 1 week, bees begin to perform new roles, such as nursing, storing, and processing food (e.g., turning nectar into honey) (Trhlin and Rajchard, 2011). Most bees begin foraging for pollen and nectar around 3 weeks of age (Leoncini et al., 2004; Ben-Shahar, 2005; Whitfield et al., 2006). The *Acobp5* gene was significantly expressed in 30-day-old workers. Although *Acobp5* gene expression was also relatively high in 25-day-old workers, it was not significantly higher than the expression at other ages. In *A. cerana*, the *Acobp5* gene was expressed at high levels during the foraging stage, which mainly involves out-of-hive tasks. The highest levels of the *A. cerana* antenna special protein gene expression were in 27-day-old workers (Li et al., 2008), which is similar to the time of *obp5* gene expression in this study. Although we could not determine the functional significance of the lower transcript levels in 10-, 15-, and 20-day-old workers, we presume that the *Amobp5* gene in *A. mellifera* and the *Acobp5* gene in *A. cerana* could assume different functions. Our results suggest that the *Acobp5* gene may play a critical role in acquiring the odorant molecules related to foraging for nectar and pollen or other general odorants. The *Amobp5* gene may be involved in tasks in the hive, such as cleaning or uncapping cells.

In summary, AmOBP5 and AcOBP5 were obtained by heterologous expression in *E. coli*. They showed differential binding affinities to 3 volatile compounds in competitive binding assays. The *Amobp5* and *Acobp5* genes had different temporal expression patterns. The *Amobp5* gene was expressed significantly higher in 10- and 15-day-old workers than in workers of other ages. However, the highest expression level of the *Acobp5* gene was in 30-day-old workers. The *Amobp5* and *Acobp5* genes had the lowest expression level in 1-day-old workers. We presume that the *obp5* gene plays different roles in *A. mellifera* and *A. cerana*. The *Amobp5* gene may be correlated with nurse and cleaning activities in the hive, while the *Acobp5* gene may be related to foraging work.

These proteins play a crucial role in the recognition of olfactory messages. Additional studies are needed to better understand the honeybee olfactory system and behavior.

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