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The Odorant-Binding Protein *obp11* Gene Shows Different Spatiotemporal Roles in the Olfactory System of *Apis mellifera ligustica* and *Apis cerana cerana*

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Abstract
Odorant-binding proteins participate in the olfactory system of the honeybee. *Apis mellifera ligustica* and *Apis cerana cerana* are species of honeybee that have different biological functions. The two species have diversified olfactory systems, with *A. cerana* displaying sensitive olfactory involvement in collecting nectar and pollen from small plants; and *A. mellifera* collecting from large nectariferous plants. We hypothesized that, given this difference in biological activity, the *obp11* genes of *A. mellifera* and *A. cerana* may show different olfactory expression patterns. We cloned and sequenced the *obp11* genes from *A. mellifera (Amobp11)* and *A. cerana (Acobp11)*. Using quantitative real-time PCR, we demonstrated that nurse workers, which have the highest olfactory sensitivity in the *A. mellifera* hive, have the highest expression of the *Amobp11* gene; whereas 1-day- emerged workers, which have the lowest olfactory sensitivity, have correspondingly low expression. However, the highest expression of the *Acobp11* gene is observed for foragers, which display the highest olfactory sensitivity in the *A. cerana* population. The OBP11 protein from the two species is highly conserved, with an apparent molecular weight and predicted extracellular localization that is similar to other OBP proteins. The expression of the *obp11* gene in *A. mellifera* and *A. cerana* correlates with the different roles of the olfactory system for the two different species. These findings support the critical role of odorant-binding proteins in the honeybee olfactory system.

Introduction

For social insects such as the honeybee, olfactory language plays a critical role in colony life, with important functional roles for work within and outside of the hive. According to honeybee biology, newly emerged bees are developmentally immature (Calderone, 1998), and they join a caste that mainly cleans cells while awaiting functional maturity (Winston, 1987). From the ages of 4-12 days (Seeley, 1982), the nursing caste feeds larvae or the queen for about 1 week (Seeley, 1979). Middle-aged bees (12-21 days old) build and maintain the nest, and receive and process nectar (Johnson, 2003, 2008a). After 21 days, workers initiate tasks outside of the nest (foraging nectar and pollen, scouting, defending) (Seeley, 1995; Seeley & Visscher, 2004; Beekman et al., 2006; Visscher, 2007). These behaviors are correlated with the function of the olfactory system, which mediates volatile signals to workers, as opposed to contact perception (Maisonnasse, 2010). Moritz and Crewe suggested that the queen emits volatile pheromones to inhibit new queens or the ovary development of workers (Moritz & Crewe, 1991).

Honeybees have been observed as a model for the insect olfactory system (Vanesa, 2009). The odorant-binding proteins (OBPs) of honeybees are the main functional proteins in the olfactory system. OBPs recognize and distinguish volatile compounds and then transport these compounds to olfactory receptors. Honeybee OBPs are small, water soluble molecules, which are expressed as abundant extracellular proteins. The genome of the honeybee (Honeybee Genome Sequencing Consortium, 2006) contains 21 genes encoding OBPs (Forêt & Maleszka, 2006), each gene with a markedly different expression pattern. Most of the OBPs are restricted

to olfactory tissues, with particularly high expression in the antenna, while the *obp11* gene is expressed exclusively in the antenna of adult bees (Francesca et al., 2010).

Apis cerana cerana Fabricius (*A. cerana*), is an important local species in China. *A. cerana* has a keen olfactory ability, foraging small and dispersed nectariferous plants, defending strongly against ectoparasitic *V. destructor* and chalkbrood disease, and providing tolerance for low environmental temperatures (Sarah et al., 2010). On the other hand, *Apis mellifera ligustica* Spinola (*A. mellifera*) belongs to a species of honeybee that mainly forages large nectariferous plant and produces a number of bee products (royal jelly, pollen, propolis, etc). *A. mellifera* is easily infected with *Varroa destructor* and chalkbrood disease. While *A. cerana* and *A. mellifera* are two important honeybee species that exhibit long-term evolutionary divergence (Qiu et al., 2012), the colonies of *A. cerana* suffer less damage than those of *A. mellifera* from parasites and chalkbrood disease. Because of its sensitive olfactory system, *A. cerana* recognizes and distinguishes dummy larvae more efficiently than *A. mellifera*. However, to our knowledge, there have been few direct comparisons between the molecular mechanisms of olfactory sensing of the two species. Therefore, in this study, experimentation was performed to compare the *obp11* genes of *A. cerana* and *A. mellifera* at the molecular level. We examined whether the *obp11* gene expression pattern correlates with the different roles of the olfactory system in *A. mellifera* and *A. cerana*, thus providing a molecular basis for the differences in behavior of the two species. Based on our findings, we propose that the *obp11* gene expression patterns vary according to the divergent evolutionary behavior of two species.

Material and Methods

Samples collection

A. mellifera and *A. cerana* were fed in an experimental apiary of bee science at the College of Bee Science of Fujian Agriculture and Forestry University during the spring of 2011-2012. Individual insects were collected within 24h of emergence, marked with enamel paint on the thorax to identify them by age, and then introduced into the same healthy colony. When appropriate, antennae were harvested from *A. mellifera* and *A. cerana*. The samples were first collected into Trizol reagent and then stored at -70ºC until use.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from the antennae of sampled bees using Trizol reagent according to the manual's protocol (Invitrogen, USA). Subsequently, cDNA was synthesized using the Promega RT-PCR System according to the manual's protocol(Promega, USA).

Obp11 gene cloning and expression

The primers used for amplifying the *obp11* gene from *A. mellifera* and *A. cerana* were designed using Primer Primer 5.0 (Table 1). PCR was performed using the following program: denaturation at 94ºC for 1 min; 35 cycles of 94ºC for 40 s, annealing at 57ºC for 50 s, and extension at 72ºC for 50 min; and a final extension at 72ºC for 7 min. PCR products were cloned into pGEM-T vector (Promega, USA) and transformed into *E. coli* DH5α. Positive colonies were selected by identification with the restriction endonucleases *EcoR I* and *Xhol* I, and then the *obp11* gene fragments were purified using a Gel Extraction Kit (Sangon, Shanghai) and cloned into pET-28a vector, which was digested with the same restriction endonucleases, to construct recombinant expression plasmids pET-28a-*Amobp11* and pET-28a-*Acobp11*. We used IPTG to induce expression of recombinant proteins in *E. coli.*

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

Primer	Sense and antisense sequences (5′–3′)	Purpose
Aobp11	GAATTCATGAAAGCAGCAGAAATTTG / CTCGAGTCACGGAGCAATAAACGCTA	cDNA isolation (reverse transcriptase PCR)
Bobp11	TCTCGTTTATGGGGAAATCAGCGAT / TCCGTATTCCGTAGCTTCGACATCC	Expression analysis (Real-time PCR)
β-Actin	TGCCAACACTGTCCTTTCTG / AGAATTGACCCACCAATCCA	Internal control

Real-time PCR (RT-PCR)

Antennae were separately collected at 1, 4, 10, 15, 20, 25 and 30 days of age, and total RNA was extracted and reverse transcribed to synthesize cDNA, in accordance with the Promega manual. Samples were stored at -70ºC until use. Real-time PCR was performed using the Applied Biosystems StepOnePlus Real-Time PCR System with SYBR Green dye (Promega) in 96-well plates (ABI, USA). Relative quantification analysis was performed according to cycle threshold values (CT) generated from the Promega GoTaq 2-Step RT-qPCR system (Promega, USA). Standard curves were prepared using a purified PCR product for the *obp11* and *β-actin* genes. For each experiment, the endogenous *β-actin* g gene was analyzed in triplicate (internal control, Shu *et al*., 2011) with a non-template reaction (negative control) and a water only reaction (blank control). Relative quantification analysis was performed using the comparative standard method (Schefe et al., 2006).

Statistical analysis

Quantitative data are presented as mean ± standard deviation (SD) for real-time PCR experiments. One-way ANOVA (SPSS 17.0 Statistical software) was used to analyze the different expression pattern of each gene at different ages.

Results

Sequence analysis of the Amobp11 and Acobp11 genes

To characterize the *Amobp11* and *Acobp11* genes, a 432 bp open reading frame was amplified and sequenced. The *Amobp11* and *Acobp11* genes have many similar characteristics with about 99.31% identity (Fig. 1A).

The *Amobp11* gene encodes a 144 amino acid protein that contains a 23 amino acid hydrophobic signal peptide at the N-terminus. The software SignalP 4.0 (Petersen et al., 2011) predicted a molecular weight of 16.6 kDa and a pI of 5.06. The *Acobp11* gene encodes a 138 amino acid protein that has a predicted molecular weight of 16.2 kDa and a predicted pI of 4.95 (http://web.expasy.org/cgi-bin/protparam/protparam). The deduced amino acid sequences of AmOBP11 and AcOBP11 were aligned using DNAMAN software (Fig.1B). The alignment shows that the two sequences vary in sequence at the C-terminus, which is truncated by 6 cysteine residues for AcOBP11. Using TMHMM2.0 posterior probabilities for sequences outside the transmembrane, AmOBP11 and AcOBP11, like the other OBPs, are predicted to be extracellular proteins. According to the Kyte and Doolittle method, AmOBP11 and AcOBP11 are hydrophilic in property; however, these proteins have some aliphatic amino acids, with an aliphatic index of 81.88 for AcOBP11 and 83.15 for AmOBP11, as well as four region of lipophilicity (http://web.expasy.org/protparam/). Compared with 12 OBPs (1-11and 13), AmOBP11 and AcOBP11 have six conserved cysteine residues, which are shaded in dark (Fig. 1C).

Heterologous expression of the Amobp5 and Acobp5 gene

We cloned the *Amobp11* and *Acobp11* genes into plasmids, as verified by digestion with the restriction endonucleases *EcoR I* and *Xhol* I. Subsequently, cohesive termini of the target gene were inserted into the expression vector pET-28a and transformed into *E. coli* BL21/Rosetta competent cells.

Fig. 1 Analysis of the sequences of the *Amobp11* and *Acobp11* genes. A-Alignment of the *Amobp11* and *Acobp11* nucleic acid sequences. Divergent nucleic acid sequences are circled. B-Alignment of the deduced AmOBP11 and AcOBP11 protein sequences. Divergent amino acid sequences are circled. C-Alignment the CDS protein sequences of 12OBPs (1-11 and 13) and AmOBP11 and AcOBP11. The conserved cysteine residues in this alignment are shaded in dark.

Finally, we used 1 mM IPTG to induce *Amobp11* and *Acobp11* gene expression. The electrophoretic bands corresponding to recombinant OBP11 protein from pET-28a-*Amobp11-E.coli* Rosetta (AmOBP11) and pET-28a-*Amobp11-E.coli* Rosetta (AcOBP11) were verified to be approximately 16 kDa on a 12% SDS-PAGE gel (Fig.2). We used ultrasonic energy to release AmOBP11 and AcOBP11 protein, which formed an insoluble inclusion body.

Expression profiling of Amobp11 and Acobp11 genes by real-time PCR

To further characterize the functions of the respective *obp11* genes in each species of honeybee, we used real-time PCR to quantitatively assess expression levels. The PCR amplification efficiency for the *obp11* gene was 94.8% (slope = -3.453). The efficiency for the *β*-actin gene was 96.4% (slope

= -3.412). This indicates that the relative standard curve method for real-time PCR analysis with SYBR Green dye is experimentally suitable. The transcript abundance was calculated based on the difference in threshold cycle (Ct) values between the *obp11* and *β-actin* gene transcripts.

The *Amobp11* gene demonstrated the highest expression in the antennae of 10-day-old and 15-day-old workers, with nearly 200-fold increase. Other days presented relatively lower expression (Fig.3). In contrast, the *Acobp11* gene demonstrated expression that was low at 1 day with a slight increase up to 20 days. The highest expression was observed in 25-day-old and 30-day-old workers, which demonstrated approximately 9- to 12-fold increase in expression as compared to 1-day-old workers (Fig.4).

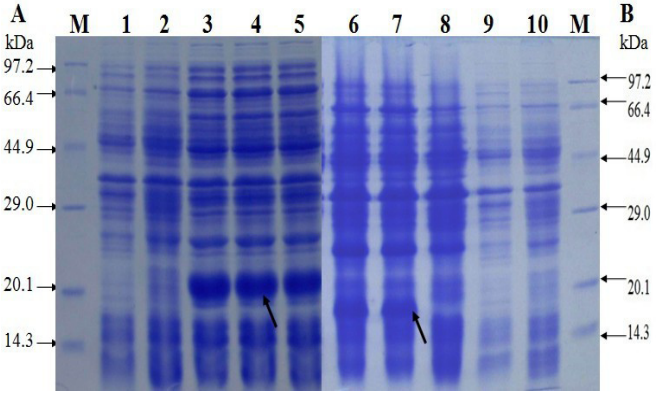

Fig. 2 SDS-PAGE analysis of recombinant AmOBP11 (A) and AcOBP11 (B). A: Lane M: Protein molecular weight marker; Lane 1: pET-28a+*E.coli* Rosetta; Lane 2: pET-28a-*Acobp11*+*E.coli* Rosetta without IPTG to induce expression; Lanes 3-5: pET-28a-*Acobp11*+*E. coli* Rosetta induced with IPTG; Lane 6-7: pET-28a-*Amobp11* +*E.coli* Rosetta induced with IPTG; Lane 8: pET-28a-*Amobp11*+*E. coli* Rosetta without IPTG; Lane 9: *E.coli* Rosetta; Lane 10: pET-28-a+*E.coli* Rosetta. Arrows show the OBP11 expression proteins.

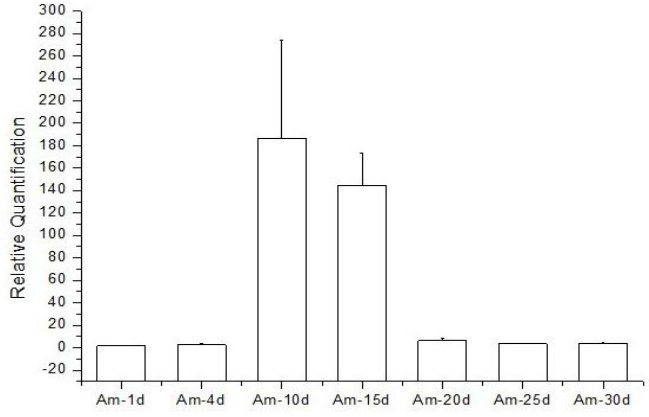

Fig.3 *Amobp11* gene expression levels in *Apis mellifera ligustica* antennae across seven ages determined by qRT-PCR Expression levels of the *Amobp11* gene were calculated relative to the control *β-actin* gene using the standard curve method. Bars on each column represent SD for three independent experiments. The *Amobp11* gene expression levels in 10-day-old and 15-day-old workers were significantly higher than others ages (F=5.269, P<0.01, n=3), and expression of the *Amobp11* gene in 1-day-old workers was the lowest. There were no significant differences between expression in 10-day-old and 15-day-old workers, or others days workers (Duncan' ANOVA test).

Discussion

In this study, we cloned and identified the *Acobp11* and *Amobp11* genes. Based on our sequence analysis, we concluded that the *obp11* genes of the two species have high similarity. The predicted protein sequences of AmOBP11 and AcOBP11 are conserved throughout the molecule, with the exception of one internal residue and six residues at the c-terminus. Furthermore, hydrophobicity analysis suggests that, similar to other honeybee OBPs, AmOBP11 and AcOBP11 are extracellular proteins. We heterologously expressed the *Amobp11* and *Acobp11* genes in *E. coli* and demonstrated an apparent molecular mass that agrees with the predicted values for the native honeybee proteins.

As a social insect, the foragers of *A. mellifera* seek large nectariferous plants, while the *A. cerana* foragers seek small and dispersed nectariferous plants. Therefore, the divergent behavior of *A. mellifera* and *A. cerana* foragers suggests a diversity of olfactory function. Based on this diversity, we proposed that the expression of the *obp11* gene may differ for *A. mellifera* and *A. cerana*. Previously, Forêt and Maleszka (2006) indicated that the *obp11* gene is expressed exclusively in the antennae of adult bees; thus, we collected different aged workers' antennae to analyze developmental variations in *obp11* gene expression. Varying expression patterns of the *obp11* gene in the life cycle of each species could lead to different behaviors within and outside of the colony.

Both *Amobp11* and *Acobp11* genes showed the lowest expression levels in 1-day-old workers. Winston (1987) and Calderone (1998) reported that newly emerged bees cannot fly or sting, and thus are developmentally immature (Winston,

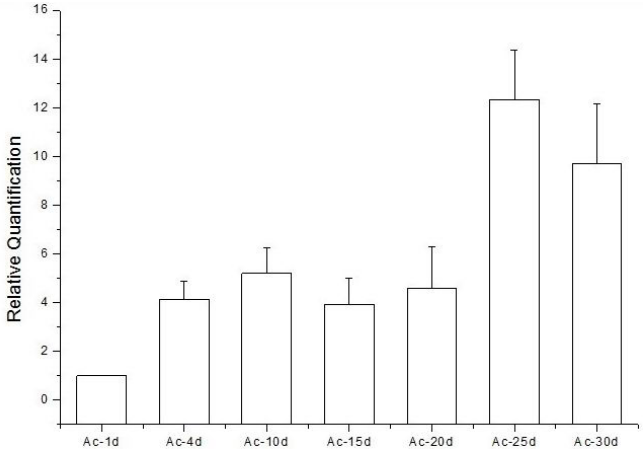

Fig.4 *Acobp11* gene expression levels in *Apis cerana cerana* antennae across seven ages determined by qRT-PCR expression levels of the *Acobp11* gene were calculated relative to the control *β-actin* gene using the standard curve method. Bars on each column represent SD for three independent experiments. The *Acobp11* gene expression levels in 25-day-old and 30-day-old workers were significantly higher than others ages (F=6.507, P<0.01, n=3), and expression of the *Acobp11* gene in 1-day-old workers was the lowest. There were no significant differences between expression in 25-day-old and 30-day-old workers, or between expression in 30-day-old and 10-day-old workers (Duncan' ANOVA test).

1987; Calderone, 1998). Therefore, the low expression of the *obp11* gene in the 1-day-old workers of both species correlates with reduced olfactory function. Olfactory sensing for the two species is closely correlated with the function of the *obp11* gene.

Our results demonstrate that the developmental and temporal expression profile of the *Amobp11* and *Acobp11* genes differs in the time and extent of induction after the first day of emergence. For *A. mellifera,* the *obp11* gene expression peaks at 10 to 15 days of age in workers, with nearly 200-fold higher expression than at others ages; whereas for *A. cerana,* the *obp11* gene, expression rises gradually with the highest expression in the 25-day-old workers. The different peaks of expression for the two species correlate with different behavioral functions in the life cycle of the honeybee. For most honeybee species, 10-day-old workers feed larvae, while 12- to 21-day-old workers build and store and process food. At the peak of *Amobp11* gene expression (10 and 15 days old workers), the honeybees perform nurse-tasks, begin to secrete beeswax, and work to clean within the hive. While performing these in-hive tasks, the 10- to 15-day-old workers are attracted by brood pheromones. For this reason, we propose that the *Amobp11* gene expression in the olfactory system is correlated with nursing tasks.

On the other hand, at the peak of *Acobp11* expression (25 days of age) most worker bees begin to forage for nectar and pollen (Johnson, 2008b, 2010). The later peak of *Acobp11* expression is similar to that of Acer-ASP2 (antenna special protein), which is expressed more highly in 27-day-old workers than at other ages (Lee et al., 2008). Acer-ASP2 is related to odorant sensors that function in the collection of certain nectars and pollens (Danty et al., 1997; Li, et al., 2008). Therefore, we propose that the *Acobp11* gene has a similar function in out-hive foraging, and that the difference in the temporal expression of the *Amobp11* and *Acobp11* genes might suggest different functional outcomes in sensing the intention of the OBP11 proteins for the two species.

The differences in the levels of induction of the *Amobp11* and *Acobp11* genes also may be indicative of diffe-rent functions. The *Amobp11* gene is induced nearly 200-fold at 10-day-old workers, while the *Acobp11* gene is induced approximately 5-fold in 10-day-old workers; and 10- to 12-fold at its peak induction in 25 to 30-day-old workers. Further experiments are needed to assess why obvious expression differences of the *Amobp11* and *Acobp11* genes, and the *obp11* gene show different roles in the olfactory system of *A. mellifera* and *A. cerana*.

The olfactory stimuli that motivate honeybees may come from outside the hive or from within the hive, and differing exposure to each of these stimuli would undoubtedly have a profound effect, which is consistent with the OBP expression and may determine the behavior of the honeybee. The olfactory system within the hive functions to motivate workers to perform different tasks based on the age of larvae (Le Conte, 2001, 2008; Maisonnasse, 2009). Larvae of different ages emit volatile compounds to adjust the behavior of the honeybees. Young larvae emit E-*ß*-ocimene, a highly volatile pheromone that is dispersed within the colony to accelerate forage behavior of in-hive workers by the olfactory system (Maisonnasse, 2010). Brood ester pheromones motivate older workers to take care of the brood (Slessor, 2005; Pankiw, 2007; Peters, 2010). Newly emerging bees engage in some nursing behaviors too, whereas the others ages, such as middle-aged and 12-21 days old workers do not engage in nursing behavior (Ben-Shahar et al., 2002; Ben-Shahar, 2005). Meanwhile, E-*ß*-ocimene is one of the monoterpene volatile organic compounds, which is emited by larvae to engage workers in nursing tasks. During the process of performing nursing tasks, OBPs play an important role in the olfactory system of the honeybee. OBPs assist E-*ß*-ocimene to transfer to the olfactory receptor, which elicits the corresponding behavior. The *Amobp11* gene belongs to one of the main antennae OBPs, The 10- to 15-day-old workers have the highest expression of *Amobp11* gene. Thus, we propose that the high *Amobp11* gene expression of 10- to 15-day-old workers is correlated with sensitive olfactory reception of volatile brood compounds, which might encourage them to engage in nursing behaviors (Maisonnasse, 2009).

On the other hand, because the peak of *Acobp11* gene expression is in the foraging stage, it is likely that the function of this *obp11* gene for *A. cerana* is more highly evolved to respond to a different set of olfactory stimuli, which are encountered outside of the hive. The olfactory system of *A. cerana* is known to be more sensitive than that of *A. mellifera*, especially in regards to the collection of honey and pollen. Therefore, it is likely that the OBP11 proteins of these two species might performing in the response to different sources of stimuli to determine the differing behavior of these two species at different stages. We conclude that the spatiotemporal expression patterns of the *obp11* gene in *A. mellifera* and *A. cerana* suggests that this gene plays different roles in the olfactory sensitivity of workers.

Conclusions

We demonstrated that the nurse workers, which have the highest olfactory sensitivity in the *A. mellifera* colony, have the highest expression of *Amobp11* gene; whereas 1-day-old workers, which have lowest olfactory sensitivity, have correspondingly low expression. However, the highest expression of *Acobp11* gene is observed for foragers, which display the highest olfactory sensitivity in the *A. cerana* population.

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