Expression Characterization and Localization of the foraging Gene in the Chinese Bee, *Apis cerana cerana* (Hymenoptera: Apidae)

WeiHua Ma,1,2,4 YuSuo Jiang,2 Jiao Meng,2 HuiTing Zhao,3 HuaLei Song,1 and JinShan Shen1

1Institute of Horticulture, Shanxi Academy of Agricultural Sciences, Taiyuan, Shanxi 030031, China, 2College of Animal Science and Veterinary Medicine, Shanxi Agricultural University, Taigu, Shanxi 030801, China, 3College of Life Science, Shanxi Agricultural University, Taiigu, Shanxi 030801, China, and 4Corresponding author, e-mail: mwhuachina@163.com

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Abstract

In social insects, the foraging gene (for) regulates insect age- and task-based foraging behaviors. We studied the expression and localization of the for gene (*Acfor*) in *Apis cerana cerana* workers to explore whether the differential regulation of this gene is associated with the behaviors of nurses and foragers. The expression profiles of *Acfor* in different tissues and at different ages were examined using real-time quantitative reverse transcription polymerase chain reaction. Cellular localization in the brain was detected using in situ hybridization. *Acfor* transcripts in different ages workers showed that *Acfor* expression was detected in all the heads of 1- to 30-d-old worker bees. *Acfor* expression reached a peak at 25 d of age, and then declined with increasing age. The results showed that *Acfor* gene expression in five tissues was respectively significantly higher in foragers than in nurses. In nurses, the relative expression of *Acfor* was the highest in the antennae. There was a highly significant difference in expression between antennae, legs, and the other three tissues. In foragers, *Acfor* expression was the highest in the thorax, which was significantly different from all other tissues. In situ hybridization showed that *Acfor* was highly expressed in the lamina of the optic lobes, in a central column of Kenyon cells in the mushroom bodies of the brain of workers, and in the antennal lobes. This suggested that *Acfor* expression affects age-related foraging behavior in *Apis cerana cerana*, and that it may be related to flight activity.

Key words: *Apis cerana cerana*, foraging, expression profiles, localization

The age-related behavior transition by honey bees from hive work to foraging is well known (Ben Shahar et al. 2002). Adult worker bees perform nursing and hive activities when they are young, whereas older bees of approximately 2–3 wk age forage outside the hive (Ben Shahar et al. 2002). The behavior transition is affected by the foraging gene (*for*) (Ben Shahar et al. 2002, 2003; Robinson 2002; Whitfield et al. 2003; Page et al. 2006; Heylen et al. 2008; Smith et al. 2008; Thamm and Scheiner 2014). The for gene has been linked to foraging-related behavior in several insect species (Osborne et al. 1997; Ben Shahar et al. 2002, 2003; Ingram 2005, 2011; Tobback 2008, 2011, 2013; Kodaira 2009; Lockett 2016). In addition, it plays a major role in behavioral plasticity. In *Drosophila melanogaster*, the for gene (*Dmfor*) was previously found in the ‘sitter’ and ‘rover’ mutants (Osborne et al. 1997). This gene encoded a cGMP-dependent protein kinase (PKG) (Osborne et al. 1997, Ben-Shahar et al. 2003) and affected *D. melanogaster* food deprivation (Osborne et al. 1997, Kaun et al. 2007). The expression of *Amfor*, a homologous gene of *Dmfor*, was upregulated in foragers as compared with that in nurse bees (Ben-Shahar et al. 2002, Heylen et al. 2008). Honeybee foraging-related behaviors were studied, including concentration of nectar collected, the amount of pollen and nectar brought back to the hive, and the functional genes involved in pollen hoarding and initiation of foraging (Hunt et al. 1995; Page et al. 2000, 2006; Rueppell et al. 2004a, b, 2009; Ruep2009). Previously, studies on *for* have mainly concentrated in the western honeybee, *Apis mellifera ligustica* (*Am*), whereas few on the eastern subspecies *Apis cerana cerana* (*Acc*). Previous reports. *Acc*, a honey bee species native to China, is widely kept, and it plays a key role in maintaining ecosystem diversity and economic benefits (Wang et al. 2012, Meng et al. 2017). *Acc* has a stronger capacity to forage sporadic nectar source flowers, cold tolerance, and learn and memorize the smell, color, and shape of flowers than *Am* (Qin et al. 2012, Wang et al. 2012, Yang et al. 2013, Meng et al. 2017). Thus, whether there has...
a difference in the expression characteristic of the foraging gene (Acfor) in Acc and Aml, even other insects.

In the present study, the different physiological stages and tissues of Acfor mRNA expression characteristics were determined and cellular localization in the brain was analyzed to lay the foundation for further studies on its function and provide a theoretical basis for foraging behavioral mechanisms of honeybees.

Materials and Methods

Bee Samples

Apis cerana cerana were obtained from the apiary of Shanxi Agriculture University, Shanxi, China from May to July in 2013. Emptied combs were placed in three normal colonies, and retrieved after the queen had laid and pupae were capped by a worker bee. Pupae were put into an incubator (33°C, 95% RH) for eclosion. We marked 150 bees per colony after emergence using nontoxic and tasteless paint (approximately 500 bees were marked in total); thereafter, the combs were returned to the hives. We began to sample just after the bees emerged, with bees just emerged recorded as 1-d old. Thereafter, we sampled every 5 d until the marked bees were not found. Ten bee heads were sampled per colony each time, with three biological replicates; i.e., 30 bee heads were collected. These samples were used for analyzing RNA expression profiles at different ages. Thirty nurse bees (7 d old) tagged and 30 pollen foragers from each colony were captured at the entrance of the hives on their way back. Thus, a total of 90 nurses and 90 foragers were sampled. The sampled were transported to the laboratory, where they were dissected on ice for their antennae, heads, thoraxes (with wing), abdomens, and legs. All the samples mentioned above were quickly frozen using liquid nitrogen, ground to a powdery form, put in an EP tube containing 1 ml Trizol, and stored at −80°C for mRNA expression pattern analysis. The brains of the 25-d-old bees, five bees sampled from each colony, were dissected for in situ hybridization. In total, 15 bees were sampled. All the samples were observed at 9:00–11:00 h on a single day.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from the ground bee samples using a Trizol reagent kit (Takara, Dalian, China) following the manufacturer's protocol, and treated using DNase I. The reaction volume of 10 μl included 5 μl total RNA, 1 μl 10x buffer, 1 μl DNase I, and 3 μl RNase-free water. The reaction conditions consisted of 37°C for 30 min, followed by the addition of 1 μl stop buffer added, and 65°C for 10 min. Total RNA concentration and purity were quantified using 1.0% denaturing and 18S rRNA reverse primer: 5′-TCACGGTCTCATCACCGGGCGA-3′

Acfor primer: 5′-TCAGGACCTCTAAGGAGGCCAAGAC-3′

Acfor forward primer: 5′-CCGTAATCGGATGATACACGTT-3′

18S rRNA forward primer: 5′-CCCAGTAATCGGAATGACT-3′

18S rRNA reverse primer: 5′-ACGCATTAGGGACTGGAATTACC-3′

Statistical Analyses

Statistical analysis was performed using the SPSS software (Stratagene). The statistical analysis was performed using the comparative 2- ΔΔCT method (Livak and Schmittgen 2001). Statistical analysis of data was performed using SPSS version 19.0 (SPSS Inc., Chicago, IL).

mRNA Expression Profiles at Different Ages

Three digoxigenin-labeled RNA sense and antisense oligonucleotide probes were synthesized using Boster (www.bosterbio.com) according to the full-length cDNA of Acfor (KP662686.1). The sequence of the antisense probes was as follows:

5′-CAAGA GTTAC GAAGC CATCT CGACA AGTGG AGTTC-3′

5′-CGCTG CCAAT TGTCG AACAA GAGGG ACAGA TCTCG-3′

5′-GATGG CCAGC TATGG GCCAT CGACC GACAA TGCTT-3′

The complete brains from 25-d-old adult worker bees were dissected and embedded in Tissue-Tek OCT Compound (Sakura Finetechical Co., Ltd, Tokyo, Japan). Cryosections (8 μm thick) were prepared at −20°C and mounted on poly-L-lysine-treated slides. The samples were air-dried for 5 min before being fixed for 24 h in 4% paraformaldehyde/0.1 M phosphate-buffered saline containing 0.1% diethyl pyrocarbonate (Zhao et al. 2014). Hybridization protocols were performed using the RISH kit (Boster, Wuhan, China) according to the manufacturer’s instructions. The sections were viewed using Olympus BX53 (Olympus Corporation, Tokyo, Japan) and the images were captured using Image-Pro Plus 7.0 (Media Cybernetics, Inc., Rockville, USA).

Results

mRNA Expression Profiles at Different Ages

The expression profiles of Acfor transcripts of different ages from worker bees were presented in Fig. 1. Acfor transcripts were detected at all ages, but the quantity varied with age. The expression of Acfor decreased from days 1 to 10, and began to increase thereafter, and it remained at peak levels until day 25. Thereafter, the Acfor expression levels declined with increasing age. The Acfor transcript in age-marked worker bees showed maximal expression (1.7957) at the age of 25 d, which was significantly different from that shown at other ages (P < 0.01), and it was 6.67-fold higher than the minimal value (at 10 d of age). The expression levels were significantly different between days 1, 20, and 30 and days 5, 10, and 15, respectively (P < 0.01).
Fig. 1. Relative amounts of *Apis cerana* cerana foraging (Acfor) mRNA in workers at different ages. Data are mean ± SEM, and different lowercase letters indicate significant differences at *P* < 0.05. The samples had been collected from emergence of the bees, which were recorded as bees that were 1-d old, and taking a sample every 5 d (*N* = 10 bees).

mRNA Expression Profiles in Different Tissues

Acfor expression profiles in different tissue of nurse and forager bees are shown in Fig. 2. Acfor mRNA expression was detected in the antenna, head, thorax, abdomen, and leg of worker bees. The expressions in five tissues of foragers were significantly higher than those in tissues of nurse bees (P < 0.01). In nurses, expression was the highest in the antennae (0.8842), followed by the legs (0.8552), although the difference was not significant (P > 0.05). However, Acfor mRNA expression showed a highly significant difference between antennae, legs, and the other three tissues studied (P < 0.01). In foragers, expression levels were the highest in the thorax (4.1451), which were significantly different from those in the other tissues (P < 0.01). The expression levels could be arranged in descending order as follows: abdomen > head > legs > antennae; however, there were no significant differences between these four tissues (P > 0.05).

Localization Analysis

To investigate the localization of Acfor mRNA in the brain, in situ hybridization was performed using digoxigenin-labeled probes. Acfor was highly expressed in Kenyon cells (KC) of the mushroom body (MB), optic lobes (OL), and antennal lobes (AL) in the brain at 25 d of age (Fig. 3). No signal was detected in the controls.

Discussion

The honeybee colony relies on age-related division of labor. Adult worker bees perform nursing and hive activities when they are young and shift to foraging for nectar and pollen outside the hive when they are approximately 2- to 3-wk old. Amfor expression peaked at 18–22 d of age (Heylen et al. 2008), and at 25 d of age in the present study. Therefore, Acfor and Amfor had similar age-related expression patterns. However, foraging peak times for Acfor and Amfor were different. The age at the onset of foraging was not exact. It depended on the needs of the colony and the nectar and pollen resources in the external environment. While the nurses showed higher expression of for than foraging workers in *Vespula vulgaris* L., *Bombus ignitus* Smith, and *Pogonomyrmex barbatus* Smith (Ingram et al. 2005, Tobback et al. 2008, Kodaira et al. 2009). Moreover, for had different expression patterns in different subspecies (Ingram et al. 2011, 2016; Tobback et al. 2011). In *Cardiocondyla obscurior* Wheeler, for affected the behavior associated with age, whereas it was not associated with task (Oettler et al. 2015). This suggested that for is conserved in foraging behaviors and gene pathways within and across species; however, the regulation of these pathways has evolved and caused different foraging behaviors in different species. The expression abundance was higher on day 1 in the present study, which may have been due to a significant change in light intensity between pupal and eclosion stages. In addition, food searching behavior in the newly eclosed workers, known as bee phototaxis, partly affected the for expression (Ben-Shahar et al. 2003).

We investigated Acfor expression in the antennae, head, thorax, abdomen, and legs of nurses and foragers. The head is a commonly sampled body part of insects, and for has been detected in the heads of fruit flies, honey bees, ants, and *Bombus* sp. (Osborne et al. 1997; Ben Shahar et al. 2002, 2003; Ingram et al. 2005, 2011, 2016; Tobback et al. 2008, 2011, 2013; Kodaira et al. 2009; Lockett et al. 2016). In *Lasiglossum laevissimum* Smith, for gene expression levels were similar in the head, thorax, and abdomen in newly eclosed males not yet capable of flight, and they were the highest in the thorax and lowest in the abdomen of queens during the first brood provisioning period in spring. This suggested that increased for expression was associated with flight activity in *L. laevissimum* (Awde et al. 2014). In our studies, Acfor mRNA expression was detected in the antennae, head, thorax, abdomen, and legs of worker bees. Foragers showed significantly higher expression in five tissues than nurse bees, which was in accordance with the findings of Ben-Shahar et al. (2002, 2003) and Heylen et al. (2008). Conversely, Liu et al. (2011) found that Amfor expression did not differ significantly between foragers and nurses, but it was associated with species, environment, and colony structure. In foragers, Acfor expression was the highest in the thorax, and it did not differ significantly from that of the other tissues. This may be because the thorax, the sports center of the forager bee, and needs more energy during flight outside the hive, which causes an increase in gene expression (Roberts and Harrison 1999, Roberts and Elekonich 2005, Awde et al. 2014).

In situ hybridization analysis showed that Acfor was highly expressed in KC of the mushroom body, the lamina of the OL, and AL in the brain at the age of 25 d, whereas Amfor in foragers was highly
expressed in the lamina of the OL and KC of the mushroom body (Ben-Shahar et al. 2002). OL are an important part of the transmission and processing of visual cues in the compound eye (Gronenberg 2001, Ehmer and Gronenberg 2002), and AL is the primary olfactory processing center (Müller 2002). In addition, MB can integrate massive amounts of information, including vision, olfaction, and tactile, from multisensory organs to form associated memories, which are transferred to the central nervous system and cause neural physiological reactions (Müller 2002). Thus, MB and AL are more active than OL in learning and memory tasks.

The transition of bees from working inside the hive to foraging outside may be affected by Acfor and other genes, and by extrinsic factors. Therefore, further studies are needed to understand how genes affect behavioral plasticity in bees.

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