LOCATION AND STRENGTH OF MALVOLIO EXPRESSION IN CHINESE HONEYBEE REVEALS ITS POTENTIAL ROLE IN LABOR DIVISION

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Abstract

The *malvolio* (*mvl*) gene plays an important role in the transition from nursing to foraging in honeybees (*Apis mellifera*). *Apis cerana cerana* (*A. c. cerana*) is a subspecies of the eastern honeybee, well-known for its pollinator role throughout China. Although *A. c. cerana* shares many characteristics with *A. mellifera*, it is unclear whether *Acmvl* plays a similar role to *Ammvl* in foraging behavior. In this study, *Acmvl* expression was quantified during the transition from nursing to foraging in *A. c. cerana*. *Acmvl* protein production was also characterized in different tissues in bees from three behavioral groups. Finally, in situ hybridization was used to describe *Acmvl* expression patterns in forager bee brains. *Acmvl* expression was low early in life but then showed a major peak, which suggests its role in labor division. Examination of tissue type revealed that *Acmvl* expression was highest in the thoraxes of nurse bees and the heads of forager bees. In bee brains, *Acmvl* was selectively expressed in the somata of Kenyon cells in the mushroom bodies, optic lobes and antennal lobes. Taken together, these findings suggest that *Acmvl* plays a role in the nurse-forager transition of *A. c. cerana*.

Keywords: aging, *Apis cerana cerana*, gene expression, location, *malvolio*, protein expression

INTRODUCTION

Social insects are of special significance in the field of social behavioral evolution due to their unique colony organization forms (Toth & Robinson, 2007). Among the social insects, honeybees are well known for their complex, age-related division of labor and have often served as a model for the study of eusociality (Weinstock et al., 2006; Lattorff & Moritz, 2013). Young worker bees begin with tasks in

the hive and then transition to nectar or pollen foraging when they are two to three weeks old (Robinson, 2002; Denison & Raymond-Delpech, 2008).

This regulation of task switching, from nursing to foraging, is determined by the complex interaction of numerous intrinsic and extrinsic factors (Robinson, 2002). Characterization of the molecular mechanisms underlying social behavior may aid in the understanding of this division in labor (Denison & Raymond-Delpech,

2008). Large-scale functional genomic analyses have revealed patterns of differential gene expression during the transition from nurse to forager, that a large number of genes were found that are associated with this behavioral transition (Heylen et al., 2008; Lattorff & Moritz, 2013). However, *malvolio* and *foraging* are the only two potential causal genes to be identified (Heylen et al., 2008).

Malvolio (*mvI*) was first described in *Drosophila melanogaster*, where it plays a role in feeding behavior (Rodrigues et al., 1995). The *mvI* transporter affects food choice behaviors via the regulation of dopaminergic innervation in the mushroom bodies of *Drosophila* (Søvik et al., 2017), and *mvI* expression is correlated with feeding preferences and various metabolic processes in Calliphoridae (Diptera: Calyptratae) (Cardoso et al., 2016). Both patterns of *AmmvI* mRNA expressions and sucrose responsiveness experiments suggested the role of *mvI* in the honeybee foraging behavior (Ben-Shahar et al., 2004; Pankiw & Page, 2003; Denison & Raymond-Delpech, 2008; Toth & Robinson, 2009; Zayed & Robinson, 2012).

The Chinese honeybee (*Apis cerana cerana*) is a subspecies of the eastern honeybee well-known for pollinating flowering plants throughout China and collecting nectar from scattered floral resources (Wang et al., 2012). However, few studies have been conducted on its foraging behavior and molecular mechanisms. *Apis cerana* collects more pear pollen than *Apis mellifera*, the western honeybee, under natural conditions, which may lead to increased pear pollination near *A. cerana* hives (Gemeda et al., 2017). The expression and localization of the *for* gene (*Acfor*) have also been explored in *Apis cerana cerana* Fabricius workers. Further study of the *for* gene function is expected to provide a theoretical basis for honeybee foraging behavior mechanisms (Ma et al., 2018).

Although *A. c. cerana* is similar to *A. mellifera* (Oldroyd & Wongsiri, 2006), it is unclear whether *mvI* plays the same role in foraging behavior for both species. Previously, our teams had cloned the cDNA sequence of the *mvI* gene in *A. c. cerana*, analyzed its biological properties,

and determined its expression level in different tissues (Meng et al., 2015). More detailed studies of the molecular mechanisms underlying *A. c. cerana* foraging are still required.

In this study, real-time quantitative PCR (qPCR) was employed to investigate *mvI* expression throughout the behavioral transition of *A. c. cerana* bees from nursing to foraging. Additionally, western blots were used to quantify *AcmvI* expression in dissected body parts from three honeybee social groups, and *AcmvI*-expressing regions were identified in forager honeybee brains with the use of in-situ hybridization with digoxin-labeled probes. The study goal was to characterize *mvI* expression patterns and possible functions in order to better understand its role in the early task transition of *A. c. cerana*.

MATERIAL AND METHODS

Bee samples

Chinese honeybees, *Apis cerana cerana*, were collected from the apiary of Shanxi Agricultural University in Shanxi, China. Three healthy colonies were selected for study; each colony had a new queen and six frames of bees in Chinese bee (*Apis cerana*) ten-frame hives. One capped comb was obtained from each colony, transferred to an incubator and warmed at 34.5°C and 95% RH until pupal emergence. The day-old bees were marked (100 bees per colony; 300 bees in total) and returned to the hives. The colonies were sampled from May to June when *Robinia pseudoacacia* L. was in bloom. Thus, the bees were fed with *Robinia* honey until their thirtieth day. Bees utilized in the mRNA expression analysis were collected at different ages. Each day, emerging worker bees were painted uniquely to mark their day of emergence. At 1, 5, 15, 20, 25 and 30 days of age, ten adult bees were collected from each of the three colonies. This time period was expected to span the transition from nurse to forager in *A. c. cerana*, and therefore to show differential *AcmvI* expression. Meanwhile, bees from different behavioral groups – nurse bees, pollen foragers and nectar foragers were collected

Table 1.

Primer and Probe sequences of *Acmvl* and house gene *18S* rRNA

Name for gene and probe	Sequence
<i>Acmvl</i>	Forward: TGCATACGTTCTCTGTCATTGTGG Reverse: AGGTGGCATATTTCTCGGTTGTG
<i>18S</i> rRNA	Forward: CCCGTAATCGGAATGAGTACACTTT Reverse: ACGCTATTGGAGCTGGAATTACC
<i>Acmvl</i> anti-sense probe	CAAGAGTTACGAAGCCATCTCGACAAGTTT CGCTGCCAATTGTGCAACAAGAGGGACAGA GATTGCCAGCTATGGGCCATCGACCGACAA

according to the protocol of Ben-Shahar et al. (2002, 2004) and Meng et al. (2015). During the 9:00-11:00 am foraging peak on sunny days, forty nurse bees (7 days old) were tagged and taken from within each colony, forty pollen foragers (foragers with pollen) were taken from the entrance of each hive and forty nectar foragers (foragers with distended abdomens) were captured on their way back to the hives (Ben-Shahar et al., 2004; Meng et al., 2015). Bee samples were transported to the laboratory and then dissected on ice; head, thorax (wings included), abdomen and leg samples were stored separately. Tissue samples were stored at -80°C until protein extraction. The brains of five pollen foragers from each colony were also dissected, and each was cut into two to four square cubic milliliters for in-situ hybridization.

Quantitative real-time PCR and *Acmvl* expression analysis

Acmvl-specific primers were designed according to the full-length *Ammvl* cDNA sequence (GenBank accession no. XM_623943) using the Primer 5 software. *18S* rRNA was selected as the reference gene for the quantification of *Acmvl* gene expression (Tab. 1).

Total RNA was extracted from bee tissues according to standard protocols using TRIzol (Invitrogen Life Technologies, USA). Total RNA integrity was determined via 1.0% agarose electrophoresis, and RNA was quantified at 260 nm. One µg of mRNA was then reverse transcribed into cDNA using PrimeScript® RT Master Mix (Perfect Real Time) (Takara, Dalian, China).

The qPCR reactions were carried out in a 20 µL

solution, including 10 µL SYBR Premix Ex Taq, 0.8 µL forward and reverse primer, 2 µL cDNA, 0.4 µL ROX Reference Dye II and 6 µL H₂O, on a MX 3500P system (Stratagene, La Jolla, CA, USA). The amplification procedure was as follows: initial denaturation at 95°C for 30 s; forty cycles at 95°C for 5 s, at 63°C for 35 s, and at 72°C for 30 s; then a 10 min elongation at 72°C. A melting curve analysis was performed at the end of each qPCR reaction to confirm that only one PCR product had been amplified. The $2^{-\Delta\Delta CT}$ method was used to quantify relative gene expression (Livak & Schmittgen, 2001).

Acmvl polyclonal antibody preparation and protein expression analysis

Rabbit polyclonal antibodies for the *Acmvl* protein were successfully prepared using AbMax Biotechnology Co., LTD (Beijing, China) with a previously reported full-length cDNA sequence for *Acmvl* (GenBank accession no. KP662687).

For protein extraction, honeybee tissues were first blended with a pre-cooled lysis buffer (tissue weight: lysis buffer volume=1:10) and then homogenized in an ultrasonic homogenizer. The homogenized samples were centrifuged at 12 000 rpm at 4°C for 10 min, and the supernatant was retained. A bicinchoninic acid assay kit (P0011, Beyotime Biotechnology, China) was used to quantify extracted proteins. From each sample, equal amounts of denatured protein were loaded on a SDS-polyacrylamide gel composed of a 5% stacking gel and a 10% separating gel; 80V was applied for one hour and 120V for three hours. After electrophoresis, the proteins were transferred to a nitrocellulose

(NC) filter membrane. Rabbit anti-*Acmv* serum was used as the primary antibody, and goat anti-rabbit IgG-horseradish peroxidase (Tiangen, Beijing, China) was used as the secondary antibody to detect *Acmv* expression. Rabbit polyclonal β -actin antibodies (Sangon) were used as a control. The primary and secondary antibodies were diluted at a ratio of 1:3000.

The *Acmv* content of different tissues was quantified using the Bio-Rad Bole ChemiDoc MP Chemiluminescence Imaging System (Bio-Rad, USA). Image J was used to calculate the ratio of the signal (or gray value) from the target bands to that of β -actin.

Statistical analysis

Statistical analysis of the data was performed using SPSS 17.0 (SPSS Inc. Chicago, IL, USA) (Zhao et al., 2014). All experiments were performed in triplicate ($n=3$) and data was summarized as mean $\pm$ SE. Differences in expression were compared using one-way ANOVAs. $p < 0.05$ was deemed statistically significant.

Location of *Acmv* expressing regions in forager honeybee brains using in-situ hybridization

Procedures and conditions for the in-situ hybridization of *Acmv* in honeybee brains were followed according to a previously described protocol (Ben-Shahar et al., 2002). Three digoxin-labeled RNA sense and antisense oligonucleotide probes were designed using the full-length *Acmv* cDNA sequence (Tab. 1). Freshly dissected brains were fixed, sectioned (12 μ m thick) and mounted on slides. Hybridization was performed at 60°C in a 50% formamide buffer with a digoxin-labeled anti-sense RNA probe (Boster Biotech, Wuhan, China). The sense probe was used as the control. This hybridization protocol was consistent with that of a previous study (Ma et al., 2018). The slides were imaged, and the images were processed using Image-Pro Plus 7.0 (Media Cybernetics, Inc., Rockville, Maryland, USA).

RESULTS

Acmv expression analysis

The dynamics of *Acmv* mRNA expression during life-stage transitions are summarized and depicted in Fig. 1. *Acmv* gene expression first declined from day 1 to 10 (0.317 fold) and then (2.830 fold) peaked on day 25. In older worker bees, expression declined again (0.214 fold) until reaching a minimum on day 30. Thus, *Acmv* expression decreased until day 10, increased from day 15 to 25, and then subsequently dropped. The *Acmv* transcript in age-marked worker bees showed maximal expression (1.247) at 25 days of age, which was significantly different from the levels on days 1, 5, 10, 15 and 30 ($p < 0.05$), and was 12.21 times higher than the minimum value at 30 days of age.

Fig. 1. *Acmv* expression profile in age-marked worker bees. Bees were sampled at seven different time points separated by five-day intervals. Expression is relative to the value at day 30 (assigned the arbitrary value of one). Bars represent standard errors. Different letters indicate statistical differences.

Protein expression analysis

Acmv protein was expressed in the head, thorax, and legs of both the nurse bee and the foraging bee, but little or no *Acmv* expression was observed in the abdomen tissue samples (Fig. 2A). The Western blot results (relative gray value) revealed tissue-specific *Acmv* production in bee heads, thoraxes and legs across the three behavioral groups (Fig. 2B).

In nurse bees, the *Acmv* protein expression was highest in the thoraxes, significantly higher than in the head and legs ($p < 0.05$), but the levels in

Fig. 2. *Acmvl* protein production in the tissues of nurse, nectar forager and pollen forager *A. c. cerana* bees.

A. The strip of *Acmvl* protein in four tissues. 1: Head (antennas included); 2: Thorax (wings included); 3: Abdomen; 4: Leg. β -actin was used as the internal control. B. The bar of *Acmvl* protein expression in three tissues. Image analysis was conducted using Image J. Bars represent the means of three independent experiments. Uppercase letters indicate differences between bee behavioral groups in protein production within the same tissue. Lowercase letters indicate differences between tissues within a behavioral group. Bars represent standard errors. Different letters represent statistical differences.

the head and legs did not differ significantly from each other ($p > 0.05$). In nectar foragers, *Acmvl* protein expression was highest in the head and significantly higher than in the thorax and legs ($p < 0.05$). In pollen foragers, *Acmvl* protein expression was significantly higher in the head and thorax than the foot ($p < 0.05$), and the difference between the head and thorax was not significant.

Acmvl protein expression in the different tissues of the three kinds of bees is shown in Fig. 2B. In the head, the expression was highest in the nectar foragers, significantly higher than in the nurses and pollen foragers ($p < 0.05$), but the nurses and pollen foragers did not differ significantly in their head expression ($p > 0.05$). In the thorax, expression was highest in the nurses

and significantly higher than in the nectar and pollen foragers ($p < 0.05$). In the leg, expression was significantly higher in the nectar foragers and the nurses than the pollen foragers ($p < 0.05$). Overall, *Acmvl* levels were very high in thorax samples from nurse bees and in head samples from forager bees. The greatest *Acmvl* level occurred in head samples from nectar foragers; this was 2.018 and 1.696 times higher than that measured in thorax and leg samples.

Location of *Acmvl*-expressing regions in forager honeybee brains

The western-blot results suggest that the *Acmvl* gene is predominantly expressed in the heads of *A. c. cerana* foragers. To more precisely identify *Acmvl*-expressing regions in forager brains, in-situ hybridization was performed on fresh brain tissue from pollen foragers marked with digoxin (Fig. 3). Hybridization signals were strongest in the Kenyon cells (KC) of the mushroom bodies, optic lobes (OL) and antennal lobes (AL) in the brain. Very weak signals were detected in all experiments when a sense probe was used as the control.

DISCUSSION

Malvolio, a key Nramp (natural resistance-associated macrophage protein) family member in insects, can encode manganese, iron, and copper transporters, and also regulates the sensory perception of sweet tastes (Folwell et al., 2006; Rodrigues et al., 1995; Southon et al., 2008; Ben-Shahar et al., 2004; Orgad et al., 1998). Behavioral studies have revealed an association between *mv1* and food choice in *D. melanogaster* (Orgad et al., 1998; Søvik et al., 2017), and also between *mv1* and task transitions in worker bees (Ben-Shahar et al., 2004; Søvik et al., 2015). Thus, *mv1* is considered a candidate gene for influencing the age at which honeybees (*Apis mellifera*) transition from working in the hive to outdoor foraging (Toth & Robinson, 2009).

This study described the temporal and spatial expression of *Acmvl* in honeybees using qPCR, western blotting and immunohistochemistry techniques. *Acmvl* mRNA expression was

Fig. 3. *AcmvI* expression in pollen forager brains. Antennal lobes (AL); Kenyon cells (KC); Optic Lobes (OL). (A) Treatment group hybridized with antisense probe; dark yellow particles are positive signals. (B) Control group hybridized with the sense probe. B, C, and D depict positive signals in Kenyon cells of the mushroom body, optic lobes, and antennal lobes, respectively, while b, c, and d depict negative controls for the corresponding regions.

observed during bee development here. Before day 20, mRNA levels were low, peaked on day 25, and then decreased to the minimum on day 30. Thus, expression was low when bees were focused on in-hive tasks during the two to three weeks post-emergence, but expression peaked when these bees performed outdoor activities and then decreased again (Seeley, 1982; Page Jr et al., 2006). The occurrence of a peak in *AcmvI* expression during the time series suggests that *AcmvI* influences the transition from nurse to forager in *A. c. cerana*. Previous research on *Apis mellifera* had also reported higher levels of *mvI* mRNA in the brains of pollen foragers than those of nurse bees (Ben-Shahar et al., 2004). Tissue-specific expression of *mvI* protein was also compared among the three behavioral or task groups in *A. c. cerana*. Four tissues were tested, but *AcmvI* was only expressed in three: head, thorax and legs. *AcmvI* expression was highest in the thoraxes of nurse bees and the heads of forager bees. Meanwhile, *AcmvI* expression was not detected in bee abdomens, perhaps due to the relatively low proportion of midgut and malpighian tubules in the abdomen, although Folwell et al. (2006) and Rodrigues et al. (1995) have previously reported high expression in

these two tissues. Relatively higher transcript levels were found in the thoraxes of nurse than forager bees, which suggests that high *AcmvI* expression in the thorax may play an important role in bee breeding. The highest *AcmvI* content occurred in nectar forager heads, which may be a response to the sucrose reward. Higher expression of *AcmvI* was also observed in the heads of *A. c. cerana* foragers than nurses, which is consistent with observations of western honeybee workers (Ben-Shahar et al., 2004). The bee head, including the brain and antennae, is used to critically evaluate food resources and to regulate outdoor feeding and foraging activities. Foraging is associated with high levels of transcription in the brain, as seen in bee (*A. mellifera*) and bumblebee (*Bombus terrestris*) foragers (Ben-Shahar et al., 2002; Tobback et al., 2011).

In-situ hybridization was performed to identify *AcmvI*-expressing regions in forager bee brains. *AcmvI* was widely expressed in the honeybee brain, which is consistent with patterns seen for *AmmvI* in *Apis mellifera* (Ben-Shahar et al., 2004). A strong *AcmvI* signal was detected in the Kenyon cells of the mushroom bodies (MB), optic lobes (OL) and lateral region of the

antennal lobes (AL). The OL receive and process visual information from the compound eyes, allowing for such higher order visual functions as motion detection, distance perception and color vision (Ehmer & Gronenberg, 2002; Gronenberg, 2001; Nrie & Desplan, 2016). AL comprises the primary olfactory processing center (Mller, 2002). Adult worker honey bees may alter their behaviour with age but still rely strongly on sensory information from the antennae throughout their lives (Winnington et al., 1996). The mushroom bodies (MB) are involved in the integration of higher-order processing, including sensory information, vision, olfaction, tactile, learning and memory, which are then transferred to the central nervous system where they cause neural physiological reactions (Scheiner et al., 2001; Takeuchi et al., 2001; Mller, 2002; Paul et al., 2005; Strausfeld, 2002). As a part of MB, Kenyon cells receive input from visual and olfactory areas (Gronenberg, 1986, 1999, 2001) and play an important role in learning and memory (Menzel 1999). *Mvl* influences behavioral maturation in part via effects on response to the sucrose reward (Ben-Shahar et al., 2004), which is correlated with sensitivities to pollen, odours and light (Scheiner et al., 2004), as well as to antennal scanning activity (Scheiner et al., 2005).

In this study, a major peak of *Acmvl* gene and protein expression occurred in foraging bee heads, especially in nectar foragers, and was located in the somata of the Kenyon cells of the MB, optic lobes and antennal lobe in the brains. Taken together, these findings suggest that *Acmvl* are highly correlated with nurse-forage activity in *A. c. cerana* via responsiveness to the sucrose reward. However, further research is required to investigate the potential mechanisms involved and to describe how *Acmvl* regulates the division of labor.

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