Sociobiology
An international journal on social insects

RESEARCH ARTICLE - BEES

Quantitative analysis of the genes affecting development of the hypopharyngeal gland in honey bees (*Apis mellifera* L.)

H Liu, ZL Wang, LB Zhou, ZJ Zeng

Jiangxi Agricultural University, Nanchang, Jiangxi, China

Article History

Edited by
Evandro doNascimento Silva, UEFS, Brazil
Received 01 February 2015
Initial acceptance 27 April 2015
Final acceptance 04 June 2015

Keywords
Honeybee, hypopharyngeal gland (HG), gene expression, quantitative real-time PCR (q-PCR).

Corresponding author
Zhi Jiang Zeng
Honeybee Research Institute
Jiangxi Agricultural University
Nanchang, Jiangxi, 330045, China
E-Mail: bees1965@sina.com

Abstract

Royal jelly has many important biological functions, however the potential molecular mechanisms of royal jelly secretion in hypopharyngeal gland (HG) are still not well understood. In our previous study, six genes (SV2C, eIF-4E, PDK1, IMP, CGNP and TGF-βR1) have been shown to be associated with royal jelly secretion. In this study, the relative expression levels of these genes were further determined in the hypopharyngeal gland of worker bees (*Apis mellifera* L.) at different developmental stages (nurse, forager and reversed nurse stages) with quantitative real-time PCR (q-PCR) assay. Our results indicated that the relative expression levels of SV2C, eIF-4E, IMP, CGNP and TGF-βR1 were decreased at forager stage relative to nurse stage, while were reversed at reversed nurse stage compared to forager stage. For the PDK1, it reduced continuously with the developmental stages. We concluded that SV2C, eIF-4E, IMP, CGNP and TGF-βR1, but not PDK1, were possibly potential candidate genes related to HG development and/or royal jelly secretion.

Introduction

Age-dependent division of labor is one of the most typical features of the social insect honeybees (Robinson et al., 2005). Some tissues such as the hypopharyngeal gland (HG), which is located in the head of bees and proven to produce and secrete the protein components of royal jelly, will change in parallel with the role change of the bees. Young bees, before 6 days after eclosion, perform tasks inside the hive, including brooding, cleaning cells and feeding larvae within 3 days after eclosion in hives, and their HGs are small and not fully developed during this period. Nurse bees (6-12 days) are mainly responsible for feeding queen and larvae within 3 days, in the meanwhile, the secretory activity of HGs reach a peak, facilitating the synthesis and secretion of royal jelly. After that, most of the worker bees generally mature into foragers to collect nectar outside the hive (Michener, 1974; Pankiw & Page Jr ,2000; Brillet et al., 2002; Seeley, 2009). The HGs gradually degrades during this stage, and the secretory protein components changed into digestive enzymes such as alpha-glucosidase, amylase and glucose oxidase for brewing honey (Kubo et al., 1996; Ohashi et al., 1997, 1999).

In addition, a large number of researches has demonstrated that the degeneration and secretory activity of HGs are quite flexible. A prior study has found that the HGs are well-developed but no royal jelly secretion occurs in most of the workers when a few brood need to be nursed in the winter (Fluri et al., 1982). Moreover, its growth degree depends on the needs of feeding larvae (Free, 1961) and consumption rate of protein diets (Al-Ghamdi et al., 2011). Besides that, Huang and Robinson (1996) proved that foragers would reverse to nurses (reversed nurses) and their HGs could also redevelop if there are no young bees or queen in the colony.

Previously, we performed a digital gene expression (DGE) analysis of the HGs at three developmental stages (newly emerged worker, nurse and forager) between *Apis mellifera* and *Apis cerana*, the two sibling honeybee species representing the most typical subspecies for western and eastern bee, respectively, with the aim of searching for the DEGs affecting the development of HG and/or the

secretion of royal jelly (Liu et al., 2014). As a result, synaptic vesicle glycoprotein 2C (SV2C) was found to be expressed significantly high amounts in nurses among three developmental stages in both species and it was significantly higher in HGs of *Apis mellifera* than that of *Apis cerana* at the nurse stage. Furthermore, there was no significant difference at newly emerged worker or forager stage between two species. Additionally, there were another 5 genes displaying significantly abundant expressions at the newly emerged worker and nurse stages while reduced rapidly at forager stage, including eukaryotic translation initiation factor 4E (eIF-4E), 3-phosphoinositide-dependent protein kinase 1 (PDK1), IGF-II mRNA-binding protein (IMP), cell growth-regulating nucleolar protein-like (CGNP) and TGF-β receptor 1 gene (TGF-βR1). Furthermore, the five genes exhibited higher expressions in the HGs of *Apis mellifera* than that of *Apis cerana* at both the newly emerged worker and nurse stages.

Combined with the observations of expression patterns of these genes and the corresponding development stages in worker bees, we hypothesized that these genes might be associated with royal jelly secretion and/or hypopharyngeal gland development. In this study, quantitative real-time PCR (q-PCR) was used to further analyze the differential expression levels of these genes among the HGs of nurses, foragers and reversed nurses.

Materials and Methods

Sampling collection

The honeybees (*Apis mellifera ligustica*) were bred in the Honeybee Research Institute, Jiangxi Agricultural University, China (28.46 °N, 115.49 °E) using standard beekeeping techniques. Three categories of workers (nurses, foragers and reversed nurses) were gathered alive from two colonies as independent biological replicates, immediately frozen in liquid nitrogen and then stored at -80 ºC until further processing. The nurses were caught at the time when they entered the cells and were nursing the larvae; the foragers could be easily identified by the pollen loads on their hind legs; and the reversed nurses were collected according to the method of Huang and Robinson (1996). A total of 360 workers were sampled randomly. The HGs were dissected in normal saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) under a binocular stereo microscope. After rinsed with DEPC-treated water, the HGs from 20 individuals per colony were pooled together in a tube for subsequent processes.

Total RNA extraction and cDNA synthesis

Total RNA was extracted using 1 mL of Trizol Reagent (Invitrogen, USA) according to the manufacturer's instructions. After subjected to quality inspection (absorption ratio 260 nm/280 nm) and concentration quantification by NanoPhotometer™ P 300 (IMPLEN, Germany), cDNA was synthesized with 1 μg RNA with 3 μl (50 μM) oligo-dT 18 primer (Invitrogen, USA) in 23 μL DEPC-treated water and incubated at 70 ºC for 5 min and then immediately placed on ice for 5 min. Afterwards it was added 10 μL RT M-MLV buffer (5×, Takara), 10μL dNTP mixture (2.5 mM each, Takara), 2 μL M-MLV Revertase (50 U/μL, Takara), 2 μL RNase inhibitor (50 U/μL, TransGen) into the RNA mixture and incubated at 42 ºC for 1 h, and then at 75 ºC for 5 min. Finally, the synthesized cDNA was diluted with 50 μL DEPC-treated water. The products were kept at -80 ºC for subsequent q-PCR reactions.

Primer design and quantitative real-time PCR assays

The primers used for amplifying the 6 specific genes and two house keeping genes Gpdh (Huang et al., 2012) and Arp1 (Lourenço et al., 2008) were designed based on mRNA using Primer 5.0 software, the primers were listed in Table 1.

The q-PCR reactions were cycled in a Thermal Cycler iQ5 (Bio-Rad, USA) using the following conditions: 5 μL SYBR® *Premix Ex Taq*™ (Takara), 3.2 μL sterile water, 0.4 μL (10 μM /L, invitrogen) forward primer, 0.4 μL (10 μM /L, invitrogen) reverse primer and 1 μL cDNA template. The q-PCR assay was performed using the following program: initial denaturation 95 ºC for 30 s, 40 cycles including 95 ºC for 10 s and 60 ºC for 1 min. Finally, melting curves were recorded by increasing the temperature from 55 ºC to 95 ºC (increased 0.5 ºC per 10 s) subsequently to 95 ºC for 1 min and 55 ºC for 1 min. Each sample had three technical replicates and biological replicates, respectively. The specificity of the PCR reaction was verified by melting curve analysis.

Table 1. The q-PCR primer sequences used in this study.

Gene name	Accession Number	Sense and antisense sequences (5′–3′)
SV2C	XM_394660.4	ACTCGGCTTGTGCTCGGTATT / CTGCTTTGATTTATTCTTTTGCG
eIF-4E	XM_624287.3	ATCAAACATCCACTTCAACATACA / TACATTTACAACAGCCCCACAA
PDK1	XM_394208.4	TCACCACCATCAACCCG / GTAGGACAGGACGAAATAGAGT
IMP	XM_393878.4	AGACGCAGGAACAAGCACAAC / CGAGAATACGAAGCGGGAAGT
CGNP	XM_623800.3	GTGAAAGAAAGCAACAAGAATG/ GA ACTGCTGATGGTATTTTGTGAC
TGF-βR1	XM_003251608.1	GAGGTGTTACGATAAGGTGCG / CGTGAGGAGGAATAAAGGGC
Gpdh	NM_001014994.1	GCTGGTTTCATCGATGGTTT / ACGATTTCGACCACCGTAAC
Arp1	NM_001185146.1	TCCTGGAATCGCAGATAGAATG / GGAAGGTGGACAAAGAAGCAAG

Statistical analysis

The threshold values (Ct) were analyzed by using Bio-Rad iQ5 2.1 Standard Edition Optical System Software. And the amplification efficiency (E) of each gene was obtained using the q-PCR package (Spiess & Ritz, 2010; Hornik, 2011).

Relative quantification analysis with two internal control genes was performed using the formula according to Huang (2012), the computational formula as follows:

$$r = \frac{\sqrt[n]{\prod_{i=1}^{n} E_i^{C_t(i)}}}{E^{C_t(\mathrm{target})}}$$

where E is the PCR amplification efficiency; C_t is cycle threshold; i is i-th reference gene; n is number of reference gene; r is the relative gene expression. The significance of the differential expressions was analyzed by variance analysis (ANOVA) using StatView (v 5.01, USA). P-value less than 0.05 was regarded as statistically significant.

Results

Our experiments were carried out to examine whether the 6 genes were associated with the secretory activity of HGs, and the results are showed in Figure 1. The relative expression level of SV2C significantly reduced at forager stage compared to nurse stage ($P< 0.05$), and then significantly recovered at reversed nurse stage ($P< 0.05$). Contrary to our expectation, the expression of PDK1 decreased at forager stage and then further decreased at reversed nurse stage ($P> 0.05$). Furthermore, eIF-4E and CGNP showed much reduced at forager stage ($P< 0.05$) and then slightly recovered at reversed nurse stage ($P> 0.05$). In addition, the expression patterns of TGF-βR1 and IMP indicated they reduced at forager stage ($P> 0.05$) and then recovered at reversed nurse stage ($P> 0.05$).

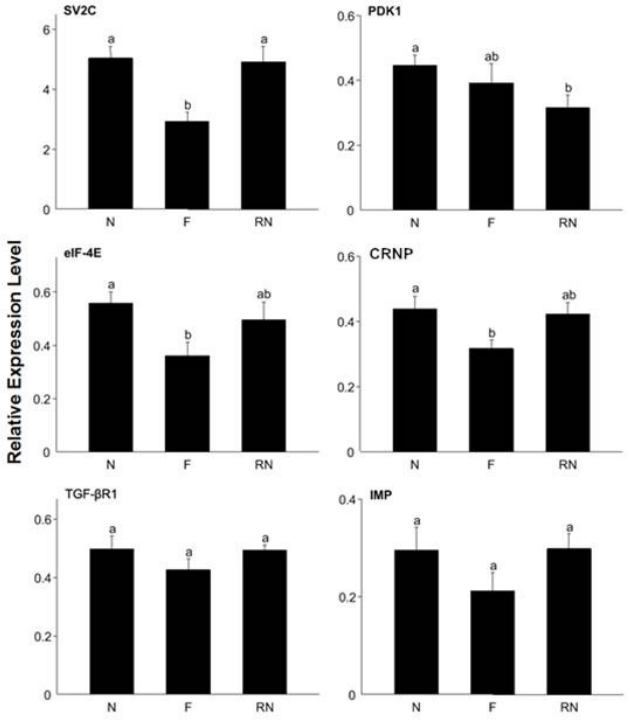

Fig 1. Relative expression levels of the *SV2C*, *PDK1*, *eIF-4E*, *CGNP*, *TGF-βR1* and *IMP* genes in HGs of honey. N: nurse stage; F: forager stage; RN: reversed nurse stage; SV2C: synaptic vesicle glycoprotein 2C; PDK1: 3-phosphoinositide-dependent protein kinase 1; eIF-4E: eukaryotic translation initiation factor 4E; CRNP: cell growth-regulating nucleolar protein-like; TGF-βR1: TGF-β receptor 1; IMP: IGF-II mRNA-binding protein. Different letters on top of bars indicate significant difference ($P< 0.05$) between the groups. Values are expressed as mean ± SEM.

Discussion

Our results revealed that the relative expression levels of the SV2C, eIF-4E, PDK1, IMP, CGNP and TGF-βR1 genes were reduced from nurse to forager, which is consistent with our previous DGE analysis (Liu et al., 2014), suggesting that our previous DGE results are reliable.

SV2C is a member of transmembrane glycoproteins almost existing in all of the neuronal and neuroendocrine cells, which are closely related to the normal secretion of neurotransmitters. Besides that, SV2 could be also found to be expressed in the neuroendocrine cells (Feany et al., 1993). Considering the expression pattern of SV2C in HGs of the three development stages, we speculated that SV2C might be associated with the secretion activity of royal jelly, and it could be considered as a marker for genetic improvement programs. However, this speculation should be treated cautiously as no supportive evidence has been reported in prior studies. It needs to be verified through subsequent.

The IMP, TGF, PDK1 and eIF-4E belong to IGF, TGF, PKB/Akt and mTOR signaling pathway respectively, which has the main role involved in the regulation of cell growth, proliferation, differentiation, cell death and motility (Hafen 2004; Ikushima & Miyazono, 2011). The IMP and TGF-βR1 could activate IGF-II and TGF-β respectively, the latters would activate phosphatidyl inositol 3-kinase (PI3K) signaling and finally lead to PKB/Akt signaling, via PDK1-dependent or -independent manner. Upon the activation of PKB/Akt regulates, a large number of genes with multifarious physiological functions would be expressed and exert their corresponding functions. In addition, the nucleolar proteins and CGNP are respectively involved in the biosynthesis of ribosomes and cell proliferation and death. In brief, these genes co-regulate multiple cellular and physiological processes including the metabolisms of carbohydrate, lipid and protein, cell differentiation, proliferation, apoptosis, lifespan, and so on (Sarbassov et al., 2005; Fanayan et al., 2000).

Both the present study and our prior observation (Liu et al., 2014) suggested that the expression levels of eIF-4E, CGNP, IMP and TGF-βR1 expressing higher at newly emerged worker, nurse and reversed nurse stages compared to forager stage which is in parallel with the physiological conditions of HGs: the HGs grew rapidly at newly emerged worker stage and synthesized a large amount of royal jelly

at the nurse stage, so many genes related to the biological metabolism and cell growth over expressed at these two stages and then reduced their expression quickly at forager stage in view of the degeneration of HGs and the cell apoptosis. However, the HGs re-growed and speeded up biological metabolism at reversed nurse stage lead to increased expression of many relevant genes. Therefore, according to their biological functions in other organisms and expression patterns detected in this study, we speculated that these four genes might participate in the development of HGs.

Contrary to our expectations, PDK1 reduced continuously ($P > 0.05$) with the developmental stages. Actually in our previous study, PDK1 declined continuously all the time (Liu et al., 2014). Hence we speculated that PDK1 has the function of controlling the lifespan of HGs. In fact, Reddy et al. (2009) found PDK1 in mice could control oocytes lifespan by PDK1 - PKB/Akt - p70 S6 kinase 1 (S6K1) - ribosomal protein S6 (rpS6) signaling pathway.

In summary, we used q-PCR to investigate the differential expression levels of SV2C, eIF-4E, PDK1, IMP, CGNP and TGF-βR1 genes among the HGs of nurses, foragers and reversed nurses. Our results indicated that SV2C might be a potentially strong candidate gene associated with the secretory activity of royal jelly in HGs, in addition, eIF-4E, TGF-β, CGNP and IMP might be also associated with the development of HGs, while PDK1 might be not involved in this process. In addition, we generalized three signal pathways in Figure 2 for better understanding.

Acknowledgements

We thank Li-Zheng Zhang, An Yuan, and You Li for their help in conducting experiments. This work was supported by the earmarked fund for China agriculture research system (No.CARS-45-KXJ12), the 555 talents project of GanPo of JiangXi province and the Natural Science Foundation of Jiangxi Province (No. 20114BAB214001).

References

Al-Ghamdi, A. A., Al-Khaibari, A. M., & Omar, M. O. (2011). Consumption rate of some proteinic diets affecting hypopharyngeal glands development in honeybee workers. Saudi Journal of Biological Sciences, 18: 73-77.

Brillet, C., Robinson, G. E., Bues, R., & Le Conte, Y. (2002). Racial differences in division of labor in colonies of the honey bee (*Apis mellifera*). Ethology, 108: 115-126.

Fanayan, S., Firth, S. M., Butt, A. J., & Baxter, R. C. (2000). Growth inhibition by insulin-like growth factor-binding protein-3 in T47D breast cancer cells requires transforming growth factor-β (TGF-β) and the type II TGF-β receptor. Journal of Biological Chemistry, 275(50): 39146-39151.

Feany, M. B., Yee, A. G., Delvy, M. L., & Buckley, K. M. (1993). The synaptic vesicle proteins SV2, synaptotagmin and synaptophysin are sorted to separate cellular compartments in CHO fibroblasts. The Journal of Cell Biology, 123: 575-584.

Fluri, P., Lüscher, M., Wille, H., & Gerig, L. (1982). Changes in weight of the pharyngeal gland and haemolymph titres of juvenile hormone, protein and vitellogenin in worker honey bees. Journal of Insect Physiology, 28: 61-68.

Free, J. B. (1961). Hypopharyngeal gland development and division of labour in honey bee (*Apis mellifera L.*) colonies, Proceedings of the Entomological Society of Washington B., 36: 5-8.

Hafen, E. (2004). Interplay between growth factor and nutrient signaling: lessons from Drosophila TOR. TOR. Springer Berlin Heidelberg, 153-167.

Hornik, K. (2014). The R FAQ. ISBN: 3-900051-08-9. <http://ringo.ams.sunysb.edu/images/c/c2/R-FAQ.pdf>

Huang, Q., Kryger, P., Le Conte, Y., & Moritz, R. F. (2012). Survival and immune response of drones of a *Nosemosis*

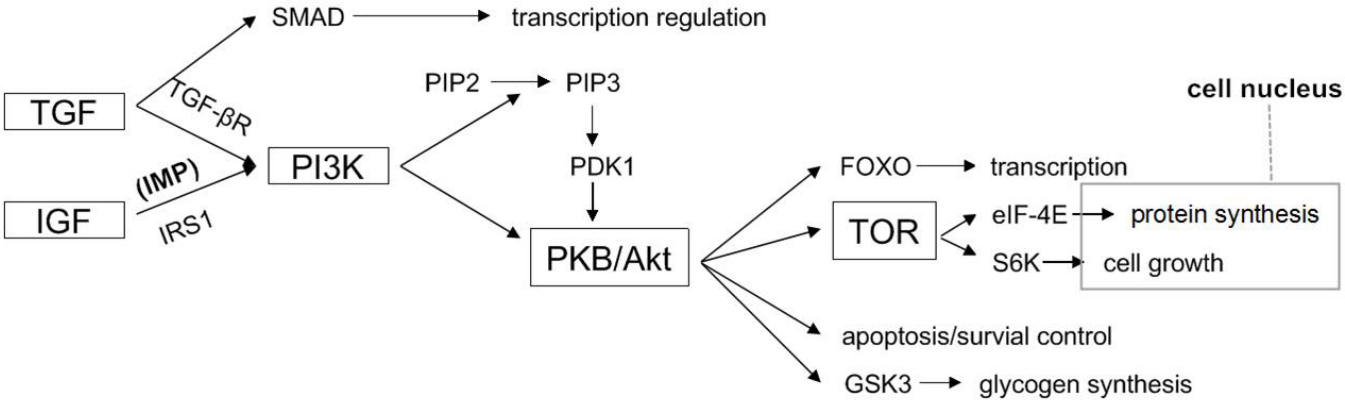

Fig 2. The sketch of signaling pathway network. TGF: transforming growth factor; TGF-βR: TGF-β receptor gene; SMAD: Mothers against decapentaplegic homolog; IGF: insulin-like growth factor; IMP: IGF-II mRNA-binding protein; IRS1: insulin receptor substrate 1; PI3K: phosphoinositide 3-kinase; PIP2: phosphatidylinosital biphosphate; PIP3: phosphatidylinositol trisphosphate; PDK1: 3-phosphoinositide-dependent protein kinase 1; PKB/Akt: protein kinase B; FOXO: forkhead box; TOR: mammalian target of rapamycin; eIF-4E: eukaryotic translation initiation factor 4E; S6K: ribosomal protein S6 kinase; GSK3: glycogen synthase kinase 3.

tolerant honey bee strain towards *N. ceranae* infections. Journal of Invertebrate Pathology, 109: 297-302.

Huang, Z. Y., & Robinson, G. E. (1996). Regulation of honey bee division of labor by colony age demography. Behavioral Ecology and Sociobiology, 39: 147-158.

Ikushima, H., & Miyazono, K. (2011). Biology of transforming growth factor-β signaling. Current Pharmaceutical Biotechnology, 12: 2099-2107.

Kubo, T., Sasaki, M., Nakamura, J., Sasagawa, H., Ohashi, K., Takeuchi, H., & Natori, S. (1996). Change in the Expression of Hypopharyngeal-Gland Proteins of the Worker Honeybees (*Apis melliferaL.*) with Age and/or Role. Journal of Biochemistry, 119: 291-295.

Liu, H., Wang, Z. L., Tian, L. Q., Qin, Q. H., Wu, X. B., Yan, W. Y., & Zeng, Z. J. (2014). Transcriptome differences in the hypopharyngeal gland between Western Honeybees (*Apis mellifera*) and Eastern Honeybees (*Apis cerana*). BMC Genomics, 15: 744.

Lourenço, A. P., Mackert, A., dos Santos Cristino, A., & Simões, Z. L. P. (2008). Validation of reference genes for gene expression studies in the honey bee, *Apis mellifera*, by quantitative real-time RT-PCR. Apidologie, 39: 372-385.

Michener, C. D. (1974). The social behavior of the bees: a comparative study. Harvard University Press, 73.

Ohashi, K., Natori, S., & Kubo, T. (1997). Change in the Mode of Gene Expression of the Hypopharyngeal Gland Cells with an Age-dependent Role Change of the Worker Honeybee *Apis mellifera L*. European Journal of Biochemistry, 249: 797-802.

Ohashi, K., Natori, S., & Kubo, T. (1999). Expression of amylase and glucose oxidase in the hypopharyngeal gland with an age-dependent role change of the worker honeybee (*Apis mellifera L.*). European Journal of Biochemistry, 265: 127-133.

Pankiw, T., & Page Jr, R. E. (2000). Response thresholds to sucrose predict foraging division of labor in honeybees. Behavioral Ecology and Sociobiology, 47: 265-267.

Reddy P, Adhikari D, Zheng W, et al. (2009). PDK1 signaling in oocytes controls reproductive aging and lifespan by manipulating the survival of primordial follicles. Molecular Genetics, 18: 2813-2824.

Robinson, G. E., Grozinger, C. M., & Whitfield, C. W. (2005). Sociogenomics: social life in molecular terms. Nature Reviews Genetics, 6: 257-270.

Sarbassov, D. D., Guertin, D. A., Ali, S. M., & Sabatini, D. M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science, 307(5712): 1098-1101.

Seeley, T. D. (2009). The wisdom of the hive: the social physiology of honey bee colonies. Harvard University Press.

Spiess, A. N., & Ritz, C. (2010). qpcR: Modelling and analysis of real-time PCR data. R package version, 1-3.