Journal of Asia-Pacific Entomology 20 (2017) 299–303

Contents lists available at ScienceDirect

Journal of Asia-Pacific Entomology

journal homepage: www.elsevier.com/locate/jape

DNA methylation comparison between 4-day-old queen and worker larvae of honey bee

Yuan-Yuan Shi [a,b], Hao Liu [a,b], Ya-Feng Qiu [a], Zhi-Yong Ma [a], Zhi-Jiang Zeng [b,*]

[a] Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai 200241, China
[b] Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, Jiangxi 330045, China

ARTICLE INFO

Article history:
Received 11 November 2016
Revised 13 January 2017
Accepted 27 January 2017
Available online 31 January 2017

Keywords:
Honey bee
Queen larvae
Worker larvae
Caste differentiation
Differentially methylated gene

ABSTRACT

The honey bee is a social insect that is famous for queen-worker differentiation. Numerous studies indicate that queen larvae (QL) and worker larvae (WL) have different expressed genes and proteins. DNA methylation has been found to play an important role in regulating gene expression. To further explore the roles of the methylated genes in queen-worker differentiation, we analyzed DNA methylome profiles of 4-day-old QL and WL (*Apis mellifera*). The results demonstrated that there were 7.2 gigabases of sequence data from six methylated DNA immunoprecipitation libraries, and provided a genome-wide DNA methylation map as well as a gene expression map for 4-day-old QL and WL. The genome coverage of every sample was 4.79. According to CpG representation, all promoters in the *A. mellifera* genome were classified into high CpG promoters, intermediate CpG promoters and low CpG promoters. The methylated cytosines of larvae were enriched in introns, followed by coding sequence regions, 2 K downstream of genes, 5′ untranslated regions (UTRs), 2 K upstream of genes, and 3′ UTRs. Compared with 4-day-old WL, a number of genes in QL were down-methylated that were involved in biological regulation, immune system and metabolic regulation. In addition, these DMGs were involved in many signal pathways of caste differentiation such as Mitogen-activated protein kinase (MAPK), Notch, Insulin and Wnt signaling pathways.

Introduction

Because of its precise labor division and high levels of social cohesion, the honey bee (*Apis mellifera*) is the most important model organism to study the animal behaviour (Seeley, 1989; Zeng, 2009). An integrated honey bee colony is made up of three castes: one single reproductive queen, hundreds of haploid drones, and thousands of nearly sterile workers (Winston, 1987; Seeley, 1989). Despite having an identical genome, queen and workers exhibit striking differences in behaviour, morphology, longevity and reproduction (Weaver, 1957; Zeng, 2009).

The mechanism for queen-worker differentiation has been relatively well-studied (Weaver, 1966; Hartfelder et al., 1993; Kucharski et al., 2008; Kamakura, 2011; Leimar et al., 2012; Buttstedt et al., 2016) and the main factor regulating this phenomenon is known to the quality of the food fed to queen larvae (Winston, 1987; Buttstedt et al., 2016). Caste differentiation is more affected by sugars and the actual amount of food available to the developing larvae (Buttstedt et al., 2016). The major royal jelly proteins also play a role in reproductive maturation and brain development in queen larvae (Drapeau et al., 2006). Royalactin activates the ribosomal protein S6 K kinase by activation of epidermal growth factor receptors in the fat body, resulting queen characteristics in the larvae (Kamakura, 2011). Queen larvae (QL) and worker larvae (WL) exhibit differential genes (Evans and Wheeler, 2000; Hepperle and Hartfelder, 2001; Barchuk et al., 2007; Chen et al., 2012; Cameron et al., 2013), proteins (Wu and Li, 2010), and microRNAs (Chen et al., 2010; Shi et al., 2015).

DNA methylation has been shown to be closely associated with queen-worker determination (Wang et al., 2006; Schaefer and Lyko, 2007; Kucharski et al., 2008; Elango et al., 2009; Foret et al., 2009; Lyko et al., 2010; Foret et al., 2012; Kucharski et al., 2016). *A. mellifera* become the first insect known to have three varieties of DNA methyltransferase (Wang et al., 2006). The reduction of DNA methyltransferase 3 expression may enhance queen production (Kucharski et al., 2008). Both diet and cell size can affect queen-worker differentiation through DNA methylation (Shi et al., 2011). Methylated genes in *A. mellifera* are divided into CpG low-content and CpG high-content genes, with caste specific genes tending to have high CpG content (Elango et al., 2009). The majority of methylated cytosines are located in the exon regions of the genes in brains of queens and workers (Lyko et al., 2010). There are 2399 differentially methylated genes (DMGs) in brains of 4-day-old QL and WL, with 82% of DMGs up-methylated in WL (Foret et

* Corresponding author at: Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, Jiangxi 330045, China.
E-mail address: bees1965@sina.com (Z.-J. Zeng).

http://dx.doi.org/10.1016/j.aspen.2017.01.014
1226-8615/

al., 2012). In adult queens and workers, 561 DMGs are found, with 56% of DMGs up-methylated in workers (Lyko et al., 2010).

In our previous study, we demonstrated that DNA methylation of QL and WL were <20%, and that differences were most apparent in 4-day-old larvae. In order to confirm our findings and to provide clearer information about the epigenetic factors affecting caste differentiation, we used three biological replicates to compare the DNA methylation of 4-day-old QL and WL by methylated DNA immunoprecipitation-sequencing analysis (MeDIP-seq). MeDIP-seq recently became an efficient method of analysis for large-scale DNA methylation, and has been used successfully in studies of plant genomics (Zhang et al., 2006) and pig muscle tissues (Li et al., 2012). 4-day-old QL and WL have differential expressed genes, proteins and microRNAs, however, there is little study on the differences in methylated genes between these two castes. In the present study, we sought to describe the distribution of methylation modifications in the *A. mellifera* genome, and to identify the DMGs that are involved in caste differentiation between 4-day-old QL and WL.

Materials and methods

Experimental honey bee colonies

According to standard beekeeping techniques, the *A. mellifera* colonies were kept at honey bee research institute, Jiangxi agricultural university, Nanchang, China.

Honey bee queen and worker larvae

The two castes were sampled from three *A. mellifera* colonies by having three biological replicates (10 larvae for each replicate) for 4-day-old QL and WL. All samples (QL1, QL2, QL3, WL1, WL2, and WL3) were flash frozen in liquid nitrogen until use.

Methylated DNA immunoprecipitation-sequencing analysis

The whole larval DNA was isolated and purified by Universal Genomic DNA Extraction Kit (TaKaRa, DV811A), and then was sent to BGI (Beijing Genomics Institute at Shenzhen, China) for meDIP-seq analysis by using Illumina HiSeq™ 2000 (Illumina Inc., CA, USA). The detailed protocol was published in Li and Shi (Li et al., 2012), these clean reads were deposited in the National Center for Biotechnology Information sequence read archive (SRR4897308 for QL1, QL2, and QL3, SRR4897290 for WL1, WL2, and WL3).

To compare DNA methylation rates among samples of 4-day-old larvae, read depth was normalized by averaging the number of reads in every group. A 1 Mb sliding window was then used to smooth the distributions. The CpGo/e ratio, density of SNPs, genes, repeats and CpG islands were all calculated using a 1 Mb sliding window. Measurement of DNA methylation levels along chromosomes revealed that in every chromosome there was a different pattern of DNA methylation in 4-day-old QL compared with 4-day-old WL (Fig. S1). According to CpG representation (Weber et al., 2007; Li et al., 2012), the promoters in the *A. mellifera* genome were also classified into high CpG (HCPs), intermediate CpG (ICPs), and low CpG promoters (LCPs) (Fig. S2).

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes annotation

The reference genome of *A. mellifera*, together with gene information, was downloaded from the NCBI database (ftp://ftp.ncbi.nih.gov/genomes/*Apis_mellifera*/). The information about Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms was downloaded from the UniProtKB-GOA database. Both GO and KEGG with $p < 0.05$ were considered as significant enriched.

Real-time quantitative PCR

According to manufacturer's instructions, total RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). 200 ng of RNA was reverse-transcribed to cDNA with Prime Script™ RT Master Mix kit (TaKaRa). Real-time quantitative PCR was performed on Step One real-time PCR system using the Real Time SYBR master mix kit (TaKaRa). Gene-specific primers were listed in Table 1.

Data analyses

The nucleotides of every sample library were 1.2 Gb, and the genome size of *A. mellifera* was 250.287 Mb (ftp://ftp.ncbi.nih.gov/genomes/*Apis_mellifera*/). The following formula was used to calculate the genome coverage of every sample library:

$$\text{The genome coverage} = (\text{nucleotides per library})/(\text{genome size})$$

Model-based analysis of chip sequencing (MACS) was used to scan the methylated levels in *A. mellifera* genome. The genes with DNA methylation went through GO analysis. The number of up- and down-methylated genes was the result of comparisons between QL and WL. The following formulae were used to calculate fold-changes and p-values from the normalized gene methylation of QL and WL samples:

The normalized gene methylation of QL = clean reads of the methylated gene in QL / total clean reads in QL * 1000000,

The normalized gene methylation of WL = clean reads of the methylated gene in WL / total clean reads in WL * 1000000,

Fold-change = $\log_2$ (the normalized gene methylation of QL / the normalized gene methylation of WL) (Guan et al., 2013),

$$p(x|y) = \left(\frac{N_2}{N_1}\right)^y \frac{(x+y)!}{x!y!\left(1+\frac{N_2}{N_1}\right)^{(x+y+1)}} \quad C(y \le y_{\min}|x) = \sum_{y=0}^{y \le y\ \min} p(y|x) \quad D(y \ge y_{\max}|x) = \sum_{y \ge y_{\max}}^{\infty} p(y|x)$$

where x and y indicate the mapped clean reads number for the same methylated gene in QL and WL library, N_1 and N_2 represent the total reads number for the two libraries respectively. In this study, absolute $\log_2$ (fold change) > 1 and $p < 0.05$ were considered to represent significant differences in methylated gene (Guan et al., 2013).

The real-time PCR results were examined by analysis of variance (ANOVA) using StatView (v 5.01, SAS Institute, Gary, NC, USA). Multiple comparisons of the mean values were performed using Fisher's Protected Least Significant Difference only after ANOVA showed a significant effect ($p < 0.05$).

Results

Global mapping of DNA methylation in 4-day-old QL and WL

We generated 7.2 gigabases (Gb) methylated DNA MeDIP-seq data from six samples, of which 5.8 Gb (80.5%) clean reads were aligned on the *A. mellifera* genome. The genome coverage of every sample was 4.79. After removing the ambiguously mapped reads and reads which may have come from duplicate clones, we used 5.1 Gb (70.8%) uniquely aligned non-duplicate reads in the following analysis (Table S1). To avoid false positives in enrichment, we required >10 reads to determine a methylated CpG in a sample.

Differentially methylated genes in 4-day-old QL and WL

Distributions of up- or down-methylated genes among gene functional elements in 4-day-old QL are presented in Fig. S3. The methylated cytosines of larvae were enriched in introns, followed by coding sequence (CDS) regions, 2 K downstream of genes, 5′ untranslated regions

Table 1
Gene-specific primers used in real-time quantitative PCR.

Gene name	Gene description	Gene ID	Sense and antisense sequences (5′–3′)
LOC410122	GAPDH	GB50902	CTCAGGTTGTTGCCATTA/TTGCCTCTCGTTCACTAA
LOC411086	Protein jagged-1	GB48639	GTGAATGAGTGCGTGTAA/ATTATGTCGTTGTCGTTGT
LOC413289	Notch protein	GB49149	TTCCAACTCCAATATCCAA/GTCGCAATGATGATTACC
LOC100578232	Notch 2 like protein	GB44370	AGAGGTAAGCGTAGCGATA/GTTCCTTCACAGCGACAT
LOC410351	Neurogenic locus protein delta	GB45005	CGCTAGTGTTGCCTATTAG/CTATCCTTCTCCGTTCTCT
LOC100578826	Protein notch-like	GB41174	CCTTGACTCTTCTGTAATCG/TCATCACTGCTGCTATCT
LOC552725	*N*-acetylglucosamine-1-phosphotransferase	GB49324	ACTTAGAACGAGACAACTT/GCATATCAGCAACAATCC
LOC412638	Kinesin-3C	GB48375	GTTATTGTTACTTGTGAATCT/ACCAGTTTGTCCATAAG
LOC411915	p38b	GB43914	TGACTGGTTATGTTGCTA/TAAGACTCGTGTTAGATGAT
LOC413502	Wnt1	GB45510	AGAGTACGCTTTGCTAAA/GGATTACGAAGAACATCAC
LOC411919	Wnt7	GB44402	CCTTCTTCTTCTCCTTCT/GTTCATTCGTTTCGTTTC
LOC725827	InR-2	GB55245	GATAGATACGAGCAAGATT/GTTCATTCCTTCCTTCTC
LOC409523	RL	GB51503	GTATTACATAGAGACTTG/CCTTGGAATTAAGCATTA

(UTRs), 2 K upstream of genes, and 3′ UTRs (Fig. S3). We found that >7355 methylated genes were in the larval body (Table S2). Down-methylated genes outnumbered up-methylated genes in QL (Table S2). The index number of the linear relationships about DNA methylation of the genes in QL and WL replicates were $0.88 < R^2 < 0.94$ and $0.84 < R^2 < 0.94$, respectively.

By use of GO analysis, we found that a large number of genes in QL were down-methylated involved in many processes including biological regulation, growth, immune system process, and metabolic regulation (Fig. 1 and Table S3). After mapping all of the DMGs to terms in the KEGG database (Ogata et al., 1999), we found that the DMGs participated in "Calcium signaling pathway", "Phosphatidylinositol signaling system", "Purine metabolism", "Long-term potentiation", "Regulation of actin cytoskeleton", "Notch signaling pathway", "Wnt signaling pathway", "Tight junction", "Taste transduction" and "Pyrimidine metabolism" (Table 2).

By use of the NCBI annotations, we found 39 DMGs of QL and WL that had been established in adult honey bees (Wheeler et al., 2006; Barchuk et al., 2007; Ikeda et al., 2011; Wolschin et al., 2011) were related to caste differentiation pathways, namely, the insulin (InR-2, Kinesin-3C, Sirt6-PA), mitogen-activated protein kinase (MAPK) (p38b, RL), Wnt (Hex110, Wnt1, Wnt4, Wnt6, Wnt7), and notch (LOC411086, LOC413289, LOC100578232, LOC410351, LOC100578826, LOC552725) signal pathway (Table 3). In addition, we also found many differentially methylated miRNAs in QL and WL (Table 3). Compared with WL, Dnmt1, Dnmt3, Wnt4, and Wnt6 were up-methylated, while Dynactin, Hex110, His3.3A-PA, mRpL45, Sir2, Sirt6-PA, Trap1-PA, Wnt1, Wnt7 were down-methylated in QL (Table 3).

DNA methylation has negative correlations with gene expression (Li et al., 2012). In order to validate the sequencing results, LOC725827 and LOC412638 in the insulin signaling pathway, LOC411915 and LOC409523 in MAPK signaling pathway, LOC413502, LOC411919 in Wnt signaling, LOC411086, LOC413289, LOC100578232, LOC410351, LOC100578826, LOC552725 in Notch signaling pathway were selected for real-time quantitative PCR analysis (Fig. 2). These results showed that the real-time PCR results of all these genes were consistent with the meDIP-seq data.

Discussion

It is widely believed that only *A. mellifera* WL that are <3 days old are able to become queens (Weaver, 1966), and our previous results supported to the notion that 3.5 day old larvae were at a critical juncture in caste development. By using digital gene expression (DGE) tag profile, many DEGs between 4-day-old QL and WL were identified (Barchuk et al., 2007; Chen et al., 2012). In this study, we used three biological replicates to compare the DNA methylation of 4-day-old QL and WL by MeDIP-seq in order to confirm our previous findings that those results were based on a sample size of one, and to provide clearer information about the epigenetic factors affecting the developing larvae. We found that every chromosome had different DNA methylation in QL

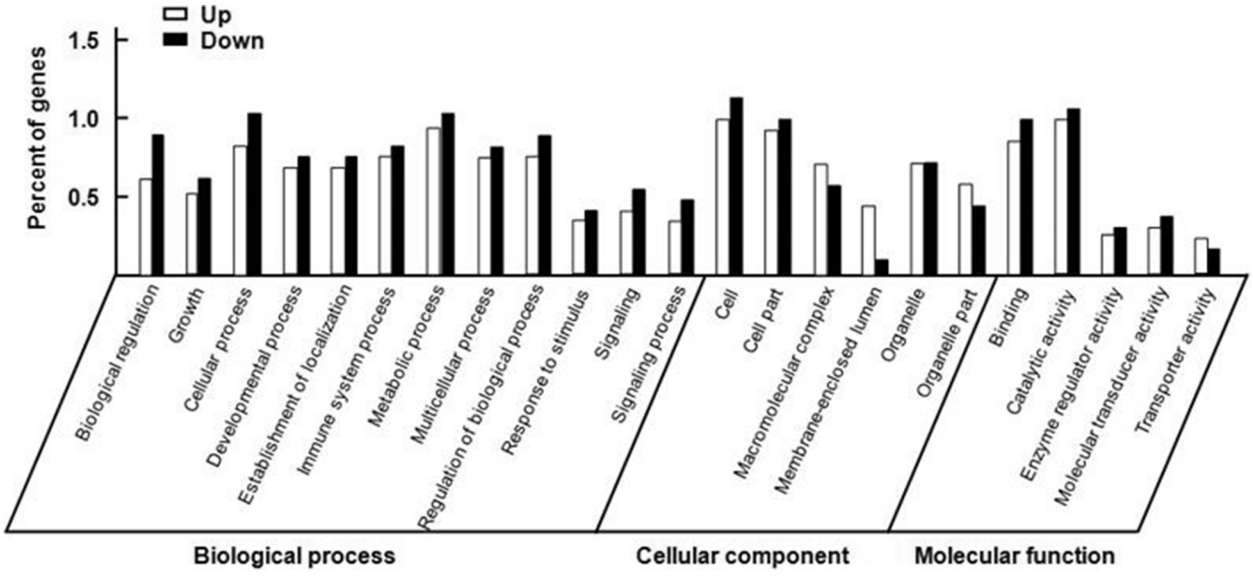

Fig. 1. Gene Ontology classification of down- and up-methylated genes in 4-day-old queen larvae, relative to worker larvae. The results are summarized in three main categories: biological process, cellular component and molecular function. The Y-axis indicates the percentage of genes in a category. The X-axis indicates category.

Table 2
KEGG analysis of the differentially methylated genes in 4-day-old queen larvae, relative to worker larvae.

Pathway	DMGs in pathway	All genes in pathway	p-value	Q-value	Pathway ID
Calcium signaling pathway	21 (6.18%)	193 (2.04%)	5.74E-06	0.000816	ko04020
Phosphatidylinositol signaling system	11 (3.24%)	76 (0.8%)	7.88E-05	0.005595	ko04070
Long-term potentiation	10 (2.94%)	70 (0.74%)	0.000186	0.00879	ko04720
Purine metabolism	16 (4.71%)	171 (1.81%)	0.000435	0.015046	ko00230
Regulation of actin cytoskeleton	19 (5.59%)	230 (2.44%)	0.000631	0.015046	ko04810
Notch signaling pathway	8 (2.35%)	54 (0.57%)	0.000636	0.015046	ko04330
Wnt signaling pathway	9 (2.65%)	70 (0.74%)	0.000856	0.017362	ko04310
Tight junction	13 (3.82%)	135 (1.43%)	0.001131	0.020069	ko04530
Taste transduction	9 (2.65%)	74 (0.78%)	0.001282	0.020233	ko04742
Pyrimidine metabolism	10 (2.94%)	98 (1.04%)	0.002695	0.038273	ko00240

compared to WL (Fig. S1). All promoters in the *A. mellifera* genome were classified into HCPs, ICPs and LCPs (Fig. S2).

Several studies have proved that in *A. mellifera* methylated genes are predominantly clustered in CDS regions (Wang et al., 2006; Kucharski et al., 2008; Lyko et al., 2010; Foret et al., 2012) and exons (Lyko et al., 2010; Cingolani et al., 2013). In this study, DNA methylation occurred preferentially in introns, CDS regions and promoter regions, consistent with our previous research (Fig. S3). Comparing with previously published larval head and adult brain methylation patterns, we found that the number of methylated genes was highest in larval body: >7355 methylated genes (Table S2) were in the larval body, 6086 methylated genes in the larval head, and 5854 in an adult brain (Lyko et al., 2010; Foret et al., 2012). These marked differences might be due to the different levels of growth and functioning between larval tissues and the adult brain. For the whole larval body, higher numbers of tissues were involved in the methylation analysis compared to the adult brain. In addition, larval tissues are closely related to protein turnover and high growth, whereas brain cells are postmitotic.

Contrary to our findings in 4-day-old WL, our results indicated that some genes in QL showed a decrease in DNA methylation. The genes Hex110, His3.3A-PA, mRpL45, Sirt6-PA, Trap1-PA (Table 3) have been shown previously to be methylated in adult honey bees (Wheeler et al., 2006; Barchuk et al., 2007; Wolschin et al., 2011), while our study indicated that these genes were also methylated at the larval stage. It is well known that individual development involves many signaling pathways, such as insulin (Wolschin et al., 2011), MAPK (Kamakura, 2011), Target of Rapamycin (TOR) (Patel et al., 2007), Notch and Wnt signaling pathways (Li et al., 2011). In the present study, we found that InR-2 and Kinesin-3C in insulin signaling pathway, p38b and RL in MAPK signaling pathway, Wnt1, Wnt7 in Wnt signaling, LOC411086, LOC413289, LOC100578232, LOC410351, LOC100578826, LOC552725 in Notch signaling pathway (Table 3) were down-methylated in QL compared to WL.

Caste differentiation is a significant and unique natural phenomenon. DNA methylation provides new ideas for the explanation of honey bee larval development (Kucharski et al., 2008; Lyko et al., 2010; Shi et al., 2011; Foret et al., 2012; Weiner and Toth, 2012) after its initial discovery in the system (Wang et al., 2006). Gene expression regulation due to the nutrition intake might be the major contribution (Shi et al., 2011; Leimar et al., 2012). We measured, for the first time, the DNA methylation profiles of QL and WL in every chromosome. Further studies are needed to understand how DMGs are translated into physiological changes in the developing queens and workers of the honey bee.

Conclusions

In a word, our results provide clearer information about the epigenetic factors affecting caste differentiation, and used three biological replicates to compare the DNA methylation profiles of 4-day-old QL and WL by MeDIP-seq. There is a different pattern of DNA methylation in every chromosome between 4-day-old QL and WL. This study suggests that differentially methylated genes in the larvae might also function in caste differentiation.

Table 3
Differentially methylated genes in 4-day-old queen larvae, relative to worker larvae.

Gene	Gene annotation/pathway	DNA methylation (up/down)			Gene	Gene annotation/pathway	DNA methylation (up/down)		
		4 d QL1	4 d QL2	4 d QL3			4 d QL1	4 d QL2	4 d QL3
5-HT1	G protein coupled receptor	Down	Down	Down	Let-7	mircoRNA	Down	Down	Down
5-HT2beta		Down	Down	Down	Mir184		Down	Down	Down
AChE-1		Down	Down	Down	mir71		Up	Down	Down
DopR2		Down	Down	Down	mir927		Down	Down	Down
nAChRa1		Down	Down	Down	LOC411086	Notch signaling pathway	Down	Down	Down
nAChRa4		Down	Down	Down	LOC413289		Down	Down	Down
nAChRa6		Down	Down	Down	LOC100578232		Down	Down	Down
Atg13	Adenosine triphosphate (ATP)	Down	Down	Up	LOC410351		Down	Down	Down
Atg2		Down	Up	Down	LOC100578826		Down	Down	Down
ATP7		Down	Down	Down	LOC552725		Down	Down	Down
Dnmt1	DNA methyltransferases	Up	Up	Up	p38b	MAPK signaling pathway	Down	Down	Down
Dnmt3		Up	Up	Up	RL		Down	Down	Down
Dynactin	Calmodulin-binding protein	Down	Down	Down	RpL4	Tim 44-like domain	Down	Down	Down
HDAC1	Histone deacetylation	Down	Down	Down	Rpn5	Code ribosomal protein	Down	Down	Down
HDAC6		Down	Down	Down	Rpn6		Down	Down	Down
Hex110	Wnt signaling pathway	Down	Down	Down	Sir2	Sir2 family insulin signaling pathway	Down	Down	Down
His3.3A–PA	Histone deacetylation	Down	Down	Down	Sirt6-PA		Down	Down	Down
InR-2	Insulin signaling pathway	Down	Down	Down	Trip1-PA	TRAF zinc finger	Down	Down	Down
Kinesin-3A	Calmodulin-binding protein	Down	Down	Down	Wnt1	Wnt signaling pathway	Down	Down	Down
Kinesin-3C	Insulin signaling pathway	Down	Down	Down	Wnt4		Up	Up	Up
mRpL45	Tim 44-like domain	Down	Down	Down	Wnt6		Up	Up	Up
mRpS22		Down	Down	Down	Wnt7		Down	Down	Down

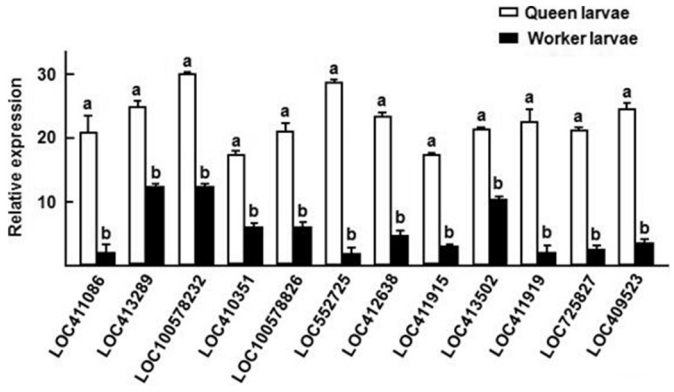

Fig. 2. Verification of ten differentially methylated genes between 4-day-old queen larvae and worker larvae by RT-PCR. Different letters on top of bars indicate significant difference ($p < 0.05$) with Fisher's Protected Least Significant Difference. Each bar corresponds to a single group represented as the mean ± S.E. of its biological replicates.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.aspen.2017.01.014.

Acknowledgements

We thank Doctor Xu-Jiang He for reviewing this manuscript. This work was supported by the National Natural Science Foundation of China (No. 31502046) and the Earmarked Fund for the China Agriculture Research System (No. CARS-45-KXJ12).

References

Barchuk, A.R., Cristino, A.S., Kucharski, R., Costa, L.F., Simões, Z.L., Maleszka, R., 2007. Molecular determinants of caste differentiation in the highly eusocial honey bee *Apis mellifera*. BMC Dev. Biol. 7, 70.

Buttstedt, A., Ihling, C.H., Pietzsch, M., Moritz, R.F., 2016. Royalactin is not a royal making of a queen. Nature 537 (7621), E10–E12.

Cameron, R.C., Duncan, E.J., Dearden, P.K., 2013. Biased gene expression in early honeybee larval development. BMC Genomics 14, 903.

Chen, X., Yu, X., Cai, Y., Zheng, H., Yu, D., Liu, G., Zhou, Q., Hu, S., Hu, F., 2010. Next-generation small RNA sequencing for microRNAs profiling in the honey bee *Apis mellifera*. Insect Mol. Biol. 19 (6), 799–805.

Chen, X., Hu, Y., Zheng, H., Cao, L., Niu, D., Yu, D., Sun, Y., Hu, S., Hu, F., 2012. Transcriptome comparison between honey bee queen- and worker-destined larvae. Insect Biochem. Mol. Biol. 42 (9), 665–673.

Cingolani, P., Cao, X., Khetani, R.S., Chen, C.C., Coon, M., Sammak, A., Bollig-Fischer, A., Land, S., Huang, Y., Hudson, M.E., Garfinkel, M.D., Zhong, S., Robinson, G.E., Ruden, D.M., 2013. Intronic non-CG DNA hydroxymethylation and alternative mRNA splicing in honey bees. BMC Genomics 14, 666.

Drapeau, M.D., Albert, S., Kucharski, R., Prusko, C., Maleszka, R., 2006. Evolution of the yellow/major royal jelly protein family and the emergence of social behavior in honeybees. Genome Res. 16 (11), 1385–1394.

Elango, N., Hunt, B.G., Goodisman, M.A., Yi, S.V., 2009. DNA methylation is widespread and associated with differential gene expression in castes of the honeybee, *Apis mellifera*. Proc. Natl. Acad. Sci. U. S. A. 106 (27), 11206–11211.

Evans, J.D., Wheeler, D.E., 2000. Expression profiles during honeybee caste determination. Genome Biol. 2 (1), 1–0006 (research0001).

Foret, S., Kucharski, R., Pittelkow, Y., Lockett, G.A., Maleszk, R., 2009. Epigenetic regulation of the honey bee transcriptome: unravelling the nature of methylated genes. BMC Genomics 10, 472.

Foret, S., Kucharski, R., Pellegrini, M., Feng, S., Jacobsen, S.E., Robinson, G.E., Maleszka, R., 2012. DNA methylation dynamics, metabolic fluxes, gene splicing, and alternative phenotypes in honeybees. Proc. Natl. Acad. Sci. U. S. A. 109 (13), 4968–4973.

Guan, C., Barron, A.B., He, X.J., Wang, Z.L., Yan, W.Y., Zeng, Z.J., 2013. A comparison of digital gene expression profiling and methyl DNA immunoprecipitation as methods for gene discovery in honeybee (*Apis mellifera*) behavioural genomic analyses. PLoS One 8 (9), e73628.

Hartfelder, K., Tozetto, S.O., Rachinsky, A., 1993. Sex-specific developmental profiles of juvenile hormone synthesis in honeybee larvae. Rouxs Arch. Dev. Biol. 202 (3), 176–180.

Hepperle, C., Hartfelder, K., 2001. Differentially expressed regulatory genes in honeybee caste development. Naturwissenschaften 88 (3), 113–116.

Ikeda, T., Furukawa, S., Nakamura, J., Sasaki, M., Sasaki, T., 2011. CpG methylation in the hexamerin 110 gene in the European honeybee, *Apis mellifera*. J. Insect Sci. 11.

Kamakura, M., 2011. Royalactin induces queen differentiation in honeybees. Nature 473 (7348), 478–483.

Kucharski, J.R., Maleszka, J., Fore, S., Maleszka, R., 2008. Nutritional control of reproductive status in honeybees via DNA methylation. Science 319 (5871), 1827–1829.

Kucharski, R., Maleszka, J., Maleszka, R., 2016. A possible role of DNA methylation in functional divergence of a fast evolving duplicate gene encoding odorant binding protein 11 in the honeybee. Proc. Biol. Sci. 283 (1833) (pii:20160558).

Leimar, O., Hartfelder, K., Laubichler, M.D., Page Jr., R.E., 2012. Development and evolution of caste dimorphism in honeybees-a modeling approach. Ecol. Evol. 2 (12), 3098–3109.

Li, Z., Nie, F., Wang, S., Li, L., 2011. Histone H4 Lys 20 monomethylation by histone methylase SET8 mediates Wnt target gene activation. Proc. Natl. Acad. Sci. U. S. A. 108 (8), 3116–3123.

Li, M., Wu, H., Luo, Z., Xia, Y., Guan, J., Wang, T., Li, R., 2012. An atlas of DNA methylomes in porcine adipose and muscle tissues. Nat. Commun. 3, 850.

Lyko, F., Foret, S., Kucharski, R., Wolf, S., Falckenhayn, C., Maleszka, R., 2010. The honeybee epigenomes: differential methylation of brain DNA in queens and workers. PLoS Biol. 9 (11), e1000506.

Ogata, H., Goto, S., Sato, K., Fujibuchi, W., Bono, H., Kanehisa, M., 1999. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 27 (1), 29–34.

Patel, A., Fondrk, M.K., Kaftanoglu, O., Emore, C., Hunt, G., Frederick, K., Amdam, G.V., 2007. The making of a queen: TOR pathway is a key player in diphenic caste development. PLoS One 2 (6), e509.

Schaefer, M., Lyko, F., 2007. DNA methylation with a sting: an active DNA methylation system in the honeybee. BioEssays 29 (3), 208–221.

Seeley, T.D., 1989. The honey bee colony as a superorganism. Am. Sci. 77, 546–553.

Shi, Y.Y., Huang, Z.Y., Zeng, Z.J., Wang, Z.L., Wu, X.B., Yan, W.Y., 2011. Diet and cell size both affect queen-worker differentiation through DNA methylation in honeybees (*Apis mellifera*, Apidae). PLoS One 6 (4), e18808.

Shi, Y.Y., Zheng, H.J., Pan, Q.Z., Wang, Z.L., Zeng, Z.J., 2015. Differentially expressed microRNAs between queen and worker larvae of honey bee (*Apis mellifera*). Apidologie 46 (1), 35–45.

Wang, Y., Jorda, M., Jones, P.L., Maleszk, R., Ling, X., Robertson, H.M., Mizzen, C.A., Peinado, M.A., Robinson, G.E., 2006. Functional CpG methylation system in a social insect. Science 314 (5799), 645–647.

Weaver, N., 1957. Effects of larval honey bee age on dimorphic differentiation of the female honeybee. Ann. Entomol. Soc. Am. 50, 283–294.

Weaver, N., 1966. Physiology of caste determination. Annu. Rev. Entomol. 11, 79–102.

Weber, M., Hellmann, I., Stadler, M.B., Ramos, L., Pääbo, S., Rebhan, M., Schübeler, D., 2007. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat. Genet. 39 (4), 457–466.

Weiner, S.A., Toth, A.L., 2012. Epigenetics in social insects: a new direction for understanding the evolution of castes. Genet. Res. Int. 2012, 609810.

Wheeler, D.E., Buck, N., Evans, J.D., 2006. Expression of insulin pathway genes during the period of caste determination in the honeybee, *Apis mellifera*. Insect Mol. Biol. 15, 597–602.

Winston, M.L., 1987. Biology of the Honey Bee. Harvard University Press, Cambridge.

Wolschin, F., Mutti, N.S., Amdam, G.V., 2011. Insulin receptor substrate influences female caste development in honeybees. Biol. Lett. 7 (1), 112–115.

Wu, J., Li, J.K., 2010. Proteomic analysis of the honeybee (*Apis mellifera* L.) caste differentiation between worker and queen larvae. Sci. Agric. Sin. 43 (5), 176–181.

Zeng, Z.J., 2009. Apiculture. China Agriculture Press, Beijing.

Zhang, X., Yazaki, J., Sundaresan, A., 2006. Genome-wide high-resolution rapping and functional analysis of DNA methylation in *Arabidopsis*. Cell 126 (6), 1189–1201.