SHORT COMMUNICATION

Genomewide analysis indicates that queen larvae have lower methylation levels in the honey bee (*Apis mellifera*)

Yuan Yuan Shi · Wei Yu Yan · Zachary Y. Huang · Zi Long Wang · Xiao Bo Wu · Zhi Jiang Zeng

Abstract The honey bee is a social insect characterized by caste differentiation, by which a young larva can develop into either a queen or a worker. Despite possessing the same genome, queen and workers display marked differences in reproductive capacity, physiology, and behavior. Recent studies have shown that DNA methylation plays important roles in caste differentiation. To further explore the roles of DNA methylation in this process, we analyzed DNA methylome profiles of both queen larvae (QL) and worker larvae (WL) of different ages (2, 4, and 6 day old), by using methylated DNA immunoprecipitation-sequencing (meDIP-seq) technique. The global DNA methylation levels varied between the larvae of two castes. DNA methylation increased from 2-day- to 4-day-old QL and then decreased in 6-day-old larvae. In WL, methylation levels increased with age. The methylcytosines in both larvae were enriched in introns, followed by coding sequence (CDS) regions, CpG islands, 2 kbp downstream and upstream of genes, and 5′ and 3′ untranslated regions (UTRs). The number of differentially methylated genes (DMGs) in 2-, 4-, and 6-day-old QL and WL was 725, 3,013, and 5,049, respectively. Compared to 4- and 6-day-old WL, a large number of genes in QL were downmethylated, which were involved in many processes including development, reproduction, and metabolic regulation. In addition, some DMGs were concerned with caste differentiation.

Keywords Honey bee · Queen larvae · Worker larvae · DNA methylation · Differentially methylated genes · Caste differentiation

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Introduction

A honey bee colony is composed of a single reproductive queen, thousands of nearly sterile workers, and hundreds of haploid drones (Flanders 1960; Seeley 1989; Smith et al. 2008). One fascinating phenomenon in the honey bee is caste differentiation, by which queen and workers exhibit striking differences in anatomy, physiology, and behavior, despite having an identical genome (Weaver 1957, 1966). The mechanism for this differentiation has been well studied. Higher juvenile hormone synthesis by the corpora allata results in reduced apoptosis and the expression of queen-specific genes, resulting in the queen phenotype (Hartfelder et al. 1993). Queen larvae (QL) and worker larvae (WL) have different activated genes (Evans and Wheeler 2000; Hepperle and Hartfelder 2001) as well as protein profiles (Wu and Li 2010). A recent study revealed that a single protein in royal jelly, royalactin, plays a critical role in caste determination (Kamakura 2011).

DNA methylation, one main way to induce epigenetic changes, has been shown to be closely associated with caste determination. The honey bee became the first insect known to have a fully functioning DNA methylation system (Wang et al. 2006). RNA interference of DNA methyltransferase 3, a key methylation gene, enhanced queen production (Kucharski et al. 2008). The longer duration of royal jelly and the larger queen cells decreased methyltransferase

enzyme activity, methyltransferase gene expression, and methylation in the gene *dynactin p62*, which, in turn, led to a higher percentage of queens or intercastes (Shi et al. 2011). Brains of 4-day-old larvae have 2,399 differentially methylated genes (DMGs) in the two castes, with 82 % of DMGs upmethylated in WL (Foret et al. 2012). In adult queens and workers, 561 DMGs were found, with 56 % of DMGs upmethylated in workers (Lyko et al. 2010). It is widely believed that only WL younger than 3 days are able to become queens (Weaver 1966), and whether DNA methylation contributes to this phenomenon is not clear. We are interested to determine the age-dependent changes in methylome in the whole larval body of the two castes, with the expectation that 4- and 6-day-old larvae show substantial differences between the two castes, while methylation levels should be more similar in 2-day-old larvae.

Methylated DNA immunoprecipitation-sequencing (meDIP-seq) recently became available for large-scale DNA methylation analysis. The most attractive advantage of this method is that it can enrich sequences containing methylated cytosine instead of detecting methylation at loci by oligonucleotides on a microarray (Li et al. 2010; Taiwo et al. 2012), and has been used in plant genomics (Zhang et al. 2006), chicken genomics (Li et al. 2011a), pig muscle tissues (Li et al. 2012), and human breast cancer cells (Ruike et al. 2010). To explore the dynamics of DNA methylation of different aged larvae, we determined the DNA methylomes of QL and WL with different ages (2, 4, and 6 days posthatching) by meDIP-seq.

Materials and methods

Honey bee larvae

Honey bee larvae were reared inside plastic queen cups, or regular wax worker cells, inside bee colonies. The queens from two single drone inseminated (SDI) colonies were each caged for 12 h to obtain eggs in queen cups and then eggs in worker cells. SDI could decrease the genetic variation and, therefore, reduce noise. The sampling time of the two castes were staggered to reflect this 12-h difference in ages. Queen cups were arranged in a special cage with attachments, which made the queen perceive the cells as worker cells (Zeng 2008). QL and WL ($N=10$ larvae per sample) were sampled at 2, 4, and 6 days posthatching. Each sample was flash frozen in liquid nitrogen and then stored at −80 °C. Larvae samples at the three different time points within each caste were pooled over two SDI colonies, creating six total samples for sequencing, with three for queen (2, 4, and 6 days old) and three for workers.

DNA preparation and meDIP-seq

DNA was isolated by Universal Genomic DNA Extraction Kit (TaKaRa, DV811A). Purified DNA (>50 ng per sample) was then sent to Beijing Genomics Institute (BGI at Shenzhen, China) for meDIP-seq analysis by a Illumina HiSeq™ 2000 (Illumina Inc., CA, USA), and their detailed protocol was published in Li et al. (2011a). Model-based analysis of chIP-seq (MACS) was used to scan the methylated levels in *Apis mellifera* genome. Subsequently, the genes with DNA methylation went through GO analysis. The number of up- and downmethylated genes was the result of comparisons between the two samples.

Public data used and gene ontology annotation

The *A. mellifera* reference genome, together with gene information, was downloaded from the National Center for Biotechnology Information (NCBI) database (ftp://ftp.ncbi.nih.gov/genomes/*Apis_mellifera*/). The information about GO terms was downloaded from the UniProtKB-GOA database. GO terms with $p<0.05$ were considered as significantly enriched (the enrichment in a category was considered significant when $p<0.05$).

Data analyses

Data were analyzed using analysis of variance (ANOVA) or Contingency Table Analysis by StatView (v 5.01, SAS Institute, Gary, NC, USA). Multiple comparisons of the means were carried out using Fisher's Protected Least Significant Difference only after ANOVA showed a significant effect ($P<0.01$). For each age/caste, the data analysis was based on a sample size of one.

Results

Global mapping of DNA methylation in queen and worker larvae

The number of raw reads in the six samples ranged from 19,894,058 to 20,792,961. After mapping to the honey bee genome (The Honey Bee Genome Sequencing Consortium 2006), percentage of total mapped reads and uniquely mapped reads are presented in Table S1. Rates of DNA methylation were the highest in introns (36.89±0.23), followed by coding sequence (CDS) regions (18.90±1.22) and CpG island (13.12±0.86) (Table 1).

DNA methylation levels in QL and WL are shown in Fig. 1. Rates of DNA methylation increased first from 2- to 4-day-old larvae and then decreased in 6-day-old larvae in QL. Rates of DNA methylation in WL increased with age at

Table 1 Frequency distributions of methylation sites among gene functional elements (%)

Sample	CpG island	Downstream 2 k	5′-UTR	CDS	Intron	3′-UTR	Upstream 2 k
2-Day-old larvae							
Queen	12.19	10.97	4.78	16.37	37.91	4.59	9.64
Worker	13.21	13.09	5.00	18.47	36.81	4.73	12.77
4-Day-old larvae							
Queen	13.80	13.07	4.92	19.38	37.11	4.68	10.96
Worker	15.80	13.23	5.02	21.35	36.33	4.73	12.13
6-Day-old larvae							
Queen	10.61	8.92	4.92	14.11	36.75	4.74	10.89
Worker	18.56	13.49	5.00	24.07	36.42	4.73	13.19

all three time points. Distributions of methylated genes in QL and WL changed significantly from 2 to 4 days, 4 to 6 days, and also, 2 to 6 days (Contingency Table Analysis, X^2=32.5, 68.9, and 199.0, respectively, for the three comparisons, $P<0.01$ for all the tests).

DNA-methylated genes in QL and WL are shown in Table 2. We found that the number of total genes was 11,736 in each sample. Over 7,245 genes showed DNA methylation in the six samples. The number of DMGs ranged from 725 to 5,049 in QL and WL of different ages.

Differentially methylated genes in queen and worker larvae

Distributions of up- or downmethylated genes among gene functional elements in 4- and 6-day-old QL are presented in Fig. 2a and b, respectively. In both ages, downmethylated genes outnumbered upmethylated genes (paired t tests, t=6.03, $P<0.01$, t=4.34, $P<0.01$, for 4- and 6–day-old larvae, respectively).

After gene ontology (GO) assignments, we found that a large number of larvae DMGs were related to development, growth, metabolic process, reproduction, signaling, and binding (Tables S2 and S3). Compared with WL, *Wnt7, Par-1, Sin3A, Kinesin 2A, Sir2, RhoGAPp190, p38b* were downmethylated, while *Myo7, Wnt4, Drl2, Myo6*, and *Lis-1*

were upmethylated in QL. By use of the NCBI annotations, we found that in QL and WL, 38 DMGs 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 (*IGFn, Kinesin-3C, Sirt6-PA*), mitogen-activated protein kinase (MAPK) (*p38b*), and Wnt (*Hex110, Wnt1, Wnt4, Wnt6, Wnt7*) signal pathway (Tables S4 and S5). In QL, *Dynactin, Hex110, His3.3A-PA, mRpL45, Sir2, Sirt6-PA*, and *Trap1-PA* were downmethylated and *Dnmt1, Dnmt3, p38, Wnt4*, and *Wnt6* were upmethylated (Table S4).

Discussion

We show that DNA methylome profiles are different between QL and WL in different ages. By using meDIP-seq, we found, for the first time, that DNA methylation levels in QL and WL were lower than 20 %. As shown in the Fig. 1, the differences in methylation level mostly occurred in 4-day-old larvae. After the fourth day, DNA methylation of QL was decreased, while that of WL continued to increase. This provides support to the notion that larvae at 3.5 days old were at a critical juncture
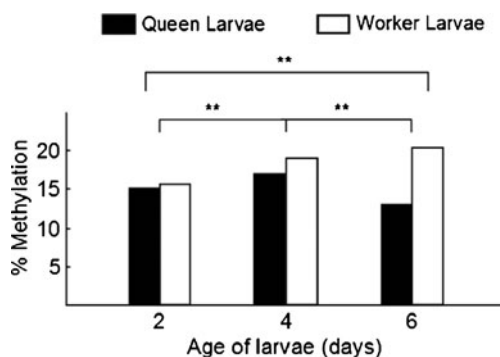

Fig. 1 Rates of DNA methylation of 2-, 4-, and 6-day-old queen and worker larvae. The 2-, 4-, and 6-day-old larval methylation were significantly different (*P*<0.01 for all the tests)

Table 2 Comparison of methylomes in queens and workers

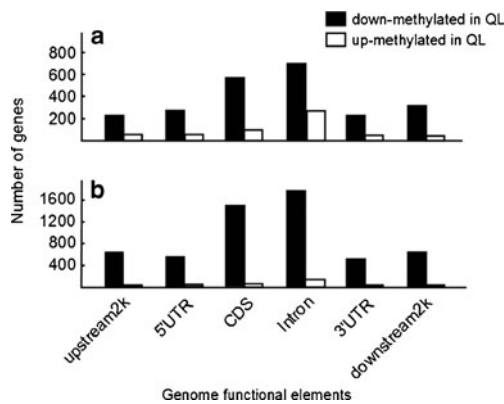| Sample	No. of total genes	No. of methylated genes		No. of DMGs	% upmethylated DMGs	
		Q	W		Q	W
Larvae body						
2 day old	11,736	7,335	7,381	725	50	50
4 day old	11,736	7,355	7,537	3,013	30	70
6 day old	11,736	7,245	7,731	5,049	10	90
Larvae head (Foret et al. 2012)						
4 day old	6,086	2,399			18	82
Adult brain (Lyko et al. 2010)						
2.5 week old	5,854	561			44	56

Fig. 2 Distributions of up- or downmethylated genes among gene functional elements in 4- (**a**) or 6-day-old (**b**) queen larvae. The WL acted as control group; if a gene is upmethylated in QL, then it is, by definition, downmethylated in WL

(Weaver 1966), and WL older than 4 days were not able to switch to becoming queens.

In a honey bee colony, QL are generally larger than WL at the same age. There are many reasons accounting for this phenomenon, a lot of experiments identified that DNA methylation plays an important role in development, reproduction, and metabolic regulation (Kucharski et al. 2008; Lyko et al. 2010; Shi et al. 2011; Foret et al. 2012; Weiner and Toth 2012). In our previous study, we found that both diet and cell size can affect queen–worker differentiation through DNA methylation (Shi et al. 2011). These two factors may be the main cause of the genomewide methylation difference detected in this study. Even though, we don't know how diet and cell size can result in lower rates of methylation in QL, respectively, since the samples used in this study are from the natural colonies. Further researches are needed to detect their respective effect on the genome-wide methylation changes in QL and WL.

Previous studies showed that in honey bee, methylated genes are predominantly clustered in CDS regions (Wang et al. 2006; Kucharski et al. 2008; Lyko et al. 2010, Foret et al. 2012). In our study, DNA methylation mainly occurred in CDS regions; however, introns and CpG islands also had DNA methylation. The primary difference between larval and adult methylomes was the number of DMGs. Comparing our results with previously published larval head (Foret et al. 2012) and adult brain (Lyko et al. 2010) methylation patterns, we found that the number of methylated genes was highest in larval body: more than 7,245 methylated genes were in the larval body, 6,086 methylated genes in the larval head, and 5,854 in an adult brain. We detected 3,013 and 5,049 in 4- and 6-day-old whole larval body (including head), compared with 2,399 and 561 in the 4-day-old larval head, and a 2.5-week-old adult brain (Table 2). This difference might be due to the difference between larval tissues and the adult brain. Larval tissues are closely related to high

growth and protein turnover, whereas brain cells are post-mitotic. In addition, for the whole larval body, higher numbers of tissues were involved in the methylation analysis compared to the adult brain.

Compared to 4- and 6-day-old WL, some genes in QL showed different DNA methylation (Table S4), which were concerned with caste differentiation. The genes *Hex110*, *His3.3A-PA*, *mRpL45*, *Sirt6-PA*, and *Trap1-PA* have been shown previously to be methylated in adult queens and workers (Wheeler et al. 2006; Barchuk et al. 2007; Ikeda et al. 2011; Wolschin et al. 2011), while our study indicated that these same genes were also methylated at the larval stage. Most of these genes that were related to caste differentiation showed no difference in DNA methylation of 2-day-old QL and WL. However, the methylation of the genes related to caste differentiation became different after the fourth day. It is well-known that individual development involves many signaling pathways, such as insulin (Wolschin et al. 2011), MAPK (Kamakura 2011), and Wnt signaling pathways (Li et al. 2011b). In our study, *IGFn* in the insulin signaling pathway, *p38b* in MAPK signaling pathway, and Wnt group (*Wnt1*, *Wnt7*) involved in Wnt signaling were downmethylated in QL. Our results suggested that methylation difference is an important pattern for the involvement of these signaling pathways in queen/worker caste differentiation. We found that some microRNAs (Chen et al. 2009) (*mir184*, *mir2–3*, *mir71*, and *mir927*) were also downmethylated in 6-day-old QL. A previous study reported an inverse correlation between DNA methylation and gene expression (Tate and Bird 1993); this regulation pattern might also apply to the miRNAs, that is to say, downmethylated miRNAs might express at high level in 6-day-old QL and activate or repress specific target genes. These results further enhance the notion that 6-day-old QL were already fixed on their trajectory to become queens.

In a word, our study measured, for the first time, the DNA methylome profiles between QL and WL in different ages using a meDIP-seq approach. Further studies are needed to understand how DNA methylation is transduced into physiological changes in the two castes.

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