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Mating flight causes genome-wide transcriptional changes in sexually mature honeybee queens

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ABSTRACT

In this study, we analyzed the gene and miRNA expression differences between the courted virgin queen (CVQ) and non-courted virgin queen (NCVQ) of *Apis mellifera* using a high-throughput sequencing method. Through Digital Gene Expression (DGE) sequencing, 452 genes were differentially expressed, out of which, 90 genes were up-regulated and 362 genes were down-regulated in CVQ compared with NCVQ. Through small RNA sequencing, 27 miRNAs showed significant expression difference between these two samples. Moreover, 9 of the differentially expressed genes are the targets of the 11 differentially expressed miRNAs. Besides, 47 novel miRNA candidates were predicted in these two samples. Our results provided valuable information for understanding the molecular mechanism of the transition to functional queens.

Introduction

Breeding offspring is an important instinct of all animals. In order to breed high-quality offsprings, animals have evolved different sexual selection and mating behaviors to find a high quality mate (Maan and Seehausen, 2011). Studies of transcription changes during the mating process in females have been conducted in *Drosophila melanogaster* (Lawniczak and Begun, 2004; Mack et al., 2006; Dalton et al., 2010), and several differentially expressed genes between unmated and mated females have been identified by microarray analysis.

The honeybee is an important economic insect. Usually, a honeybee colony comprises a queen, thousands of drones and hundreds of thousands of workers. The queen is mainly responsible for reproduction, and virgin queens reach sexual maturity at 5–10 days after emergence. But the sexual maturity virgin queens will not attract drones before they fly to a drone congregation area (DCA), where hundreds or thousands of unrelated drones gather for mating. Then, the drones pursue the queens and usually 7–17 drones could mate with a queen in the mating flight (Taber and Wendel, 1958; Adams et al., 1977). Obviously, the queens maybe released some kind of pheromone to attract the drones during this process. After mating, the ovaries of the mated queens become fully activated to lay eggs. From the virgin queen to functional egg laying queen, the physiological and behavioral changes are substantial and un-reversible (Winston, 1987). Previous research has shown that there are large-scale transcriptional changes in the brains and ovaries between virgin queens, mated queens and egg laying queens (Kocher et al., 2008). Moreover, transcriptional changes in the brains and the ovaries appear to be uncoupled, unlike the physiological and behavioral changes that are coupled during the mating process. Another study detected several differentially expressed genes between naturally mated queens and artificially inseminated queens, and between semen inseminated queens and those treated with a saline control (Kocher et al., 2010).

miRNAs are a special kind of molecules which induce gene silence in organisms. They are involved in the regulation of cell growth, development, gene transcription and translation, and many other life activities (Yang et al., 2005). miRNA has been reported to be present in the reproductive tract (Carletti and Christenson, 2009; Nothnick, 2012), but so far there are no reports of miRNAs being involved in mating behavior. Studies on miRNAs in the honeybee have been performed by several research groups (Weaver et al., 2007; Chen et al., 2010; Greenberg et al., 2012; Liu et al., 2012; Shi et al., 2012). Weaver et al. identified a total of 65 non-redundant candidate miRNAs from the honeybee genome and verified their expression in honeybee (Weaver et al., 2007). Chen et al. (2010) predicted 267 novel honeybee miRNAs by SOLiD sequencing. While Liu et al. (2012) discovered nine significantly differentially expressed known miRNAs between nurses and foragers and 67 novel miRNAs. Another study indicated that miRNAs are important regulators of social behavior (Greenberg et al., 2012). Such results strongly suggest that miRNA may be widely involved in the regulation of various behaviors in the honeybee. Recently, Shi et al. (2012) for the first time reported that miRNA from heterospecific royal jelly can modify gene expression in honey bees.

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Since mating flight is crucial for queens to attract drones and causes physiological changes in queens, this process may be accompanied by great changes in gene and miRNA expression. Therefore, in this study, we adopted the DGE and small RNA sequencing approaches to identify differentially expressed genes and miRNAs between CVQ and NCVQ, and survey the relationship between the differentially expressed genes and miRNAs. This work will help us to understand the molecular mechanism of the queen mating flight.

Materials and methods

Insect

Apis mellifera were sampled from the Honey bee Research Institute, Jiangxi Agricultural University, China. In order to ensure a higher genetics similarity among the experimental queens, which should increase the signal to noise ratio when searching for gene expression differences, all the queens used in this experiment were from a single drone inseminated queen and artificially bred at normal conditions. After emergence, these young queens were caged and placed in a normal sized colony without other queens. 12 days after emergence, the mating flight experiment was performed. In order to easily catch the queens after mating flight, a net (5 m × 3 m × 4 m) was installed, then, 6 sexually mature virgin queens and hundreds of sexually mature drones were placed in this net. The drones courted the queens, but in this condition, the queens didn't mate with the drone, because their flying height didn't achieve the height of the mating flight in natural condition. After 20 min of mating flight, each queen was carefully checked to make sure she hasn't mated, then, unmated queens were sampled. The whole bodies of the queens were sampled except the intestines, which were removed to prevent contamination. Finally, four courted virgin queens were sampled. At the same time, four non-courted virgin queens were directly sampled as control from the colonies. All these samples were stored at −80 °C until use.

Digital gene expression library preparation and sequencing

Total RNAs were isolated from each sampled queens, respectively. Then, total RNAs from four courted virgin queens and four non-courted virgin queens were respectively pooled, and each pooled RNA sample was divided into two portions. One portion was used to construct the DGE libraries, and another for small RNA sequencing. The protocols for DGE library construction and sequencing, and the steps and parameters for raw sequence filtration were the same as in our previous study (Wang et al., 2012). All the remained clean tags were deposited in the NCBI sequence read archive (SRR868713 for courted virgin queens and SRR868714 for non-courted virgin queens).

Mapping of DGE tags to genes and genome

Before mapping, two tag libraries containing all the possible CATG + 17 nt tag sequences were created using all the available mRNA sequences and genome sequences of *A. mellifera* downloaded from Genbank database (ftp://ftp.ncbi.nih.gov/genomes/Apis_mellifera). Then all the clean tags were first mapped to the tag database of reference mRNA sequences allowing only one nucleotide mismatch. Clean tags that mapped to multiple genes were filtered. The remained clean tags were designed as unambiguous clean tags. For gene expression analysis, the number of unambiguous clean tags for each gene was calculated and normalized to TPM (number of transcripts per million clean tags). Those tags that cannot be mapped to any gene in the tag database of mRNA sequences were then mapped to the tag database of the reference genome sequence.

Evaluation of DGE libraries

To identify the differentially expressed genes between the CVQ and NCVQ libraries, a rigorous statistical algorithm was developed, using the method described by Audic and Claverie (1997), to statistically analyze the tag frequency in each DGE library. The false discovery rate (FDR) was used to determine the threshold p-value in multiple tests. A FDR ≤ 0.001 and an absolute E-value of the $\log_2$ (CVQ/NCVQ) ≥ 1 were used as the threshold to determine significant differences in gene expression. The identified differentially expressed genes were used for GO (Gene Ontology) and KO (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis.

GO enrichment analysis firstly maps all differentially expressed genes to GO terms in the database (http://www.geneontology.org/) by BLASTX alignments, calculating gene numbers for each term, then using hypergeometric test to find significantly enriched GO terms in differentially expressed genes comparing to the reference gene background. The following formula was used to calculate the p-value of significantly enriched GO terms:

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

where N is the number of all genes with GO annotation; n is the number of differentially expressed genes in N; M is the number of all genes that are annotated to the certain GO terms; and m is the number of differentially expressed genes in M. The Bonferroni Correction was used to correct the p-value. GO terms with corrected p-value ≤ 0.05 are defined as significantly enriched in differentially expressed genes.

KEGG pathway enrichment analysis is similar to GO analysis, it firstly maps all differentially expressed genes to terms in the KEGG database (http://www.genome.jp/kegg/) by BLASTX alignments. Then use hypergeometric test to find significantly enriched terms in differentially expressed genes comparing to the whole reference gene background. The formula for calculating p-value of significantly enriched KEGG terms is the same as that of GO analysis. The Q-value (false discovery rate) was used to determine the threshold p-value in multiple tests. Q-value ≤ 0.05 is defined as significantly enriched terms.

Construction of small RNA libraries and high-throughput sequencing

To construct small RNA libraries, total RNAs were from the same queen samples as in the DGE experiment. Then, the total RNAs were subjected to 15% (w/v) denaturing by 15% TBE-Urea gel and the small RNA fragments of 10–36 nt were isolated from the gel and purified. Then, a 3′ adaptor and a 5′ adaptor were sequentially ligated to the purified small RNA molecules. Subsequently, the ligation products were reverse transcribed into cDNA and subjected to PCR amplification. The amplification products were purified using 6% TBE-Urea gel. The purified DNA fragments were used for sequencing by Hiseq™ 2000.

Data cleaning and length distribution

The sequencing-received raw image data was converted into sequence data by the base calling step. Such sequence data called raw data or raw reads and stored them in a ".fq" file. The raw data is processed by getting rid of low quality reads, reads with 5′ primer contaminants or with poly A, reads without 3′ primer or insert tag, and reads shorter than 10 nt. The remained clean tags were deposited in the NCBI sequence read archive (SRR869567 for courted virgin queens and SRR869568 for non-courted virgin queens).

Then, all the small RNA tags were mapped to the *A. mellifera* genome by SOAP software to analyze their expression and distribution on the genome.

Table 1
Statistics of DGE sequencing.

Summary		CVQ	NCVQ
Raw data	Total	5,999,932	6,090,470
Raw data	Distinct tag	215,165	220,599
Clean tag	Total number	5,887,683	5,978,453
Clean tag	Distinct tag number	104,701	110,201
All tag mapping to gene	Total number	2,464,774	2,504,345
All tag mapping to gene	Total % of clean tag	41.86%	41.89%
All tag mapping to gene	Distinct tag number	51,272	54,407
All tag mapping to gene	Distinct tag % of clean tag	48.97%	49.37%
Unambiguous tag mapping to gene	Total number	2,384,161	2,438,509
Unambiguous tag mapping to gene	Total % of clean tag	40.49%	40.79%
Unambiguous tag mapping to gene	Distinct tag number	50,400	53,475
Unambiguous tag mapping to gene	Distinct tag % of clean tag	48.14%	48.52%
All tag-mapped genes	Number	8914	9043
All tag-mapped genes	% of ref genes	80.55%	81.71%
Unambiguous tag-mapped genes	Number	8664	8785
Unambiguous tag-mapped genes	% of ref genes	78.29%	79.38%
Mapping to genome	Total number	1,026,278	1,108,304
Mapping to genome	Total % of clean tag	17.43%	18.54%
Mapping to genome	Distinct tag number	42,001	44,211
Mapping to genome	Distinct tag % of clean tag	40.12%	40.12%
Unknown tag	Total number	2,396,631	2,365,804
Unknown tag	Total % of clean tag	40.71%	39.57%
Unknown tag	Distinct tag number	11,428	11,583
Unknown tag	Distinct tag % of clean tag	10.91%	10.51%

Small RNA annotation

All the small RNA tags were annotated by aligning them against the rRNA, scRNA, snoRNA, snRNA and tRNA sequences from Genbank and Rfam and the miRNA precursor/mature miRNA of *A. mellifera* in miRBase18. In the alignment, some small RNA tags may be mapped to more than one categories. To make sure that every unique small RNA mapped to only one annotation, we followed the following priority rule: rRNAetc (in which Genbank > Rfam) > known miRNA (Calabrese et al., 2007).

Prediction of novel miRNAs

The characteristic hairpin structure of miRNA precursors was used to predict novel miRNAs. A prediction software Mireap was used to predict novel miRNA by exploring the secondary structure, the Dicer cleavage site and the minimum free energy of the unannotated small RNA tags which could be mapped to genome.

Parameters for prediction of novel miRNAs are as follows: Minimal and Maximal miRNA sequence length are 26 nt and 18 nt; minimal and maximal miRNA reference sequence length are 24 nt and 20 nt; minimal depth of Drosha/Dicer cutting site is 3 nt; maximal copy number of miRNAs on reference is 20; maximal free energy allowed for a miRNA precursor is −18 kcal/mol; maximal space between miRNA and miRNA* is 35 nt; minimal base pairs of miRNA and miRNA* is 14; maximal bulge of miRNA and miRNA* is 4; maximal asymmetry of miRNA/miRNA* duplex is 5 nt; flank sequence length of miRNA precursor is 10 nt.

Differential expression of known miRNA

The expression level of all the known miRNAs between CVQ and NCVQ was compared to find out the differentially expressed miRNA. The comparison procedures are as follows:

(1) Normalize the expression of miRNA in CVQ and NCVQ to get the expression of transcripts per million (TPM); the normalization formula used was: Normalized expression = actual miRNA count / total count of clean reads * 1,000,000;
(2) Calculate fold-change and p-value from the normalized expression. Fold-change formula: Fold_change = $\log_2$(CVQ / NCVQ)

p-Value formula:

$$p(x|y) = \left(\frac{N_2}{N_1}\right)^y \frac{(x+y)!}{x!y!\left(1+\frac{N_2}{N_1}\right)^{(x+y+1)}}$$

$$C(y \le y_{\min}|x) = \sum_{y=0}^{y \le y\min} p(y|x)$$

$$D(y \ge y_{\max}|x) = \sum_{y \ge y_{\max}}^{\infty} p(y|x)$$

(3) Generate the $\log_2$ratio plot and scatter plot.

Target prediction for known miRNAs

The target genes of novel miRNAs were predicted using Mireap software. The rules used for target prediction are based on those suggested by Allen et al. (2005), Schwab et al. (2005): (1) No more than four mismatches between sRNA & target (G-U bases count as 0.5

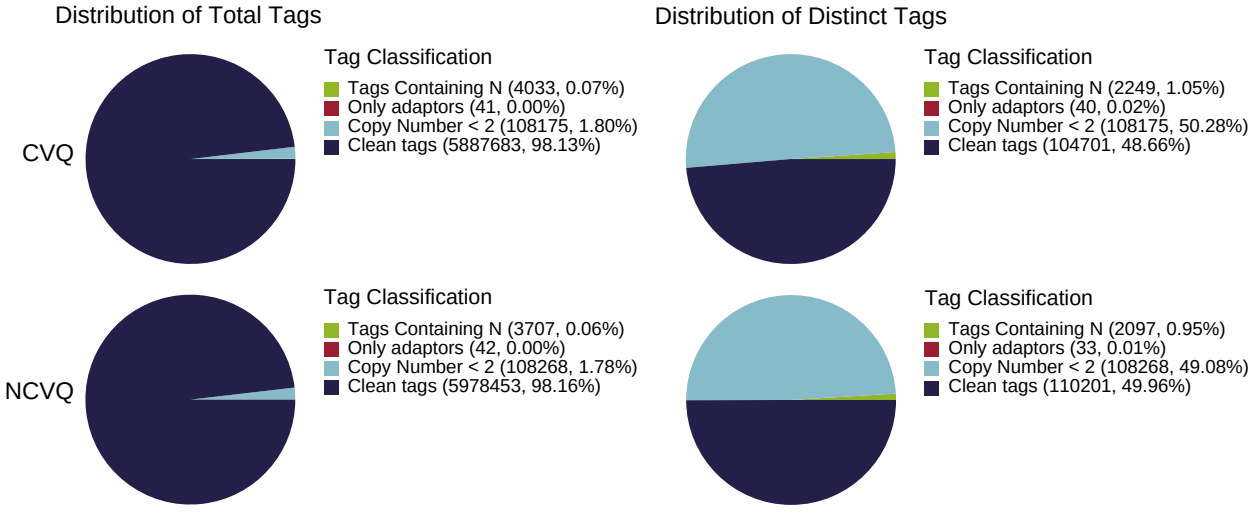

Fig. 1. Distribution of total tags and distinct tags over different tag abundance categories in each sample. The numbers and percentage of tags containing N, empty tags with adaptor only, tags with copy number <2 and clean tags are shown.

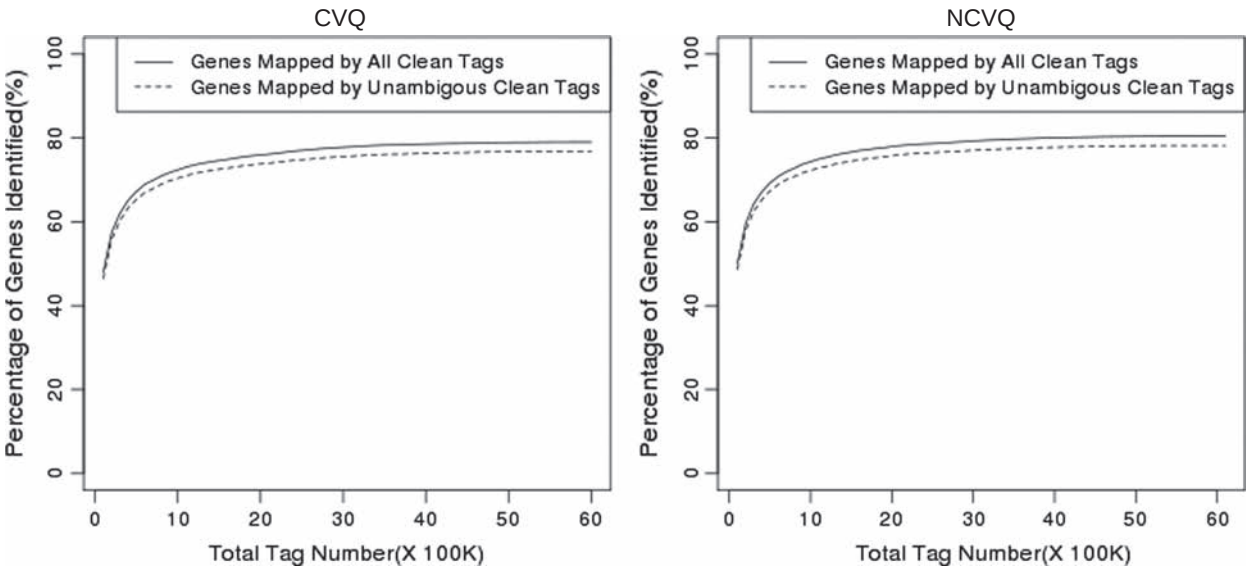

Fig. 2. Saturation analysis of clean tags. With the increase of total sequence number, the number of detected genes gradually ceased to increase.

mismatches); (2) no more than two adjacent mismatches in the miRNA/target duplex; (3) no adjacent mismatches in positions 2–12 of the miRNA/target duplex (5′ of miRNA); (4) no mismatches in positions 10–11 of miRNA/target duplex; (5) no more than 2.5 mismatches in positions 1–12 of the miRNA/target duplex (5′ of miRNA); (6) minimum free energy (MFE) of the miRNA/target duplex should be ≥75% of the MFE of the miRNA bound to its perfect complement.

Results and discussion

Digital gene expression difference between CVQ and NCVQ

To detect genes influencing mating flight behavior of *A. mellifera* courted and non-courted virgin queen, the expression profiles of all the genes were analyzed by DGE method. 6.00 million (CVQ) and 6.09 million (NCVQ) raw tags were generated in each library (Table 1, Fig. 1). The sequencing results were saturated when the sequencing amount of the two DGE libraries reached near 2 million (Fig. 2).

After filtering out low quality tags, 5.89 million (98.13%) and 5.98 million (98.16%) clean tags were remained in CVQ and NCVQ libraries (Table 1, Fig. 1). In each library, clean tags with copy numbers of more than 100 exceeded 78%, but their distribution in distinct clean tags did not exceed 6% (Fig. 3). In contrast, the tags with copy numbers between 2 and 5 showed a broad distribution of distinct clean tags. Among the clean tags, the numbers of sequences that could be mapped to reference genes were 2.46 million (41.86%) and 2.50 million (41.89%) (Table 1). Of them, 48.14% and 48.52% of the distinct clean tags were mapped to unique sequences.

A total of 452 differentially expressed genes were detected, with 90 up-regulated genes and 362 down-regulated genes in CVQ (Table S1,

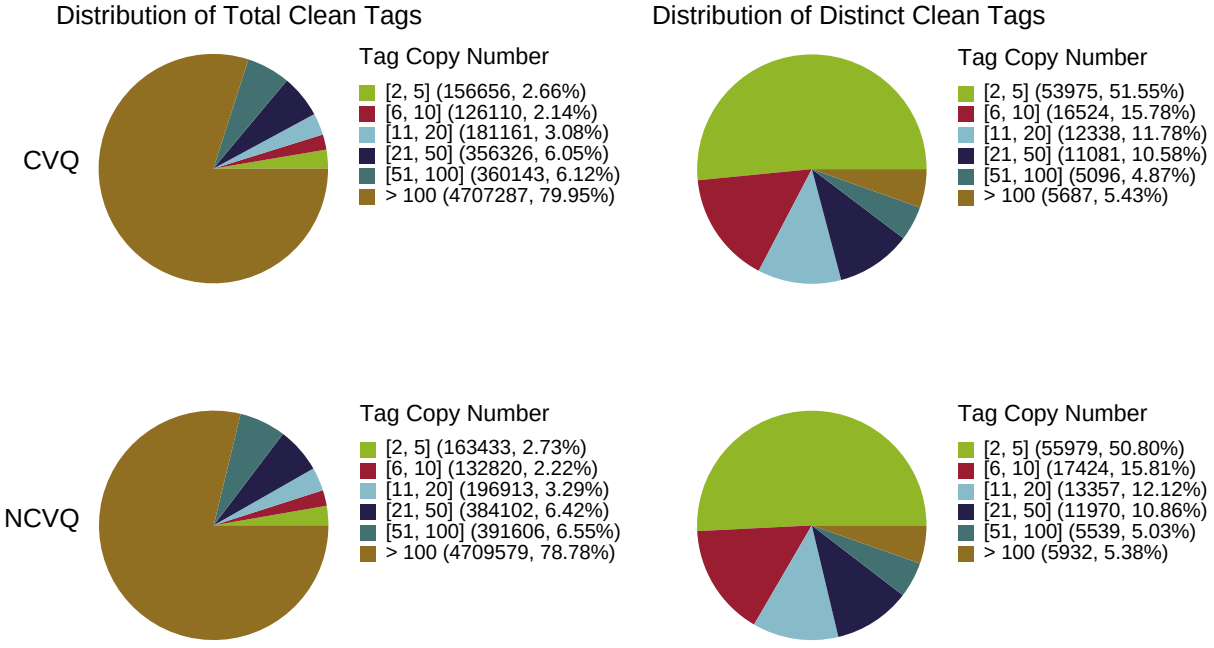

Fig. 3. Distribution of total clean tags and distinct clean tags over different tag abundance categories in each sample. Numbers in the square brackets indicate the range of copy numbers for a specific category of tags. For example, [2, 5] means all the tags in this category has 2 to 5 copies. Numbers in the parentheses of the left and right graphs respectively show the total copy number of the clean tags and the total types of clean tags in that category.

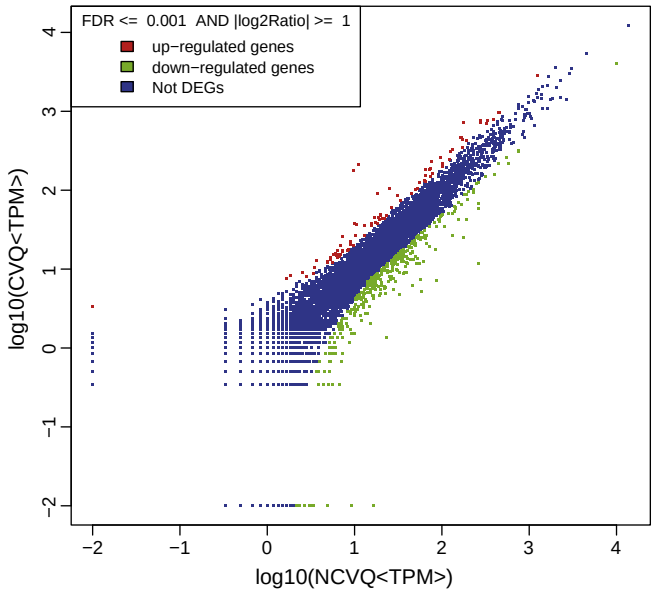

Fig. 4. Differentially expressed genes between CVQ and NCVQ. The red represents those genes up-regulated in CVQ compared to NCVQ. The green shows those genes down-regulated in CVQ. The blue shows those genes without expression difference between these two samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4). These results suggest that queens tend to suppress some genes instead of activating previously silent genes during mating flight.

To understand the functions of these differentially expressed genes, all the differentially expressed genes were mapped to terms in the GO database and compared with the background of the whole *A. mellifera* genes. Out of the 452 differentially expressed genes, 228 genes have a GO ID (Table S2) and can be categorized into 674 functional groups in three main categories. In each of the three main categories (biological process, cellular component and molecular function) of the GO classification, "cellular process," "cell" and "binding" terms were dominant. While no terms were significantly enriched in all these three main categories (p-value ≤ 0.05). To further investigate the biochemical pathways in which these differentially expressed genes were involved, we mapped all of the differentially expressed genes to terms in KEGG database and compared this with the whole genome background. Of the 452 differentially expressed genes, 138 genes had a KO ID and could be categorized into 167 pathways (Table S3); of those, no pathway was significantly enriched (Q-value ≤ 0.05).

In these differentially expressed genes, two odorant binding proteins, obp4 (NM_001040223.1), obp17 (NM_001040207.1), were up-regulated in courted virgins. Odorant binding proteins (OBPs) are water-soluble small proteins in the lymph of the olfactory neurons, and are functional component located at the start of olfactory signal transmission (Fan et al., 2011). Their function is transferring odor molecules to the sensory receptors distributed on the dendritic membrane of the olfactory nerve (Vogt, 2005). These two odorant binding proteins may allow the queens to detect the change in their chemical environment when being courted by drones during the mating flight.

We compared these differentially expressed genes with those detected in *Drosophila* in a similar study performed by Lawniczak and Begun (Dalton et al., 2010). Through BlastP alignment, we found that just 3 of the differentially expressed genes (XM_396916.4, XM_395497.4 and XM_392982.4) detected in our study have *Drosophila* homologs in those differentially expressed genes reported by Lawniczak and Begun. All these three genes are down-regulated in CVQ. It suggests that these genes maybe function by inhibiting the expression of downstream genes in queens.

We further compared our results with those differentially expressed genes between virgins and mated queens of *A. mellifera* reported by Kocher, and found that a total of 35 genes were detected both in ours and their studies. Of them, 23 genes showed an identical up- or down-regulation in courted (or mated) queens in these two studies. While, 12 of them are converse in these two studies (Table S4).

miRNA expression difference between CVQ and NCVQ

To detect differentially expressed miRNAs between CVQ and NCVQ, two small RNA libraries were constructed and sequenced using HiSeq high-throughput sequencing technology. After sequencing and removal of the low-quality tags, adaptor sequences, and tags shorter than 10 nt, 10,087,370 and 10,957,157 clean reads of 10–36 nt in length remained for analysis (Table 2). As shown in Fig. 5, most of the tags are distributed in 20–30 nt. Of these clean tags, 6,986,154 (69.26%) tags in CVQ and 8,250,416 (75.30%) tags in NCVQ can be mapped to the genome sequence of *A. mellifera*.

Then, all the clean reads were annotated by mapping to the noncoding RNAs in GenBank and the Rfam database and the known miRNA miRBase18.0. According to the annotation, these clean reads were divided into known miRNAs, rRNA, tRNA, snRNA, and sonRNA (Table 2). The total rRNA proportions, which are a mark of sample quality (usually this should be less than 40% in animal samples of high quality), are 13.83% and 7.40% in these two samples. There are 93 and 91 known miRNA in CVQ and NCVQ, and just 2 of these miRNAs were specific to CVQ, the remaining miRNAs being shared by both groups (Table S5). Of these known miRNA, 27 miRNAs showed significant expression difference between these two samples ($P < 0.01$, absolute value of $\log_2$ (CVQ/NCVQ) > 1) (Table S6, Fig. 6), 23 of them were up-regulated in CVQ and 4 down-regulated. Of them, miR-124 was reported to be expressed in many sensory neurons in *Caenorhabditis elegans* and contributes to define gene expression in sensory neurons by regulating a large number of co-expressed genes (Clark et al., 2010). This miRNA may be responsible for regulating the sensitivity of queens to the change of chemical stimulation in the environment. miR-275 was found to be indispensable for egg development in the mosquito *Aedes aegypti* (Bryant et al., 2010). It may therefore be

Table 2
Distribution of small RNA reads in the sequenced small RNA library.

	CVQ		NCVQ	
	Unique sRNAs	Total sRNAs	Unique sRNAs	Total sRNAs
Total	657,905(100%)	10,087,370(100%)	619,545(100%)	10,957,157(100%)
Map to genome	296,910(45.13%)	6,986,154(69.26%)	285,309(46.05%)	8,250,416(75.30%)
miRNA	2243(0.34%)	5,136,684(50.92%)	2063(0.33%)	2,355,197(21.49%)
rRNA	128,965(19.60%)	1,395,416(13.83%)	78,389(12.65%)	810,292(7.40%)
Repeat	3584(0.54%)	17,053(0.17%)	4849(0.78%)	34,779(0.32%)
snRNA	1955(0.30%)	8949(0.09%)	1381(0.22%)	4511(0.04%)
snoRNA	257(0.04%)	2582(0.03%)	167(0.03%)	1145(0.01%)
tRNA	15,581(2.37%)	82,757(0.82%)	10,391(1.68%)	46,922(0.43%)
Unann	489,276(74.37%)	3,400,642(33.71%)	508,578(82.09%)	7,609,887(69.45%)

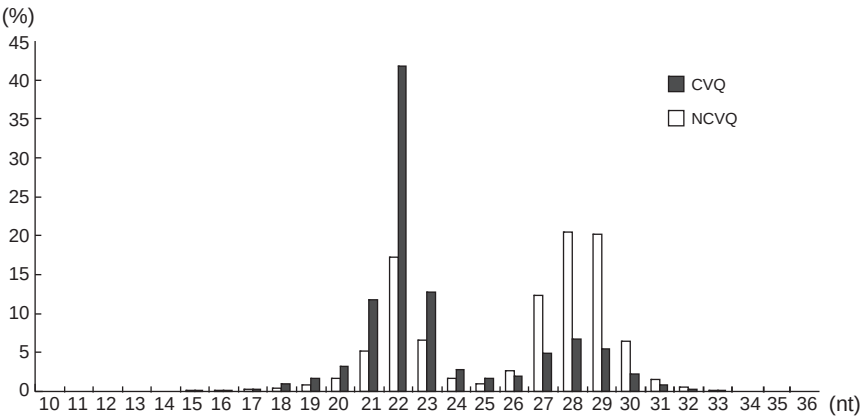

Fig. 5. Length distribution of tags produced by small RNA sequencing in each sample. The horizontal axis indicates the length of nucleic acid (nucleotides, nt), the ordinate represents distribution frequency (%).

required for egg development in activated ovaries of honeybee queens after mating.

In addition, a total of 47 novel miRNA candidates were predicted in these two samples with the software MIREAP (Table S7, Fig. S1).

microRNA target genes

We tried to predict the target genes of all the significantly differentially expressed known miRNAs. The results showed that the target genes of each miRNA range in number from several to more than one thousand (data not shown). Then, we linked these miRNA targets to the above mentioned differentially expressed genes obtained from DGE analysis, and found that 17 of the 27 (63%) differentially expressed miRNAs have targets (a total of 9 genes) in the list of the differentially expressed genes (Table S8). Most of these target genes are the common targets of several miRNAs. For example, NM_001011584.1 is the common target of 8 miRNAs. For 17 of these miRNA/gene pairs, the up- or down-regulation showed a negative correlation, while another 10 pairs showed a positive correlation. Usually, the miRNAs repress translation or leading to cleavage of its targeting mRNAs by base-pairing to the target (Bartel, 2004). But, studies reported that some miRNAs also have the function to up-regulate translation of its target mRNAs (Vasudevan and Steitz, 2007; Vasudevan et al., 2007). Maybe miRNAs can up- or down-regulate transcription of its target genes, leading to a positive relationship between the miRNA and mRNA expression level.

In conclusion, our results indicated that a large number of genes and miRNAs showed expression changes during virgin queen mating flight. These differentially expressed genes and miRNAs maybe play an important role in regulating physiological changes during mating flight of virgin queens. Moreover, as far as we know, this is the first study on

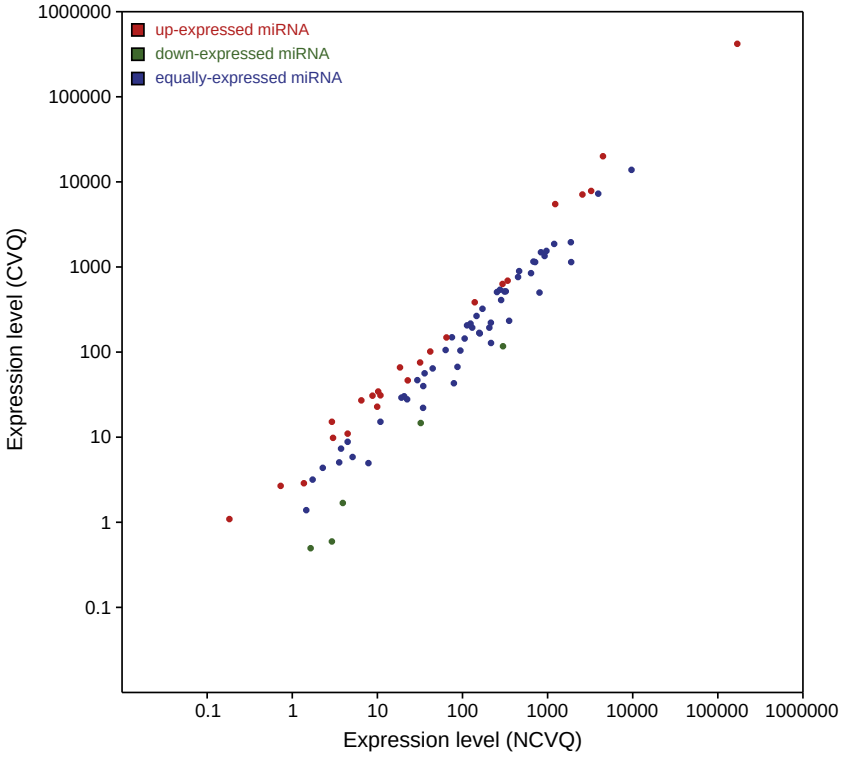

Fig. 6. Differentially expressed miRNAs between CVQ and NCVQ. The red part represents those miRNAs up-regulated in CVQ compared to NCVQ. The green part shows those miRNAs down-regulated in CVQ. The blue shows those miRNAs without expression difference between these two samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

miRNAs involved in the honeybee mating flight. Our results provide important data for studying physiological changes occurring during the mating flight of the honeybee queens.

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