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The integrative analysis of microRNA and mRNA expression in *Apis mellifera* following maze-based visual pattern learning

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Abstract The honeybee (*Apis mellifera*) is a social insect with strong sensory capacity and diverse behavioral repertoire and is recognized as a good model organism for studying the neurobiological basis of learning and memory. In this study, we analyzed the changes in microRNA (miRNA) and messenger RNA (mRNA) following maze-based visual learning using next-generation small RNA sequencing and Solexa/Illumina Digital Gene Expression tag profiling (DGE). For small RNA sequencing, we obtained 13 367 770 and 13 132 655 clean tags from the maze and control groups, respectively. A total of 40 differentially expressed known miRNAs were detected between these two samples, and all of them were up-regulated in the maze group compared to the control group. For DGE, 5 681 320 and 5 939 855 clean tags were detected from the maze and control groups, respectively. There were a total of 388 differentially expressed genes between these two samples, with 45 genes up-regulated and 343 genes down-regulated in the maze group, compared to the control group. Additionally, the expression levels of 10 differentially expressed genes were confirmed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and the expression trends of eight of them were consistent with the DGE result, although the degree of change was lower in amplitude. The integrative analysis of miRNA and mRNA expression showed that, among the 40 differentially expressed known miRNAs and 388 differentially expressed genes, 60 pairs of miRNA/mRNA were identified as co-expressed in our present study. These results suggest that both miRNA and mRNA may play a pivotal role in the process of learning and memory in honeybees. Our sequencing data provide comprehensive miRNA and gene expression information for maze-based visual learning, which will facilitate understanding of the molecular mechanisms of honeybee learning and memory.

Key words *Apis mellifera*, Y-maze, learning and memory, microRNA, DGE, differential expression

Introduction

The honeybee (*Apis mellifera*) is widely acknowledged as a model species for the study of the mechanisms and evolution of social behavior (Robinson *et al.*, 2005). Given

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their rich and highly complex life history and social organization, honeybees provide a good platform for the study of learning and memory in vertebrates and even humans. It has been found that the honeybee has an amazing ability to learn and remember tasks and objects, such as color (Menzel, 1967, 1968), orientations (van Hateren *et al.*, 1990; Srinivasan *et al.*, 1994; Zhang & Srinivasan, 1994) and odor (Menzel *et al.*, 1996). Moreover, the honeybee can also learn the relation between objects, such as sameness and differences (Giurfa *et al.*, 2001), order of objects (Zhang *et al.*, 2005), above and below relationships (Avarguès-Weber *et al.*, 2011), learn numerical attributes of objects (Gross *et al.*, 2009) and simultaneously master two abstract concepts (Avarguès-Weber *et al.*, 2012). In addition, recent studies suggest that honeybees learn what to do, where to go and when, and they are able to integrate elements of circadian time, place and visual stimuli, in a manner reminiscent of episodic memory (Zhang *et al.*, 2006; Pahl *et al.*, 2007).

MicroRNAs (miRNAs) are a kind of endogenous noncoding RNA of about 20–22 nucleotides (nt), which are emerging as key modulators of post-transcriptional gene regulation in a series of biological processes in eukaryotes, including development, metabolism and regulation of differentiation (Ambros, 2004; Bartel, 2004; He & Hannon, 2004; Filipowicz *et al.*, 2008; Wu & Belasco, 2008; Wang *et al.*, 2013a). Over recent years, emerging evidence indicates that miRNAs are actively involved in regulating gene expression patterns, and play a key role in plasticity and memory in the adult brain (Rajasethupathy *et al.*, 2009; Edbauer *et al.*, 2010; Smalheiser *et al.*, 2010; Chandrasekar & Dreyer, 2011). The first evidence in favor of a role for miRNA-mediated regulation of gene function within the context of learning and memory came from the study in *Drosophila* by Ashraf *et al.* (2006). They found that a key component of the miRNA-mediated silencing complex, Armitage, is localized to synapses under basal conditions, but is degraded upon learning. They also demonstrated that knockdown of Armitage led to an increase in the localized expression of calcium/calmodulin-dependent kinase II (CaMKII) and to enhanced long-term olfactory memory. Similarly, Konopka *et al.* (2010) suggested that cell type-specific knockout of a mammalian enzyme responsible for producing mature miRNA, Dicer, enhanced synaptic plasticity and memory in a variety of learning tasks, including spatial learning in the Morris water maze, and contextual and trace fear conditioning in mice. In addition, there are now studies demonstrating that some other miRNAs are also associated with learning and memory. For example, a study by Hansen *et al.* (2010) showed that over-expression of miR-132 impairs the formation of novel object recognition memory; Gao

et al. (2010) suggested that miR-134 is related to contextual fear memory; and Chandrasekar and Dreyer (2009, 2011) demonstrated that knockdown of each of the miR-NAs miR-124, miR-181 or Let-7d influences cocaine-conditioned place preference learning; MiR-324, miR-369 and miR-212 have also been reported to be involved in learning and memory associated with cocaine-seeking behavior (Pulipparacharuvil *et al.*, 2008; Renthal *et al.*, 2009; Hollander *et al.*, 2010). A recent report has revealed that ame-mir-276 and ame-mir-1000 are involved in neural function in the adult honeybee brain (Hori *et al.*, 2010). However, research on the miRNA activity associated with learning and memory in honeybees is slim.

As far as we know, only a small number of genes have been confirmed to be involved in honeybee learning and memory. Using short antisense oligonucleotides technique, Fiala *et al.* (1999) found that slight knockdown of protein kinase A (PKA) activity during the training procedure of PER (a classical olfactory conditioning of the proboscis extension reflex) caused an impairment of long-term memory (LTM) retention 24 h after training, from which they concluded that PKA contributes to the induction of a LTM 24 h after training when activated during learning. By injecting nicotinic antagonists, Dacher and Gauthier (2008) suggested that the honeybee acetylcholine receptors (AChRs) could be essential for triggering intracellular mechanisms involved in LTM. By means of RNA interference (RNAi), El Hassani *et al.* (2012) studied the functional role of glutamate-gated chloride channels (GluCls) in the honeybee brain following olfactory learning and memory and provided the first evidence for the involvement of GluCls in olfactory memory in an invertebrate. Besides these genes, homologues of several genes reported to be related to learning and memory in other organisms have been cloned in *A. mellifera*, including AmTYR1 receptor (one member of the tyramine receptor) (Blenau *et al.*, 2000), octopamine receptor (Grohmann *et al.*, 2003), CREB (cyclic adenosine monohsophate [cAMP] response element binding protein, the key factor involved in neuronal plasticity and memory formation) (Eisenhardt *et al.*, 2003), adenylyl cyclase (Wachten *et al.*, 2006) and AmGluRA (one member of the G-protein-coupled metabotropic glutamate receptors) (Kucharski *et al.*, 2007), all of which are speculated to be involved in learning and memory in the honeybee. Wang *et al.* (2013b) analyzed the gene expression differences following PER-based olfactory learning in *A. mellifera* using a tag-based digital gene expression (DGE) method, which facilitated our understanding of the molecular mechanism of honeybee learning and memory. In spite of this, the molecular mechanism of

learning and memory in honeybees is seldom researched when compared with mice, *Drosophila* and other model animals, and the underlying molecular mechanism still remains to be understood, especially for visual learning and memory.

Because miRNAs can down-regulate some of their targets not only at the translational, but also at the transcriptional level (Shyu *et al.*, 2008), and the expression profiles of miRNAs and of their corresponding target genes are very similar both at the tissue and cellular level (Baskerville & Bartel, 2005; Kim & Kim, 2007), it is therefore possible to use the paired expression analysis of miRNAs and mRNAs to study the molecular mechanism of a series of biological processes. In the present study, we carried out an integrative analysis of global miRNA and mRNA expression of maze-based visual learning in the honeybee through next-generation high-throughput sequencing technology. We investigated for the first time the association between differential miRNA and mRNA expression in the learning and memory processes of the honeybee. We found that both miRNA and mRNA are differentially expressed between maze learning and control honeybees, and that their expression patterns are closely related.

Materials and methods

Organism

The Western honeybee, *Apis mellifera ligustica* was used in this study. The honeybee colony was raised according to standard beekeeping techniques at the Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, China (28.46°N, 115.49°E).

Y-maze experiments

The behavioral experiments were conducted at the Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, China, on fine days in the summer of 2011. We assessed learning and memory using a twin-choice Y-maze visual association paradigm in which honeybees were required to discriminate between rewarding and unrewarding patterns. The Y-maze was located close to the window in a laboratory room and the beehive which supplied the honeybees used in the experiments was kept outside. During the experiment, honeybees entering the Y-maze were trained to choose one of two patterns, which indicated the position of the feeder reward. Training was carried out by reinforcement: if the honeybee made a positive decision by flying through the correct pattern (termed "positive"), it would find a feeder with sugar solution as a reward. If the honeybee chose the wrong pattern (termed "negative"), it would find an empty chamber without reward, and then was released to try again.

In this experiment, a pair of black/white gratings oriented at 45° versus 135° to the horizontal was used at the entrances of chambers, and we set the 45° grating pattern as "positive." During training, the positions of the patterns (left or right) were swapped every 8 min (after an average of three rewarded visits per honeybee), that is, halfway through each training block. Frequent swapping of the positions of the two comparison stimuli minimized the effects of possible biases caused by asymmetrical lighting or external landmarks. This ensured that the honeybees learned to obtain a reward by visual comparison, and not by associating the feeder location with a particular chamber.

During training, every "positive" and "negative" choice of the honeybees was recorded in each training block. The honeybees learned the visual patterns very well. In the training procedure, their pattern preference gradually moved to the "positive" pattern after several visits, and their choice frequency in favor of the "positive" stimulus was eventually significantly greater than the random choice level of 50% ($P < 0.001$). When the choice frequencies of honeybees for the "positive" pattern remained stable at above 80%, the training was ended. As this study continues our previous behavioral studies of learning and memory in honeybees, details of the maze experiments can be found in our previous paper (Qin *et al.*, 2012).

Sample preparation

Five independent replicates of the above-mentioned experiment were performed. Honeybees were sampled into two groups: the maze learning group (maze group) collected at the end of the training consisted of foragers given acquisition training as described above and learned the pattern successfully, while the control group collected at the beginning of the training consisted of foragers collecting sugar solution outside the maze and given no acquisition training. All samples were collected alive, immediately flash frozen in liquid nitrogen and then stored at −80 °C until further processing. Brain tissues were manually dissected from the heads of honeybee samples in normal saline (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, 2 mmol/L KH_2PO_4), rinsed with diethylpyrocarbonate (DEPC) treated water, and then promptly immersed in liquid nitrogen. In preparation for Illumina sequencing analysis of miRNA and DGE, equal

amounts of 25 brain tissues from maze and control groups were pooled together, respectively.

Next-generation small RNA sequencing and analysis of maze and control groups

Total RNAs of honeybee pooled brain samples of maze and control groups were extracted with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, followed by a 15% Tris-borate-ehtylenediaminetetraacetic acid (TBE) urea gel electrophoresis (Invitrogen, Carlsbad, CA, USA). For deep sequencing, the small RNA samples were prepared as follows: total RNA of each sample was size-fractionated on a 15% polyacrylamide gel electrophoresis (PAGE), and a 10–44 nt fraction was collected. After PAGE purification of small RNA molecules and ligation of a pair of Solexa adaptors to their 5' and 3' ends, the small RNA molecules were amplified. The purified DNA was used directly for cluster generation and sequencing analysis using the Illumina HiSeq 2000 (Illumina, San Diego, CA, USA). The image files generated by the sequencer were then processed to produce digital-quality data. Polymerase chain reaction (PCR) products were purified and quantified for Illumina sequencing by the Beijing Genomics Institute, Shenzhen, China.

The 50 nt sequence tags from HiSeq sequencing were put through data cleaning first, which included getting rid of the low quality tags and several kinds of contaminants from the 50 nt tags. As the length distribution analysis is helpful to see the composition of the small RNA sample, length distribution of 10–44 nt clean tags was then summarized. Afterwards, the clean tags were annotated into different categories by standard bioinformatic analyses, and those which could not be annotated to any category were taken to predict novel miRNAs. In the alignment and annotation steps, some small RNA tags might be mapped to more than one category. To ensure every unique small RNA mapped to only one annotation, we followed the following priority rule: rRNAetc (rRNA, tRNA, snRNA, scRNA and snoRNA, in which Genbank > Rfam) > known miRNA > repeat > exon > intron. The total rRNA proportion gives an indication of sample quality, which should usually be less than 40% in animal samples of high quality.

The known miRNA expressions of two samples were further analyzed to reveal the differentially expressed miRNA according to Audic and Claverie (1997). First, the expressions of miRNA in both samples were normalized to obtain the expression of transcripts per million (TPM) for each library according to the following normalization formula:

Normalized expression

$$= \text{Actual miRNA count/Total count of clean reads}$$

$$\times\ 1000000. \qquad (1)$$

Next, we calculated fold-change and *P*-value from the normalized expression to generate the $\log_2$ ratio plot and scatter plot. The following formula was used:

Fold-change $= \log_2 (\text{maze/control})$

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

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

$$D(y \geq y_{\max}|x) = \sum_{y \geq y_{\max}}^{\infty} p(y|x), \qquad (2)$$

where *x* represents the observed number of tags for a gene in one library, *y* represents the observed number of tags for the same gene in another library, and N_1 and N_2 are the total number of tags for the two libraries, respectively. In the present study, we considered that a gene was significantly differentially expressed if TPM > 1 for either library, the absolute value of fold change > 1, and $P < 0.05$.

In order to check whether novel miRNAs were present among unknown RNAs, the unannotated reads were further analyzed. There are three features in the structure of each potential miRNA precursor: highly conserved motif upstream of the hairpin precursor structure; small RNA located in two arms of the hairpin, and far from the loop; the precursor structure folded with low free energy. The characteristic hairpin structure of miRNA precursor can be used to predict novel miRNA. A prediction software Mireap was applied 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 the genome. We then summarized the prediction of novel miRNA candidates, including the base bias on the first position among small RNA candidates of a certain length and on each position among all small RNA candidates.

Digital gene expression sequencing and analysis of mRNAs

Total RNAs of maze learning and control samples were extracted by Trizol (Invitrogen, Carlsbad, CA, USA),

and their total RNA quality was found to be comparable based on analysis with a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). The mRNA library for sequencing was prepared using Gene Expression Sample Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, beads of Oligo (dT) were used to enrich mRNA from the total RNA, and then were transformed into double-stranded complementary DNA (cDNA) through reverse transcription (RT). 4-base recognition enzyme *Nla*III was used to digest this cDNA, and Illumina adaptor 1 was ligated. *Mme*I was used to digest at 17 bp downstream of the CATG site; Illumina adaptor 2 was ligated at the 3′ end. Primer GX1 and Primer GX2 were added for PCR. Then, we purified and regained 105 bp fragments through 6% TBE PAGE. After denaturation, the single-chain molecules were fixed onto the Illumina Sequencing Chip (flowcell). Each tunnel generated millions of raw reads with a sequencing length of 49 bp.

To obtain high-quality data in the Tag-seq libraries, we performed a filter of the raw reads, that is, removing the potentially erroneous tags. Briefly, by trimming the 30 adaptor sequence, filtering low-quality tags containing N, and removing small tags and one copy tags, we obtained the clean tags. 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 the Genbank database (ftp://ftp.ncbi.nih.gov/genomes/Apis_mellifera). Then all the clean tags were mapped to the reference tag database, and unambiguous tags were annotated. The initial counts of the clean tags of each gene were normalized to obtain the normalized gene expression.

To identify the differentially expressed genes between maze and control libraries, a rigorous statistical algorithm was developed by consulting 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 (corresponding to the *P*-value in differential gene expression detection) in multiple tests. A FDR $\leq$ 0.001 and an absolute *E* value of the $\log_2$ ratio $\geq$ 1 were used as the threshold to determine significant differences in gene expression. The identified differentially expressed genes were used for Gene Ontology (GO) and Kyoto Encyclopedia Of Genes And Genomes (KEGG) pathway analysis.

GO enrichment analysis of functional significance applies a hypergeometric test to map all differentially expressed genes to terms in the GO database, looking for significantly enriched GO terms in differentially expressed genes compared to the genome background. The formula used is:

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}, \qquad (3)$$

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; m is the number of differentially expressed genes in M.

KEGG pathway enrichment analysis identifies significantly enriched metabolic pathways or signal transduction pathways in differentially expressed genes compared with the whole genome background. The formula used is the same as that in GO analysis.

To validate the sequencing results, 10 genes identified as highly and significantly differentially expressed between maze group and control group were chosen for confirmation of expression differences with quantitative reverse transcription polymerase chain reaction (qRT-PCR). The qRT-PCR experiment was carried out by the Beijing Genomics Institute, Shenzhen, China. qRT-PCR primers were designed on the basis of the nucleotide sequence of the 10 chosen genes using Primer 5.0 software. Glyceraldehyde-3-phosphate dehydrogenase 1 (GAPDH1) was used as an appropriate internal control. Genes and primer sequences are summarized in Supplementary Table 1.

Analysis of miRNA targeted genes

To identify possible target sequences of differentially expressed miRNAs between maze and control groups, we used RNA hybrid software (Rehmsmeier *et al.*, 2004) and ftp://ftp.ncbi.nih.gov/genomes/Apis_mellifera/RNA/rna.fa.gz, which provided us with a single predicted site of interaction with a minimum free energy. The rules used for target prediction are based on those suggested by Allen *et al.* (2005) and Schwab *et al.* (2005). Subsequently, the predicted target genes were compared with the differentially expressed genes of DGE, to find which differentially expressed miRNAs and mRNAs were co-expressed.

Results

Differences in miRNA between maze and control honeybees

In our two libraries, there were 15 312 439 reads for the maze group and 14 580 385 reads for the control group,

Table 1 Summary of data cleaning and length distribution of tags produced by small RNA sequencing.

Group	Total reads	High quality (%)	3′adapter null (%)	Insert null (%)	5′adapter contaminants (%)	<18 nt (%)	PolyA (%)	Clean reads (%)
Maze	15 312 439	14 743 522 (100)	547 613 (3.71)	102 633 (0.70)	19 659 (0.13)	705 683 (4.79)	164 (0)	13 367 770 (90.67)
Control	14 580 385	14 067 676 (100)	459 413 (3.27)	68 558 (0.49)	12 213 (0.09)	394 654 (2.81)	183 (0)	13 132 655 (93.35)
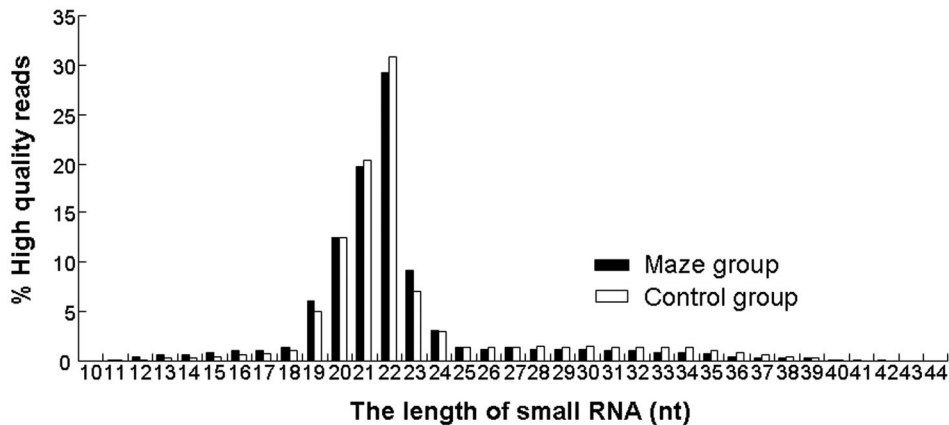
Fig. 1 Length distribution of tags produced by small RNA sequencing in the brains of maze learning and control honeybees. The horizontal axis indicates the length of the nucleic acid (nucleotides, nt), the ordinate represents distribution frequency (%).

Table 2 Mapping statistics of small RNAs in maze and control groups.

	Maze group		Control group	
	Unique (%)	Total (%)	Unique (%)	Total (%)
Total sRNAs	656 250 (100)	13 367 770 (100)	650 581 (100)	13 132 655 (100)
Mapping to genome	324 056 (49.38)	8 882 183 (66.44)	330 117 (50.74)	8 613 102 (65.59)

after discarding the empty adapters (Table 1). After filtering out those sequences that were of low-quality, shorter than 10 nt and single-read sequences, 13 367 770 and 13 132 655 clean reads of 10–44 nt in length, for the maze and control groups, respectively, remained for analysis. As the distribution chart shows (Fig. 1), the length distributions of both small RNA libraries peaked at 22 nt and were 29.27% and 30.86% of total reads in the maze and control groups, respectively, consistent with the common size of miRNAs. All clean reads were mapped to the honeybee genome, leading to 8 882 183 (66.44%) genome-matched reads in the maze group and 8 613 102 (65.59%) reads in the control group (Table 2).

Next, small RNAs were classified into different categories according to their biogenesis and annotations (Fig. 2). The most abundant (based on read count of total

small RNAs) RNA species in both libraries were classified as miRNAs, representing 64.11% of the maze library and 60.22% of the control library, which showed significantly different length distributions (Chi-square analysis, $\chi^2 > 1000$, $P < 0.001$). 23.28% and 22.10% of small RNAs were sorted as unann (unannotated) RNAs for the maze and control samples respectively. Also, rRNA, tRNA, SnRNA, snoRNA (together termed rRNAetc) were also analyzed in the library.

By referencing to the mirBase release 17.0 (http://microrna.sanger.ac.uk/), we identified 91 and 83 known miRNAs in the maze and control groups, respectively (total 93 miRNAs in both groups, Supplementary Table 2). Expression of the known miRNA in maze and control groups were compared to identify differentially expressed miRNAs, according to their absolute value of
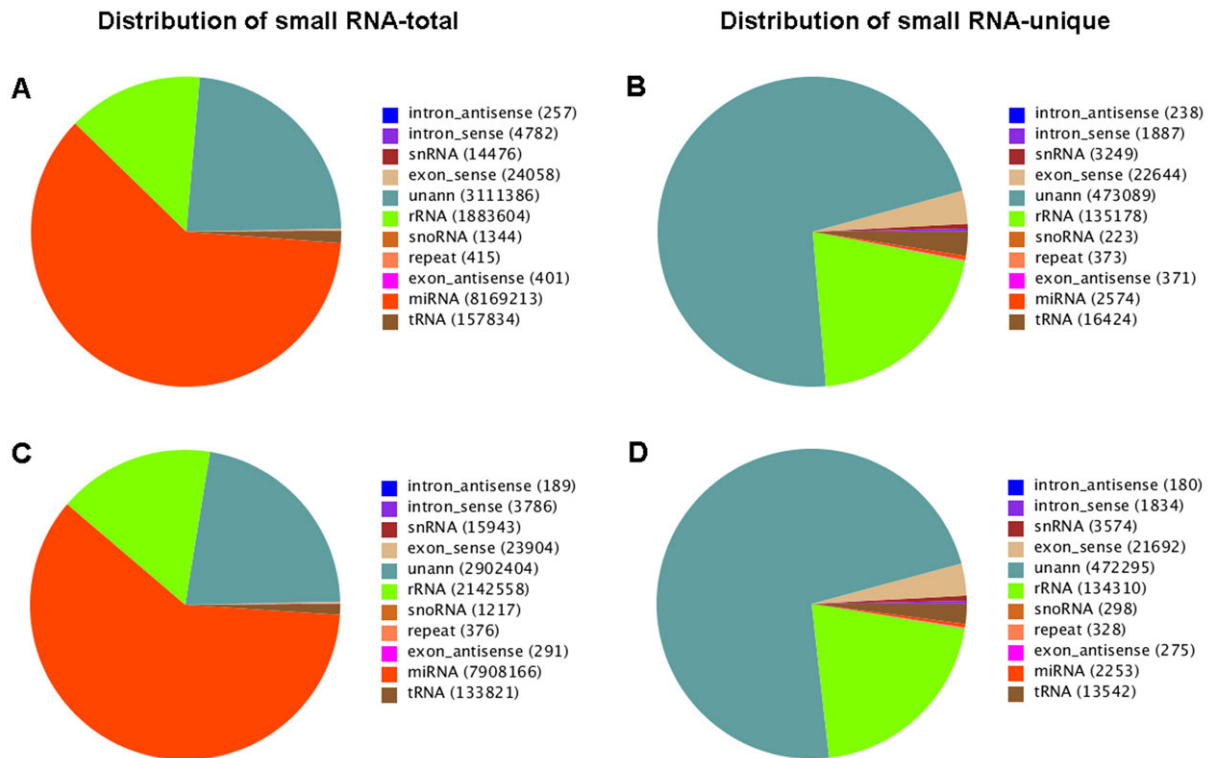
Distribution of small RNA-total

A

- intron_antisense (257)
- intron_sense (4782)
- snRNA (14476)
- exon_sense (24058)
- unann (3111386)
- rRNA (1883604)
- snoRNA (1344)
- repeat (415)
- exon_antisense (401)
- miRNA (8169213)
- tRNA (157834)

C

- intron_antisense (189)
- intron_sense (3786)
- snRNA (15943)
- exon_sense (23904)
- unann (2902404)
- rRNA (2142558)
- snoRNA (1217)
- repeat (376)
- exon_antisense (291)
- miRNA (7908166)
- tRNA (133821)

Distribution of small RNA-unique

B

- intron_antisense (238)
- intron_sense (1887)
- snRNA (3249)
- exon_sense (22644)
- unann (473089)
- rRNA (135178)
- snoRNA (223)
- repeat (373)
- exon_antisense (371)
- miRNA (2574)
- tRNA (16424)

D

- intron_antisense (180)
- intron_sense (1834)
- snRNA (3574)
- exon_sense (21692)
- unann (472295)
- rRNA (134310)
- snoRNA (298)
- repeat (328)
- exon_antisense (275)
- miRNA (2253)
- tRNA (13542)

Fig. 2 Distribution of tags produced by small RNA sequencing among different categories. The microRNA (miRNA), rRNAetc (rRNA, tRNA, snRNA, scRNA and snoRNA) and unann (unannotated), are shown. (A) Distribution of total clean tags for the maze group. (B) Distribution of distinct clean tags for the maze group. (C) Distribution of total clean tags for the control group. (D) Distribution of clean tags for the control group.

fold changes (>1 fold), TPM of either library (>1) and *P*-values (<0.05) (Fig. 3). Based on the above-mentioned standards, there were 40 miRNAs differentially expressed between the maze and control samples, and all of them were up-regulated in the maze group compared to the control group (Table 3).

In our study, as described above, a high percentage of small RNAs were sorted as unknown RNAs. We wanted to check whether novel miRNAs were present among unknown RNAs. Novel miRNAs were predicted based on the representative stem-loop hairpin structure of pre-miRNA. In total, we identified 45 novel miRNA candidates in maze and control samples (Supplementary Table 3).

Differences in mRNA between maze and control honeybees

To study gene expression and the relationship between the miRNA and their targets, we performed SAGE-Illumina sequencing to examine the transcriptional profile

of the brain from the maze and control groups. A summary of the number of DGE tags and their mapping to the reference database is presented in Table 4. In total, 5 814 195 and 6 071 332 clean tags were sequenced from maze and control brain samples, respectively, and the percentage of clean tags among the raw tags in each library were 97.71% and 97.83%. The numbers of corresponding distinct tags were 245 584 to maze brain and 246 100 to control, representing 46.35% and 47.03%, respectively, of the total (Fig. 4). In our study, the tag sequences of the two DGE libraries were mapped to *A. mellifera* transcripts and the honeybee genome. For the maze group, the highest percentage of total clean tags (47.58%) could be matched to the reference genes. Moreover, approximately 19.46% were unknown tags. As they could not be matched to any of the reference databases (genome and the mRNA transcripts), we labeled these novel genes. In the control group, the corresponding values were 47.19% and 19.66%, respectively. In each library, those tags with copy numbers of more than 100 showed percentages of greater than 76% among the clean tags, but their distribution of distinct clean tags did not exceed 9%. In contrast,
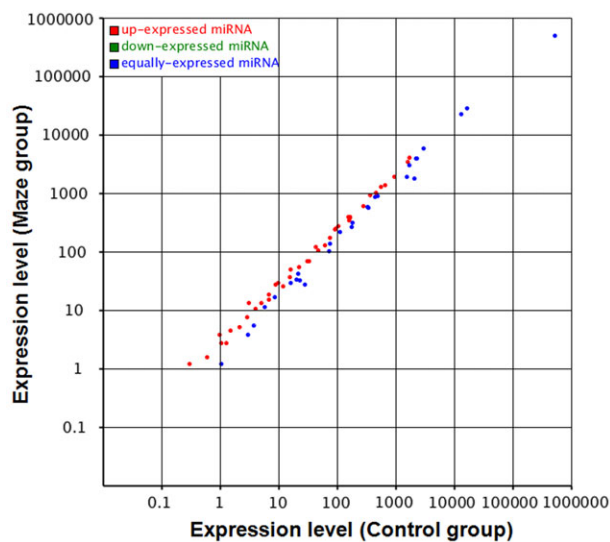
Fig. 3 Differential expression analysis of microRNA (miRNA) in the maze and control groups. Transcripts per million (TPM) clean tags means the number of a certain tag per million clean tags, which is a normalized index. Expression levels are indicated on Y (maze) or X (control). With an estimated P-value < 0.05 and $|\log_2 \text{Ratio}| > 1$ (Ratio: maze/control), the red shows the parts of up-regulated miRNAs in maze group, the blue shows the part without differentially expressed miRNAs existing in both maze and control groups; no down-regulated miRNAs (the green) were detected in our study.

those tags with copy numbers between 2 and 5 showed a broad distribution (approximately 50%) of distinct clean tags (Fig. 5).

Saturation analysis was performed to check whether the number of detected genes increased with an increase in the total tag number. As showed in Figure 6, when the sequencing amount of the two DGE libraries reaches near 1 million, the number of detected genes almost ceases to increase. This suggested that the sequencing results were saturated since the obtained clean tags in each library were around 5.68 and 5.94 million.

All annotated genes were analyzed for evidence of differential expression. A total 8 165 genes were, to some extent, differentially expressed between maze and control groups. Genes were designated to be significantly differentially expressed if the FDR value was ≤ 0.001, and there was at least an expression ratio (maze/control) of ≥ 1 in sequence counts between the two libraries. Using these standards, 388 genes were considered significantly differentially expressed, of which 45 genes were up-regulated and 343 genes were down-regulated in the maze group compared to the control group (Fig. 7, Supplementary Table 4). Of them, 303 significantly differ-

ently expressed genes were function-confirmed, while the remaining 85 genes were annotated as ''hypothetical protein'' or ''uncharacterized protein'', that is, their function is as yet unknown.

To understand the functions of these differentially expressed genes, all of them were mapped to terms in GO database and compared with the whole genome background. Of the 388 differentially expressed genes, 236 genes had an ID in GO database, and could be categorized into a total of 636 functional groups in three main categories, including cellular component, molecular function and biological process (Supplementary Table 5). When compared to the whole genome background, 149 of 388 different expressed genes were annotated as active in GO cellular component categories, and of these, 58 and 55 were enriched for the cytoplasm and the cytoplasmic part respectively ($P \leq 0.05$).

KEGG is a knowledge base for the systematic analysis of gene functions, linking genomic information with higher-order functional information which is stored in the PATHWAY database (Ogata *et al.*, 1999). To further investigate the biochemical pathways these differentially expressed genes were involved in, we mapped all of the differentially expressed genes to terms in the KEGG database and compared them with the whole genome background. Of the 388 differentially expressed genes, 184 genes had a KO (KEGG Orthology) ID and could be categorized into 159 pathways, including 25 signaling pathways such as the "Insulin signaling pathway", "MAPK signaling pathway", "GnRH signaling pathway" and "Calcium signaling pathway" (Supplementary Table 6). While compared to the whole genome background, no term was significantly enriched (Q value ≥ 0.05).

In order to validate the sequencing results, 10 differentially expressed genes were selected for qRT-PCR analysis. The result showed that the expression trends of eight of them were consistent with the DGE result, although the degree of change was lower in amplitude (Supplementary Table 7).

Integrative analysis of miRNA and mRNA expression data

Because most animals' miRNAs are intragenic, and transcribed as part of their hosting transcription units (Gennarino *et al.*, 2009), we hypothesized that the expression profiles of miRNAs and their target genes are directly correlated. As a result of this, miRNA expression was compared with their predicted target mRNA expression to see whether they were co-expressed. We identified the target genes of the 40 differentially expressed

Table 3 Significantly differential expression miRNAs in maze and control groups.

| miRNA name	Maze group (std)	Control group (std)	Fold-change (log$_2$ maze/control)	P-value	Significance	Up/ down
ame-let-7	1928.89	956.55	1.01	0	†	Up
ame-miR-100	248.28	93.89	1.40	7.92e-210	†	Up
ame-miR-1000	18.55	6.78	1.45	4.72e-18	†	Up
ame-miR-12	49.82	15.91	1.65	1.11e-54	†	Up
ame-miR-124	171.98	75.54	1.19	4.90e-113	†	Up
ame-miR-125	36.28	15.76	1.20	1.08e-25	†	Up
ame-miR-137	393.78	166.46	1.24	3.48e-275	†	Up
ame-miR-13b	25.14	11.95	1.07	1.87e-15	†	Up
ame-miR-210	103.91	47.52	1.13	7.62e-64	†	Up
ame-miR-277	69.12	33.43	1.05	2.28e-38	†	Up
ame-miR-278	2.69	1.29	1.06	0.01	‡	Up
ame-miR-279c	13.24	3.12	2.08	5.14e-21	†	Up
ame-miR-281	1.57	0.61	1.37	0.02	‡	Up
ame-miR-283	383.83	166.46	1.21	6.34e-256	†	Up
ame-miR-29b	10.47	4.04	1.38	4.52e-10	†	Up
ame-miR-3049	13.02	5.03	1.37	3.79e-12	†	Up
ame-miR-305	29.10	9.98	1.54	6.81e-30	†	Up
ame-miR-306	339.70	159.45	1.09	1.29e-193	†	Up
ame-miR-307	27.38	8.91	1.62	4.05e-30	†	Up
ame-miR-315	3378.80	1633.64	1.05	0	†	Up
ame-miR-317	4006.43	1742.83	1.20	0	†	Up
ame-miR-33	4.41	1.52	1.54	1.23e-05	†	Up
ame-miR-34	593.07	282.27	1.07	0	†	Up
ame-miR-3477	1000.99	465.79	1.10	0	†	Up
ame-miR-3719	120.81	43.86	1.46	1.49e-109	†	Up
ame-miR-3783	271.40	103.71	1.39	1.82e-225	†	Up
ame-miR-3785	54.01	22.62	1.26	5.67e-40	†	Up
ame-miR-3786	1282.04	564.78	1.18	0	†	Up
ame-miR-3791	2.69	1.07	1.34	0	†	Up
ame-miR-71	68.45	30.92	1.15	1e-43	†	Up
ame-miR-8	1367.92	657.90	1.06	0	†	Up
ame-miR-929	241.40	92.75	1.38	4.41e-199	†	Up
ame-miR-92a	1.20	0.30	1.97	0.01	†	Up
ame-miR-932	932.69	372.20	1.33	0	†	Up
ame-miR-971	7.48	2.89	1.37	1.55e-07	†	Up
ame-miR-981	128.37	62.14	1.05	1.27e-69	†	Up
ame-miR-989	387.95	155.41	1.32	1.04e-297	†	Up
ame-miR-993	5.16	2.13	1.28	3.86e-05	†	Up
ame-miR-996	3.74	0.99	1.92	2.64e-06	†	Up
ame-miR-9a	14.96	6.85	1.13	1.80e-10	†	Up

Note. Std means normalized of expression levels of miRNA in a sample. *P*-value which reflects the significance of miRNA differential expression between samples: Less *P*-value shows more significance of miRNA expression difference between samples.
†fold-change (log$_2$) > 1 or fold-change (log$_2$) < 1 and *P*-value < 0.01.
‡fold-change (log$_2$) > 1 or fold-change (log$_2$) < 1 and *P*-value < 0.05.

Table 4 Summary of digital gene expression profiles and their mapping to the reference genes.

Summary		Maze group (%)	Control group (%)
Raw data	Total	5 814 195	6 071 332
	Distinct tag	245 584	246 100
Clean tag	Total number	5 681 320 (100)	5 939 855 (100)
	Distinct tag number	113 838 (100)	115 747 (100)
All tag mapping to gene	Total number	2 703 052 (47.58)	2 803 054 (47.19)
	Distinct tag number	45 861 (40.29)	47 315 (40.88)
Unambiguous tag mapping to gene	Total number	2 409 764 (42.42)	2 497 347 (42.04)
	Distinct tag number	42 549 (37.38)	43 893 (37.92)
Mapping to genome	Total number	1 872 756 (32.96)	1 969 281 (33.15)
	Distinct tag number	50 784 (44.61)	51 447 (44.45)
Unknown tag	Total number	1 105 512 (19.46)	1 167 520 (19.66)
	Distinct tag number	17 193 (15.10)	16 985 (14.67)
All tag-mapped genes	Number (% of ref genes)	9 180 (78.22)	9 207 (78.45)
Unambiguous tag-mapped genes	Number (% of ref genes)	8 152 (69.46)	8 169 (69.61)
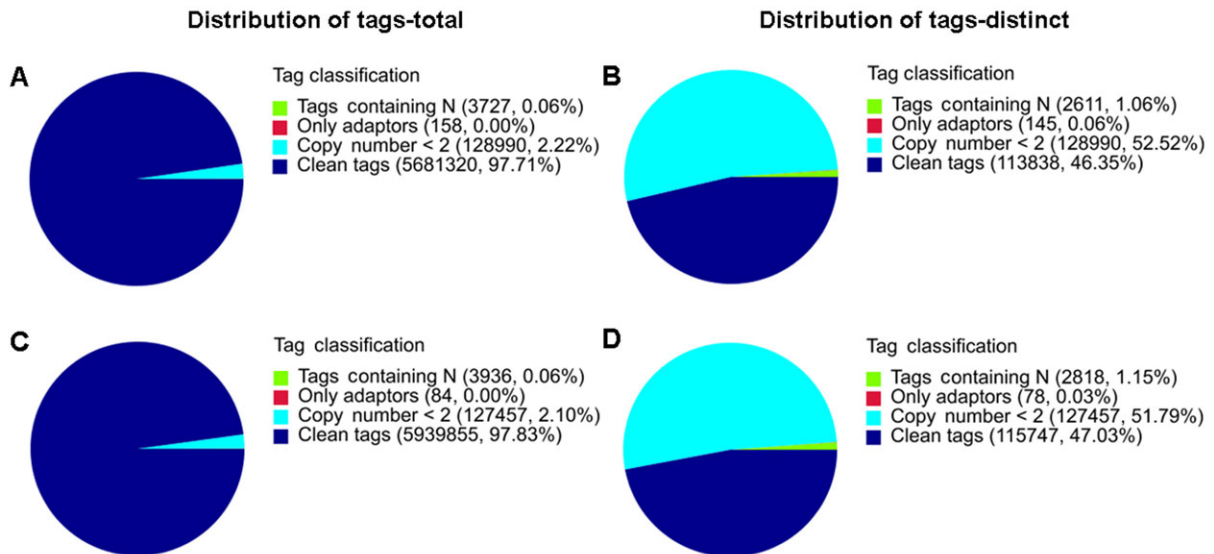
Fig. 4 Distribution of total tags and distinct tags over different tag abundance categories in each sample. The numbers and percentage of tags containing N, empty tags with adaptor only, tags with copy number < 2 and clean tags, are shown. (A) Distribution of total clean tags of the maze group. (B) Distribution of distinct clean tags of the maze group. (C) Distribution of total clean tags of the control group. (D) Distribution of clean tags of the control group.

miRNAs. All miRNAs had more than one predicted target gene, and some of the miRNAs even had hundreds, as in the case of ame-let-7 and ame-miR-13b and so on. Similarly, some genes had more than one miRNA target site. Among the 40 differentially expressed known miRNAs and 388 differentially expressed genes, 60 pairs of miRNA/mRNA were identified as co-expressed in our present study (Supplementary Table 8). Further analysis showed that the 60 related miRNA/mRNA pairs contained 33 miRNAs and 10 mRNAs (Table 5).

Discussion

Differences in miRNA between maze and control honeybees

The miRNA machinery has been shown to influence almost every cellular and developmental process investigated so far (Stefani & Slack, 2008). In recent years, specific miRNAs have been conclusively implicated in plasticity and memory in the adult brain (Ashraf *et al.*,
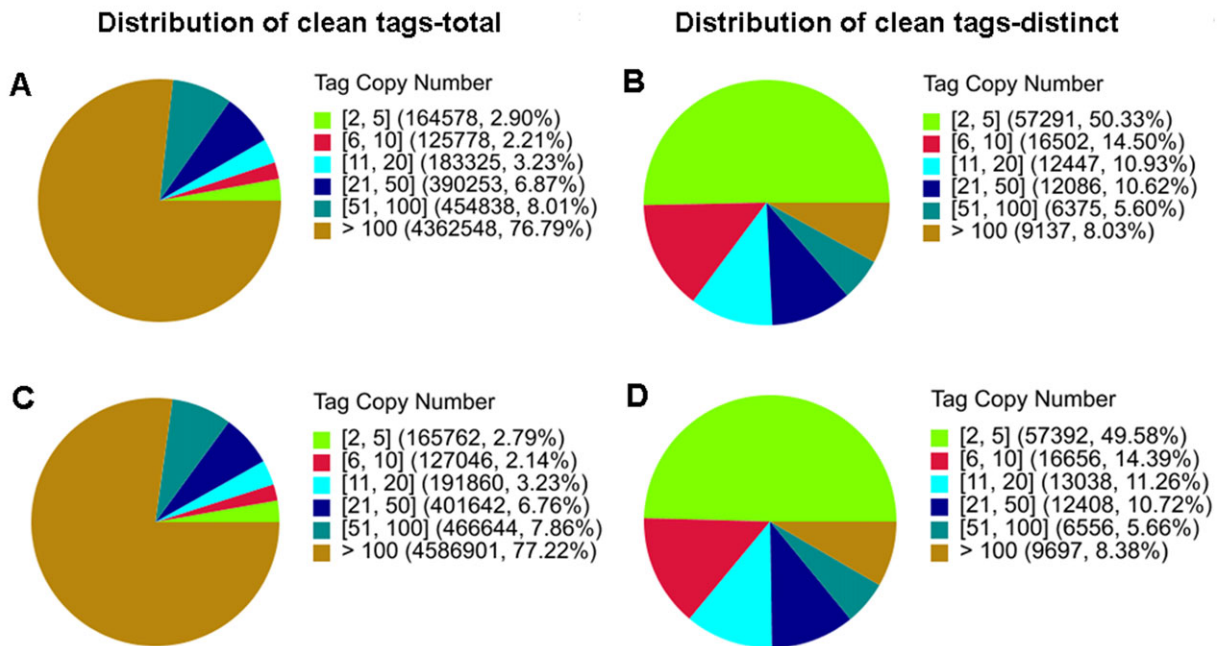
Distribution of clean tags-total

A

Tag Copy Number
- [2, 5] (164578, 2.90%)
- [6, 10] (125778, 2.21%)
- [11, 20] (183325, 3.23%)
- [21, 50] (390253, 6.87%)
- [51, 100] (454838, 8.01%)
- > 100 (4362548, 76.79%)

Distribution of clean tags-distinct

B

Tag Copy Number
- [2, 5] (57291, 50.33%)
- [6, 10] (16502, 14.50%)
- [11, 20] (12447, 10.93%)
- [21, 50] (12086, 10.62%)
- [51, 100] (6375, 5.60%)
- > 100 (9137, 8.03%)

C

Tag Copy Number
- [2, 5] (165762, 2.79%)
- [6, 10] (127046, 2.14%)
- [11, 20] (191860, 3.23%)
- [21, 50] (401642, 6.76%)
- [51, 100] (466644, 7.86%)
- > 100 (4586901, 77.22%)

D

Tag Copy Number
- [2, 5] (57392, 49.58%)
- [6, 10] (16656, 14.39%)
- [11, 20] (13038, 11.26%)
- [21, 50] (12408, 10.72%)
- [51, 100] (6556, 5.66%)
- > 100 (9697, 8.38%)

Fig. 5 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 two to five copies. Numbers in the parentheses of the left and right graphs show the total copy number of the clean tags and the total types of clean tags respectively in that category. (A) Distribution of total clean tags of the maze group. (B) Distribution of distinct clean tags of the maze group. (C) Distribution of total clean tags of the control group. (D) Distribution of clean tags of the control group.

2006; Rajasethupathy *et al.*, 2009; Edbauer *et al.*, 2010; Gao *et al.*, 2010; Smalheiser *et al.*, 2010; Chandrasekar & Dreyer, 2011). In our present study, we found several miRNAs reported to be related to neurogenesis learning and memory, including miR-124, let-7, miR-9a and mir-1000. Several reports have suggested that the neural-specific miRNA, miR-124, is an important regulator of neurogenesis in flies (Stark *et al.*, 2005), mice (Cheng *et al.*, 2009) and chickens (Cao *et al.*, 2007; Visvanathan *et al.*, 2007). The highly brain-enriched miR-124 is induced upon embryonic stem cell neuronal differentiation, and blocking their function during this process decreases neuronal differentiation at the expense of astrocytes (Krichevsky *et al.*, 2005). Furthermore, Caygill and Johnston (2008) reported that let-7 family members are required for the maturation of the *Drosophila* neuromuscular junctions (NMJs), while a recent study showed that let-7 in neural progenitors also plays a role in neural differentiation (Schwamborn *et al.*, 2009). Sensory organs in flies develop from the divisions of a single sensory organ precursor (SOP) cell. Epistasis experiments showed that miR-9a, expressed in non-SOP cells, acts via direct targeting of the transcription factor Senseless, a positive regulator of proneural genes in SOP cells. This suggests that miR-9a plays a role in cell fate specification in the developing sensory organs of *Drosophila* (Li *et al.*, 2006; Li *et al.*, 2009). Further, a recent report has provided new evidence showing that *ame*-mir-1000 was expressed in a brain-selective and preferential manner in the adult honeybee brain, and almost all of its predicted targets encode neural function-related genes (Hori *et al.*, 2010). As all of the miRNAs above-mentioned were differentially expressed between our maze and control groups, it is possible that these miRNA also play some roles in neural function as well as learning and memory in the honeybee brain.

Nevertheless, some miRNAs reported to be related to learning and memory in other organisms mentioned in the "Introduction", such as miR-132, miR-134, miR-181, miR-324, miR-369 and miR-212, were not differentially detected in our study. We suspect that this may for the reason for species specificity.

Differences in mRNA between maze and control honeybees

The results of sequencing quality assessment carried out in this study, including distributions of tag expression (Fig. 4), distribution of clean tag copy number (Fig. 5) and saturation analysis (Fig. 6), showed that our sequencing
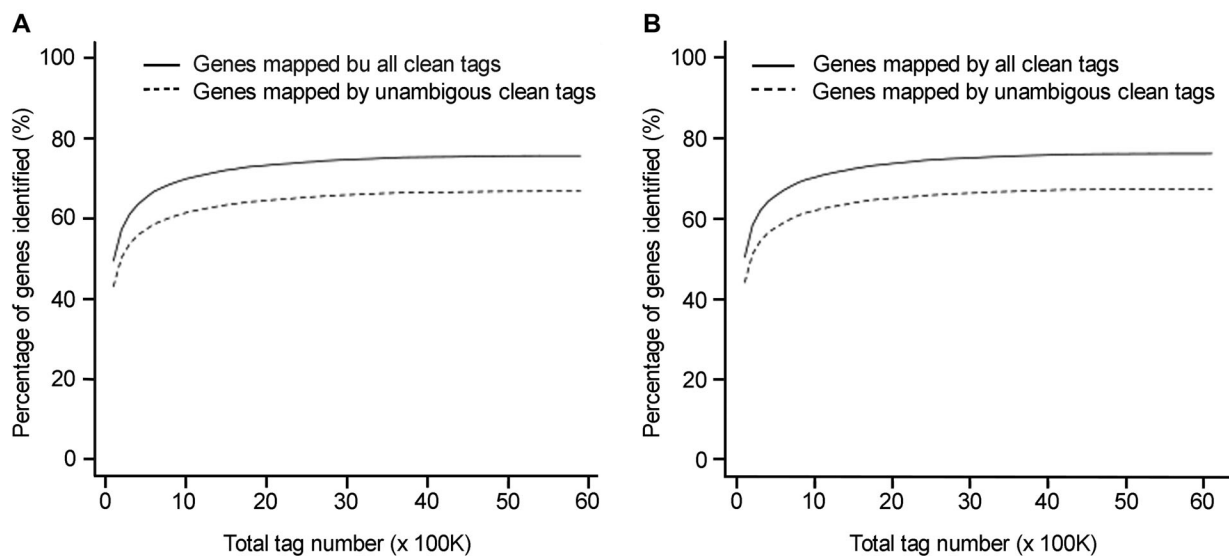
Fig. 6 Assessment of the degree of saturation in digital gene expression (DGE) sequencing. (A), (B) respectively presents the relationship between the percentage of genes identified and total tag number in the library of the maze and control groups. With the increase of total sequence number, the number of detected genes gradually stabilized.

data were accurate and credible. Moreover, 388 genes were significantly differentially expressed between the maze and control groups among which several genes were reported to be related to learning and memory. In the gene expression analysis following olfactory learning in *A. mellifera* by Wang *et al.* (2013a), the GABA$_A$ beta, nAChR, TWKSPDIVIRFa-containing neuropeptide and syntaxin binding protein 5 also showed expression differences.

The mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway is a signaling cascade controlled by the Ras family of small GTPases, which plays a crucial role in a variety of cell regulatory events (Shields *et al.*, 2000; Pearson *et al.*, 2001). A wide range of evidence using many different behavioral paradigms indicates a broad role for MAPK/ERK activation in long-term fear memory (Schafe *et al.*, 1999, 2000; Duvarci *et al.*, 2005), spatial memory (Blum *et al.*, 1999; Selcher *et al.*, 1999), and the insular cortex of long-term memories for taste (Berman *et al.*, 1998). The MAPK ERK-A gene differentially expressed in our study suggests that the MAPK/ERK cascade may also be required in maze-based visual learning of the honeybee.

Serotonin receptor, Gamma-aminobutyric acid GABA$_A$ beta and acetylcholine receptor are three kinds of important neurotransmitter receptors. Serotonin (5-hydroxytryptamine, 5-HT) is known to regulate synaptic plasticity, neurogenesis and neuronal survival in the adult brain, and emerging evidence also indicates that 5-HT systems play a pivotal role in learning and memory (Meneses, 2002, 2011; Mattson *et al.*, 2004). Previous

studies presented the notion that the activation of the 5-HT systems impairs performance, whereas reduced serotonergic function may facilitate learning (Altman & Normile, 1988; Buhot, 1997). The finding that the differentially expressed gene 5-HT1 is down-regulated in the maze group compared to the control group is in agreement with this notion. GABA receptor is the most important and most abundant inhibitory neurotransmitter in the mammalian central neural system (Sivilotti & Nistri, 1991). Previous work has suggested that it plays a key role in cognitive processes, including memory formation and consolidation (Kalueff & Nutt, 1996). Acetylcholine receptors can be divided into muscarinic and nicotinic (Purves, 1976) which are widely distributed in the central and peripheral nervous system. Gauthier *et al.* (2006) suggested that these receptors could be essential for triggering intracellular mechanisms involved in LTM. Besides, acetylcholine can be hydrolyzed by acetylcholinesterase (AChE), and it has been shown that the acetylcholine level will increase when the AChE level reduces (Winkler *et al.*, 1998). Therefore, the down-regulation of AChE-1 during learning in our study probably improves the acetylcholine level and indirectly modulates learning and memory.

Integrative analysis of miRNA and mRNA expression data

It has been suggested that miRNA and mRNA are dynamically regulated during neurogenesis (Nielsen *et al.*,

Table 5 Annotation of digital gene expressions and their related micro RNAs (miRNAs) as affected by maze and control groups.

Gene ID	Full name	The related miRNA	Gene annotation/pathway
NM_001011615.1	Hymenoptaecin	ame-mir-3786; ame-mir-993; ame-mir-9a	
XM_397432.3	Translocator protein-like	ame-let-7	Neuroactive ligand-receptor interaction
NM_001011576.1	Tachykinin	ame-miR-100; ame-mir-3049; ame-mir-306	
NM_001011583.1	Chemosensory protein 3	mir-1000; mir-3783; mir-929; mir-92a	Axon guidance; MAPK signaling pathway
NM_001011600.1	Hexamerin 70 b	ame-mir-283; ame-mir-3049; ame-mir-306; ame-mir-34; ame-mir-3719; ame-mir-932; ame-mir-981; ame-mir-9a	Tyrosine metabolism; Metabolic pathways; Melanogenesis;
NM_001040263.1	Cystathionine	ame-mir-1000; ame-mir-3049; ame-mir-8;	Riboflavin metabolism
	Beta-synthase	ame-mir-971; ame-mir-989; ame-mir-8	
NM_001042460.1	Meteorin	ame-mir-124; ame-mir-13b; ame-mir-210; ame-mir-283; ame-mir-306; ame-mir-3791; ame-mir-996	Glycerolipid metabolism; Glycerophospholipid metabolism; Metabolic pathways; Phosphatidylinositol signaling system
XM_396618.3	Transferrin 2	ame-mir-124; ame-mir-283; ame-mir-305; ame-mir-317; ame-mir-34; ame-mir-3791; ame-mir-71; ame-mir-971; ame-mir-9a	
NM_001011575.1	Nicotinic acetylcholine	ame-mir-277; ame-mir-29b; ame-mir-305;	
	Receptor alpha8 subunit	ame-mir-33; ame-mir-34; ame-mir-3719; ame-mir-3783; ame-mir-3786; ame-mir-8; ame-mir-989	
NM_001040236.1	Alpha glucosidase 2	ame-miR-100; ame-mir-1000; ame-mir-12; ame-miR-281; ame-miR-305 ame-mir-315; ame-miR-33; ame-miR-971; ame-mir-981	Starch and sucrose metabolism; Metabolic pathways; Galactose metabolism

2009). Further, there is strong evidence to suggest a role for miRNA-mediated regulation of gene function in the mature brain, which in turn influences neural plasticity and memory (Ashraf *et al.*, 2006; Hansen *et al.*, 2010; Hollander *et al.*, 2010). In our present study, we identified the target genes of the 40 differentially expressed miRNA, and compared their expression with the corresponding target genes. As shown in Supplementary Table 8, 60 pairs of miRNA/mRNA were identified as co-expressed among the 40 differentially expressed known miRNAs and 388 differentially expressed genes. Of the

60 pairs of related miRNA/mRNA, there were 10 unique genes and 33 unique miRNAs (Table 5).

Some of the 10 were reported to be important genes involved in learning and memory. As described above, the nicotinic acetylcholine receptor alpha8 subunit (nAChRa8, NM_001011575.1), one predicted target gene of several miRNAs (ame-mir-277, ame-mir-29b, ame-mir-305, ame-mir-33, ame-mir-34, ame-mir-3719, ame-mir-3783, ame-mir-3786, ame-mir-8 and ame-mir-989), is associated with learning and memory processes. Chemosensory protein 3 (NM_001011583.1), regulated
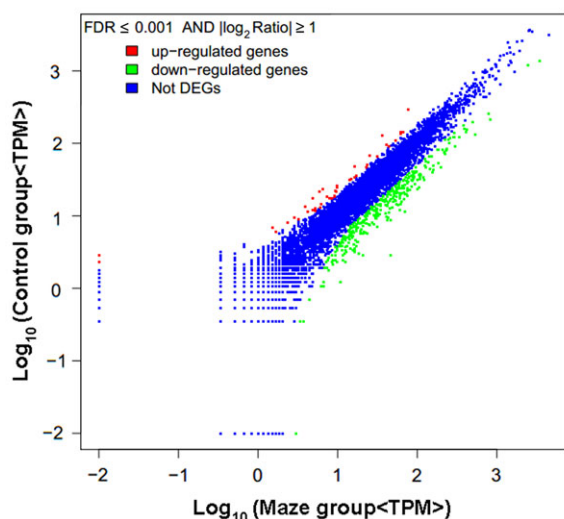
Fig. 7 Comparison of gene expression levels between maze and control groups. Transcripts per million clean tags (TPM): the number of a certain tag per million clean tags, which is a normalized index. With an estimated false-discovery rate (FDR) of ≤ 0.001 and |log$_2$Ratio| ≥ 1 (Ratio: maze/control), the red shows the proportion of up-regulated genes in brains of the maze group; the green represents down-regulated genes, and the blue shows the proportion of genes not differentially expressed.

by ame-mir-1000, ame-mir-3783, ame-mir-929 and ame-mir-92a together, was categorized into two KEGG pathways, namely "Axon guidance" and "MAPK signaling pathway", which play key roles in learning and memory processes (Park *et al.*, 2003). As a predicted target gene of ame-let-7, translocator protein-like (XM_397432.3) was categorized into the "Neuroactive ligand-receptor interaction" pathway. The neuroactive ligand–receptor interaction pathway, which is a collection of receptors located on the plasma membranes, is involved in neuronal plasticity, and in learning and memory (Su *et al.*, 2009). As it is widely distributed around the CNS and peripheral tissue, tachykinin (NM_001011576.1), one target gene of miR-100, mir-3049 and mir-306 can have neurotrophic as well as memory-promoting effects (Huston & Hasenöhrl, 1995; De Wied, 1997).

Moreover, it is clearly shown that miRNAs could down-regulate some of their targets not only at the translational but also at the transcriptional level (Shyu *et al.*, 2008; Creighton *et al.*, 2009). In our study, 40 (100%) differentially expressed miRNAs were up-regulated, while 343 (88.40%) mRNAs were down-regulated in the maze group compared to the control group after learning. In addition, among 60 pairs of co-expressed miRNA/mRNA, all of the miRNAs were up-regulated while all of the mRNAs

were down-regulated in the maze group compared to the control group after learning. This suggests an inverse expression trend between differentially expressed miRNAs and genes in our present study. Ashraf *et al.* (2006) showed that knockdown of Armitage led to an increase in the localized expression of CaMKII and to enhanced long-term olfactory memory. It was found by Cheng *et al.* (2007) and Edbauer *et al.* (2010), that miR-132 over-expression in cortical and hippocampal neurons enhances synaptic plasticity. Edbauer *et al.* (2010) suggested that this effect on plasticity (and potentially memory) may be due to an activity-dependent restructuring of the NMDA receptor by down-regulating the NR2A subunit: miR-132 potentially decreases NR2A translation during learning, which would result in enhanced sensitivity of the NMDA receptor governed by an increase in NR2B subunits (which have been shown to be critical for learning and memory). Our result showing that of 60 pairs of related miRNA/mRNA, all of the miRNAs were up-regulated, while all of their predicted target genes were down-regulated, is consistent with their findings.

As outlined above, we speculate that the miRNAs and genes may be closely related during the process of maze-based visual learning in the honeybee. However, the fact that a single miRNA can target many different mRNAs while some genes have more than one miRNA target site complicates an explanation of how miRNAs contribute to these processes.

Disclosure

The authors have no conflict of interests, including specific financial interests and relationships and affiliations relevant to the subject of the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supplementary Table 1 Description of genes and primer sequences used for qRT-PCR assays.

Supplementary Table 2 Known microRNAs in the maze and control groups.

Supplementary Table 3 Novel microRNAs in the maze and control groups.

Supplementary Table 4 The differentially expressed genes in the maze and control groups. TPM: transcript copies per million tags. Raw intensity: the total number of tags sequenced for each gene. FDR: false discovery rate. We used FDR ≤ 0.001 and the absolute value of $\log_2$ Ratio ≥ 1 as the threshold to judge the significance of gene expression difference. In order to calculate the $\log_2$ Ratio and FDR, we used a TPM value of 0.01 instead of 0 for genes that do not express in one sample.

Supplementary Table 5 Gene Ontology enrichment analysis of the differentially expressed genes. The results were summarized in three main categories: biological process, cellular component and molecular function.

Supplementary Table 6 KEGG pathway enrichment analysis of the differentially expressed genes.

Supplementary Table 7 Comparison of the same gene expression in different samples in the qRT-PCR results with the sequencing results.

Supplementary Table 8 Target genes analysis of the differentially expressed microRNAs. The target genes listed were differentially expressed genes of the DGE.