Mol Biol Rep (2013) 40:1631–1639
DOI 10.1007/s11033-012-2212-9

Gene expression analysis following olfactory learning in *Apis mellifera*

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Received: 4 June 2012 / Accepted: 9 October 2012 / Published online: 17 October 2012

Abstract The honeybee has a strong learning and memory ability, and is recognized as the best model organism for studying the neurobiological basis of learning and memory. In this study, we analyzed the gene expression difference following proboscis extension response-based olfactory learning in the *A. mellifera* using a tag-based digital gene expression (DGE) method. We obtained about 5.71 and 5.65 million clean tags from the trained group and untrained group, respectively. A total of 259 differentially expressed genes were detected between these two samples, with 30 genes up-regulated and 229 genes down-regulated in trained group compared to the untrained group. These results suggest that bees tend to actively suppress some genes instead of activating previously silent genes after olfactory learning. Our DGE data provide comprehensive gene expression information for olfactory learning, which will facilitate our understanding of the molecular mechanism of honey bee learning and memory.

Keywords *Apis mellifera* · Learning and memory · Proboscis extension response · Digital gene expression · Clean tag

Electronic supplementary material The online version of this article (doi:10.1007/s11033-012-2212-9) contains supplementary material, which is available to authorized users.

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Introduction

Honeybee is an important model organism for learning and memory research. They not only have a good learning and memory ability to color [1], pattern [2] and odor [3] of the target, but also to landmarks and time mode [4]. Moreover, the honeybee can also form a concept of the visual object [5] and discriminate landmarks [6], form long-term memory of them and optimize the flight path based on specific conditions [7]. Recent studies show that they can even generate a memory of the order of some of the tasks, and know what to do and when [8].

Honeybee has a strong olfactory learning ability. They learn odor through olfactory receptor cell distributed on its antenna, change these odor stimuli into chemical signal and finally transfer them into the mushroom body [9]. In 1961, Takeda for the first time developed the proboscis extension response (PER) experiment in the honeybee by pairing an odor stimulus with a sucrose reward [10]. This method has been gradually improved as a classical experiment model for olfactory learning [11–13].

Although much research on learning and memory has been conducted in the honeybee, the underlying molecular mechanism is still unclear, especially for the olfactory learning and memory. Up to now, only a few genes are reported to be involved in the learning and memory of honeybee [14, 15]. Using RNAi, Fiala (1999) [14] found that knockdown of PKA activity causes an impairment of long-term memory retention 24 h after training. It suggests that PKA contributes to the induction of a long-term memory 24 h after training when activated during learning. By injecting antagonists, Dacher (2008) [15] found that nicotinic receptors and NO-synthase are specifically involved in long-term memory. Besides these genes, the homologues of several genes reported to be related to

learning and memory in other organisms have been cloned in *A. mellifera*, including adenylyl cyclase [16], CREB (the key factor for learning and memory) [17], AmGluRA (one member of the G-protein-coupled metabotropic glutamate receptors) [18], Amtyr (one member of the tyramine receptor) [19] and a PKA catalytic subunit [20], all these genes are speculated to be involved in learning and memory in the honeybee. Despite of these results, genes and pathways involved in learning and memory in honeybee are far from clear.

DGE is based on the high-throughput second generation sequencing technologies. It is a tag-based transcriptome sequencing approach where short raw tags are generated. In this method, a 21 bp (specific markers of the gene) tag was obtained from the 3′ end of each mRNA molecules using endonuclease; and then a lot of the tag sequences were produced by high-throughput sequencing, the number of different tag sequences represents the expression level of corresponding genes. The expression level of virtually all genes in the sample is measured by counting the number of individual mRNA molecules produced from each gene. DGE protocol is suitable and affordable for comparative gene profiling without compromise and potential bias. This technology has been used in transcriptome profiling studies for various applications, including cellular development, cancer, and immune defence of various organisms [21–23].

In this study, we adopted the DGE approach to identify genes involved in honeybee olfactory learning and memory at the genome wide level.

Materials and methods

Insect

The *A. mellifera* were sampled from Honeybee Research Institute, Jiangxi Agricultural University, China. One colony was setup with headed by an artificially inseminated queen. The queen received semen from a single drone [single drone inseminated (SDI)]. This technique ensured a higher genetic similarity among the workers, which should increase the signal to noise ratio when searching for gene expression differences. Newly emerged bees from SDI queen were used in this experiment. The bees were gathered and breed in a rectangle box with 1 M sucrose. After 1 week they were collected from the box for experiment.

PER experiment

The PER experiment began in the morning of the eighth day. The experiment procedure was designed mainly by consulting that reported by Letzkus [24]. The bees were collected from the above mentioned boxes and randomly assigned into two groups, the trained group and untrained (control) group. The bees were briefly immobilized on ice for 5 min. Then, each bee was fixed in a metal tube with thin strips of GAFFA tape so that the whole body was immobilized but the two prolegs and head were free. Then, the bees were fed with two to three drops of 1 M sugar solution and let to recover in an incubator at a constant temperature of 28 °C.

In the evening of that day, the bees were trained. For the trained group, each bee was trained using lemon odor plus 1 M sucrose solution as the positive stimulus (reward) and strawberry odor plus saturated saline as the negative stimulus (punishment). These two stimuli were prepared by dissolving 10 μl of lemon or strawberry essence in 3 ml 50 % sugar syrup. The bees were trained to discriminate between these two different scents. During training, a suction fan was placed behind the bees to ensure a constant flow of odor during stimulus presentation and to quick remove of any lingering odor traces before the next bee was trained. Each trial consisted of a positive and a negative stimulus training. On the first trial, a droplet (about 5 μl) of positive stimulus was placed over the bee's antennae at about 1–2 cm from the antennae using a syringe needle until the bee extended its proboscis and ate a little drop of solution. If after 5 s the bee did not extend its proboscis, we briefly touched the antennae with the stimulus drop. Then, the same procedure was performed with the negative stimulus, which was a scented (strawberry odor) drop of salt water. Touching the antennae at the end of this 5 s period was intended to cause the scented drop to be associated with the unpleasant-tasting salt solution. Bees extending their proboscis after touching the antennae received a punishment in the form of the salt solution. After training, the honeybees were fed 2–3 drops of 1 M sugar solution and returned to the incubator for overnight storage. Three trials with an interval of 5 min were given.

Retention tests were carried out in the morning and evening of the subsequent 2 days after the training. The order for giving the stimuli during the test was reversed with respect to that given in the training. That is, during the tests, the negative stimulus was offered first, then, positive stimulus. As in training, the stimulus droplet was placed over the antennae of the bee at a distance of 1–2 cm for 5 s, without touching the antennae. Each bee was tested three trials with an interval of 5 min in every morning and evening. A total of 4 retention tests were performed before sampling. In the evening of the third day, after completion of the tests, the heads of the trained bees that have a good performance in all the four retention tests as well as those of untrained bees (control group) were sampled and stored in liquid nitrogen.

Digital gene expression (DGE) library preparation and sequencing

To construct DGE library, the brain was dissect from the head of the trained group and untrained group (control) samples, and total RNA was extracted using the SV Total RNA isolation System (Promega, USA) according to the manufacturer's protocol. Then, the libraries were constructed using the Illumina gene expression sample prep kit according to its protocol. Briefly, poly(A) + RNA was purified from 6 μg of total RNA using oligo(dT) magnetic beads. First strand cDNA were directly synthesized on the poly(A) + RNA-bound beads primed by oligo(dT), then, the second strand were synthesized and digested with *Nla*III, which recognize the CATG site. The digested cDNA fragments containing 3′ ends were purified from the magnetic beads, and then the Illumina adaptor1 was added to the 5′ ends of these cDNA fragments. Then these fragments were further digested by another endonuclease *Mme*I, which recognizes the junction of the Illumina adaptor1 and the CATG site, and cut at 17 bp downstream of CATG site to produce 21 bp tags containing the adaptor1 sequence. After remove the cleaved 3′ end sequences with magnetic beads precipitation, the Illumina adaptor2 was ligated to the 3′ ends of the tags to create a tag library containing a lot of tags with different adaptors on both ends. Then, the library was amplified by PCR for 15 cycles, PCR products were segregated on 6 % PAGE gel electrophoresis, and the 95 bp fragments were chosen and purified for sequencing. The double-strand DNA fragments were denatured and the single-stranded molecules were fixed onto the Illumina sequencing chip for sequencing. Each tunnel of chip (flowcell) generated millions of raw tags with a length of 49 nt.

Analysis and mapping of DGE tags to reference gene and genome data

Sequencing-received raw image data was transformed by base calling into sequence data and stored in fastq format. Raw sequences were filtered by the following steps: 1, remove adaptor sequence (since tags are only 21 nt long while the sequencing reads are 49 nt long, raw sequences are with 3′ adaptor sequences); 2, remove empty tags (no tag sequence between the adaptors); 3, remove low quality tags (tags with unknown nucleotide ''N''); 4, remove tags with only one copy number (which might result from sequencing errors); 5, remove tags which are too long or too short. After filtration, the remained clean tags containing CATG and 21 nt tag sequences. The clean tags were deposited in the NCBI sequence read archive (SRA057123).

Before mapping, two tag libraries containing all the possible CATG + 17 nt tag sequences was respectively created using all the available mRNA sequences and genome sequences of the *A. mellifera* downloaded from Genbank database (ftp://ftp.ncbi.nih.gov/genomes/Apis_mellifera/Assembled_chromosomes/seq/). Then all the clean tags were first mapped to the tag database of reference mRNA sequences with only one nucleotide mismatch is allowed. 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 can't be mapped to any gene in the tag database of mRNA sequences were continuing mapped to the tag database of reference genome sequence.

Identification of differentially expressed genes

To identify the differentially expressed genes between trained and untrained libraries, a rigorous statistical algorithm was developed by consulting the method described by Audic [25], 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 < 0.001 and an absolute E value of the log2 ratio > 1 were used as the threshold to determine significant differences in gene expression. The identified differentially expressed genes were used for GO and KEGG pathway analysis.

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

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

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; 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 comparing with the whole genome background. The calculating formula is the same as that in GO analysis.

Results and discussion

DGE library sequencing

Apis mellifera trained and untrained DGE libraries were constructed and sequenced, generating approximately 5.81 and 5.77 million raw tags in each library. After filtering the low quality tags, the total number of clean tags in each library were about 5.71 and 5.65 million (Table 1), and the percentage of clean tags among the raw tags in each library were 98.19 and 98.00 % (Fig. 1). Among the clean tags, the number of sequences that could be mapped to reference genes were about 2.10 and 2.19 million, and the percentage of these clean tags were 36.77 and 38.67 % in two libraries. The numbers of sequences that could be mapped to genome were about 1.49 and 1.78 million, occupying 26.15 and 31.39 % of the clean tags. In each library, those tags with copy numbers of more than 100 showed percentages of greater than 78 % among the clean tags, but their distribution of distinct clean tags did not exceed 7.5 %. In contrast, those tags with copy numbers between 2 and 5 showed a broad distribution (more than 50 %) of distinct clean tags (Fig. 2).

Saturation analysis of sequencing

Saturation analysis was performed to check whether the number of detected genes increased with the increase of the sequencing amount (total tag number). As showed in Fig. 3, when sequencing amount of the two DGE libraries reaches near 1 M, the number of detected genes almost ceases to increase. It suggested that the sequencing results were saturated since the obtained clean tags in each library were about 5.71 and 5.65 million.

Mapping sequences to the reference genes

To reveal the molecular events behind DGE profiles, we mapped the tag sequences of the two DGE libraries to gene reference database. This reference database contains 11,736 distinct mRNA sequences with 65,418 unambiguous reference tags. Among the 96,328 and 105,909 distinct clean tags generated from the Illumina sequencing of the two libraries, 38237 and 40,365 distinct tags were mapped to one or multiple genes in the reference database (Table 1). Tags mapped to single unique sequence are the most critical subset of the DGE libraries as they can

Table 1 Statistics of DGE sequencing

Summary		Trained	Untrained
Raw data	Total	5812242	5769851
Raw data	distinct tag	197187	217900
Clean tag	Total number	5706950	5654589
Clean tag	Distinct tag number	96328	105909
All tag mapping to gene	Total number	2098401	2186900
All tag mapping to gene	Total % of clean tag	36.77 %	38.67 %
All tag mapping to gene	Distinct tag number	38237	40365
All tag mapping to gene	Distinct tag % of clean tag	39.69 %	38.11 %
Unambiguous tag mapping to gene	Total number	1857673	1935824
Unambiguous tag mapping to gene	Total % of clean tag	32.55 %	34.23 %
Unambiguous tag mapping to gene	Distinct tag number	35493	37471
Unambiguous tag mapping to gene	Distinct tag % of clean tag	36.85 %	35.38 %
All tag-mapped genes	Number	8918	9035
All tag-mapped genes	% of ref genes	75.99 %	76.99 %
Unambiguous Tag-mapped Genes	Number	7887	8001
Unambiguous tag-mapped genes	% of ref genes	67.2 %	68.17 %
Mapping to genome	Total number	1492161	1775102
Mapping to genome	Total % of clean tag	26.15 %	31.39 %
Mapping to genome	Distinct tag number	45216	51153
Mapping to genome	Distinct tag % of clean tag	46.94 %	48.3 %
Unknown tag	Total number	2116388	1692587
Unknown tag	Total % of clean tag	37.08 %	29.93 %
Unknown Tag	Distinct tag number	12875	14391
Unknown tag	Distinct tag % of clean tag	13.37 %	13.59 %

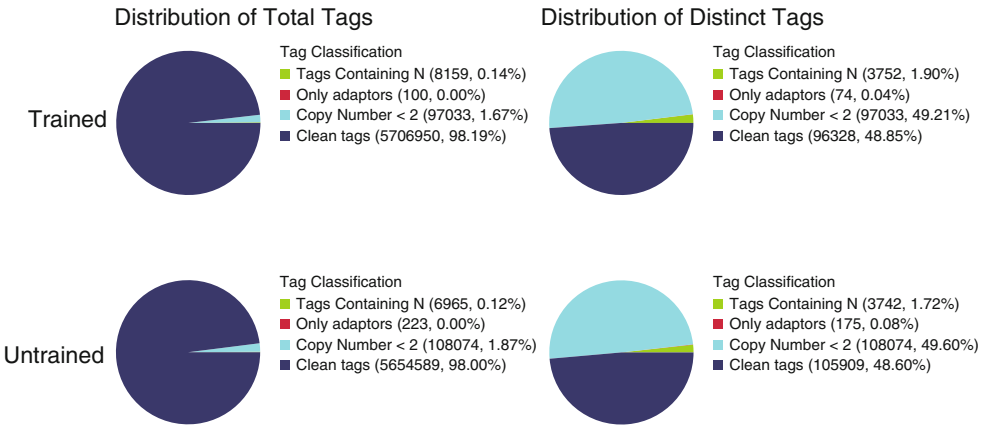

Fig. 1 Distribution of total tags and distinct tags over different tag abundance categories in each sample. The numbers and percentage of tags containing N, empty tags with adaptor only, tags with copy number <2 and clean tags, are shown

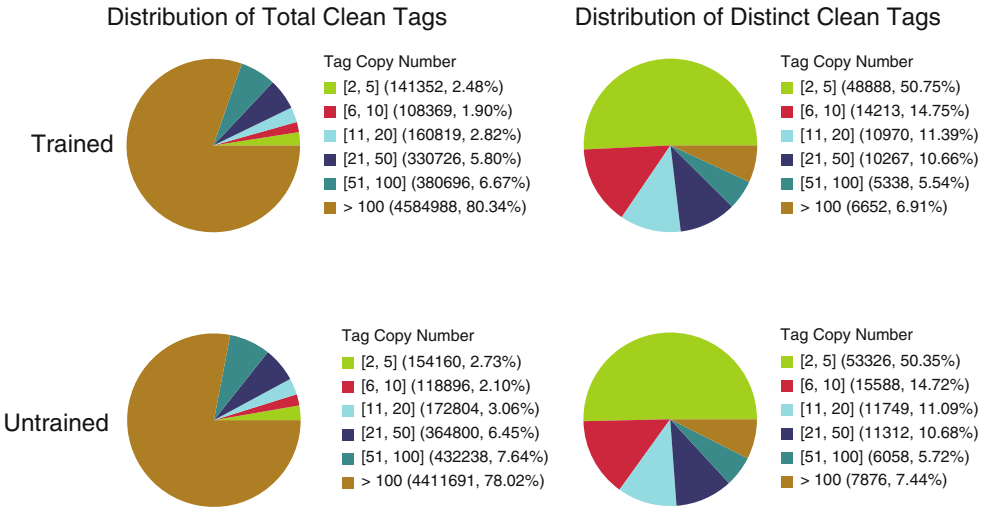

Fig. 2 Distribution of total clean tags and distinct clean tags over different tag abundance categories in each sample. *Numbers* in the *square brackets* indicate the range of copy numbers for a specific category of tags. For example, [2, 5] means all the tags in this category has 2–5 copies. *Numbers*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

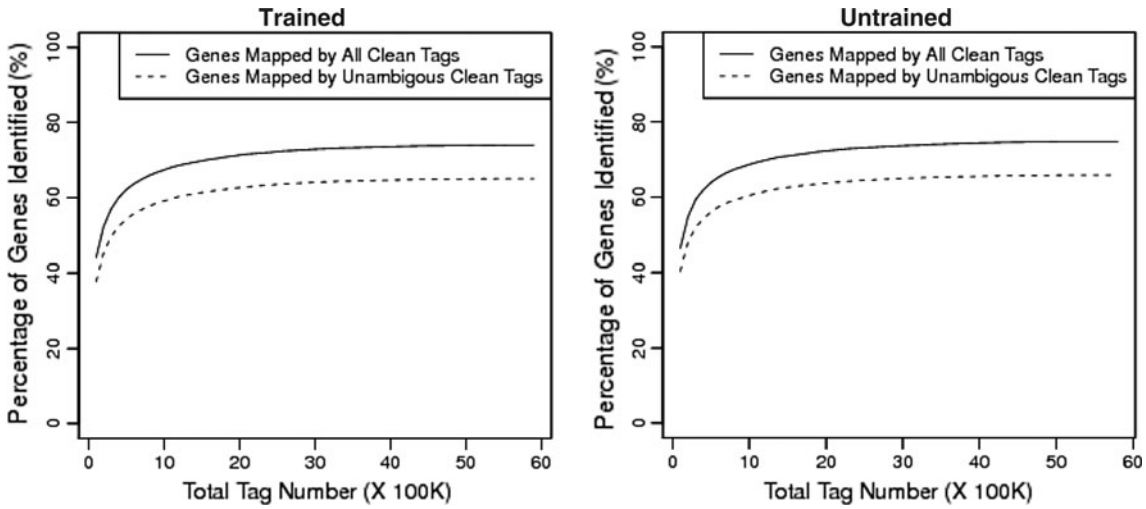

Fig. 3 Saturation analysis of clean tags. With the increase of total sequence number, the number of detected genes gradually ceased to increase

explicitly identify a transcript. In the trained and untrained libraries, 36.85 and 35.38 % of distinct clean tags were mapped to unique sequence, of them, about 60 % mapped to sense strand of the genes, and 40 % mapped to antisense strand of the genes (Fig. 4). There were 13.37 and 13.59 % of the total clean tags corresponding to 37.08 and 29.93 % of the distinct clean tags unable to be mapped to any gene. Up to 67.2 % (7,887)

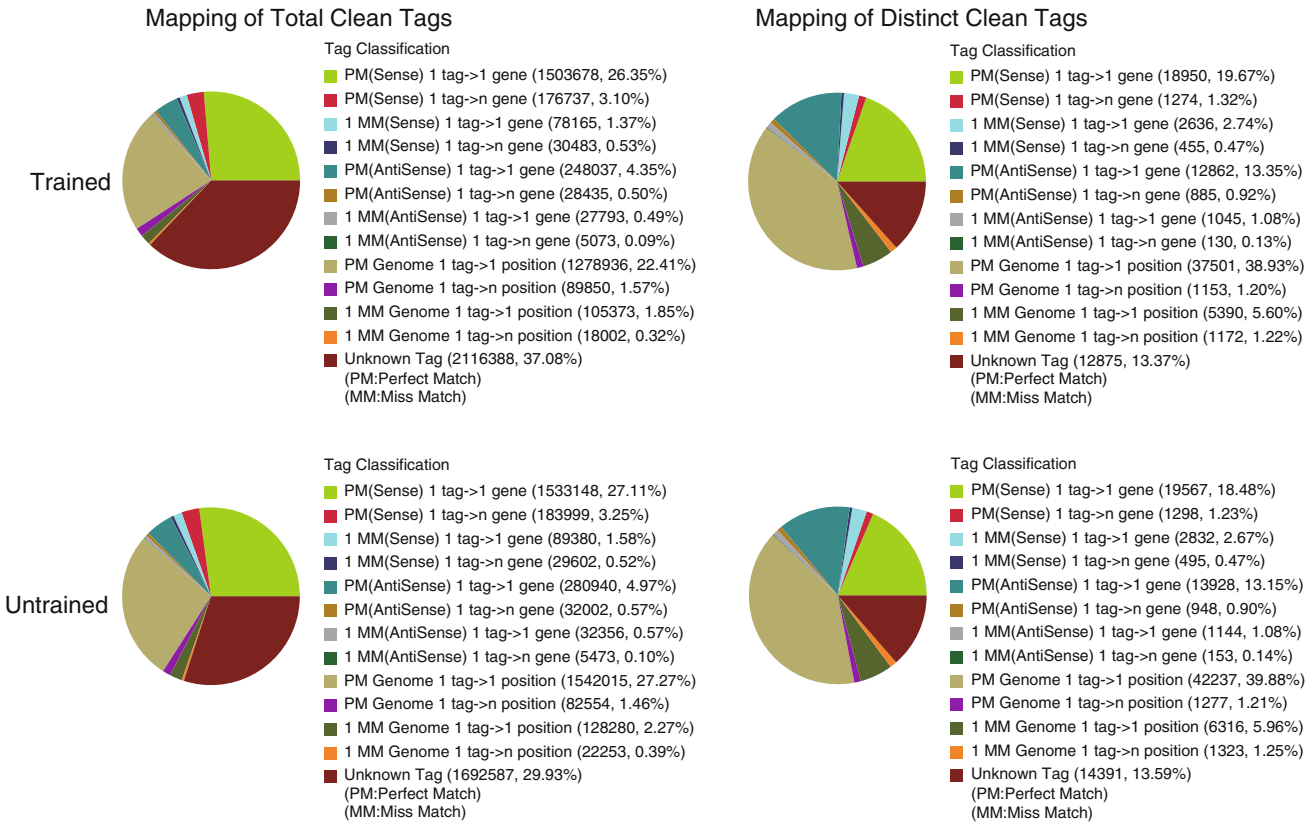

Fig. 4 Distribution of tags on genes and genome of *Apis mellifera*. *PM(Sense)* perfect match to gene (sense), *1 tag → 1 gene* one tag match to one gene, *1 tag → n gene* one tag match to more than one gene, *1 MM(Sense)* match to gene (sense) with 1 bp mismatch, *PM(AntiSense)* perfect match to anti-sense gene, *1 MM(Anti-Sense)* match to anti-sense gene with 1 bp mismatch, *PM Genome* perfect match to genome, *1 MM Genome* match to genome with 1 bp mismatch, *Unknown Tag* not match to gene (sense and anti-sense) or genome

and 68.17 % (8,001) of the genes could be unequivocally identified by unique tag (Table 1).

Differentially expressed genes

To identify genes showing a significant change in expression between the trained and untrained bees, the differentially expressed tags between these two libraries were identified by an algorithm developed by Audic et al. [25]. Between trained and untrained libraries, a total of 259 differentially expressed genes were detected, with 30 up-regulated genes and 229 down-regulated genes in trained group compared to untrained group (Fig. 5, Supplementary file 1). Of them, 89 genes annotated as "hypothetical protein" or "uncharacterized protein", that is, they are function unknown.

To understand the functions of these differentially expressed genes, all the differentially expressed genes were mapped to terms in GO database and compared with the whole genome background. Of the 259 differentially expressed genes, 121 genes have a GO ID and can be categorized into a total of 399 functional groups in three

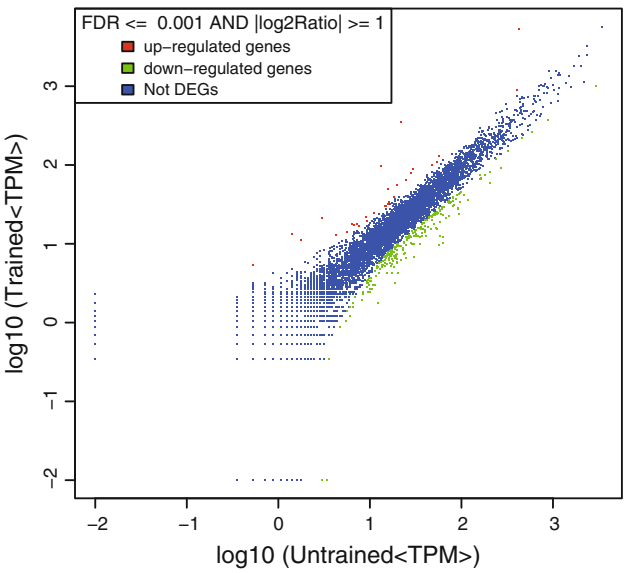

Fig. 5 Differentially expressed genes between trained and untrained groups. The *red* part represents those genes up-regulated in trained group compared to untrained group. The *green* part shows those genes down-regulated in trained group. The *blue* part shows those genes without expression difference between these two samples. (Color figure online)

main categories (Additional file 2). In each of the three main categories (biological process, cellular component and molecular function) of the GO classification, "cellular process", "cell" and "binding" terms are dominant. While compared to the whole genome background, no term was significantly enriched (P value < 0.05).

To further investigate the biochemical pathways these differentially expressed genes were involved in, we mapped all the differentially expressed genes to terms in KEGG database and compared with the whole genome background. Of the 259 differentially expressed genes, 177 genes had a KO ID and could be categorized into 132 pathways (Supplementary file 3). While compared to the whole genome background, no term was significantly enriched (Q value < 0.05).

In these differentially expressed genes, we found two odorant binding proteins (obps), obp3 (NM_001040221.1), obp17 (NM_001011583.1), and a chemosensory protein (CSP3, NM_001011583.1) showing expression difference during olfactory learning. Of them, obp3 and CSP3 were up-regulated after learning, while Obp17 was down regulated. obps are water-soluble small proteins in the lymph of the olfactory neurons with a high concentration [26]. When the odor molecules entered into the receptors from the micropores distributed on its surface, the obps combined with hydrophobic odorant molecules, and transported them to the sensory receptors distributed on the dendritic membrane of the olfactory nerve. The function of chemosensory proteins are feeling the environment of chemical stimulation and carrying non-volatile odor molecules to reach the corresponding receptor in chemoreceptors [27]. By now, there are no reports about the involvement of these genes in learning and memory storage. The up-regulation of these genes may be due to direct stimulation of the odor molecules rather than long-term memory for odors.

We found several neurotransmitter receptors showing expression difference. They are octopamine receptor (OA1, NM_001011565.1), muscarinic acetylcholine receptor (XM_395760.4), nicotinic acetylcholine receptor alpha6 subunit (nAChRa6, NM_001080095.1), gamma-aminobutyric acid receptor subunit beta ($GABA_A$ beta, XM_001120292.2). Octopamine is an important neurotransmitter in the central nervous system. In *A. mellifera*, one octopamine receptor was reported to be involved in appetitive learning [28]. Acetylcholine (acetylcholine, ACh) is another kind of important neurotransmitter. According to pharmacological response property difference to the natural alkaloids, muscarinic and nicotinic, the acetylcholine receptor can be divided into muscarinic receptors and nicotinic receptors [29]. These two types of receptors are widely distributed in the central and peripheral nervous system. The nAChR has been considered to be involved in cognition for a long time [30, 31]. The mAChR is a chemical (ligand)-gated ion channel protein, its activation can lead to cation flow, causing membrane depolarization. In recent years, a growing number of studies have shown that mAChR also plays an important role in learning and memory [32, 33]. GABA is the most important and most abundant inhibitory neurotransmitter in the mammalian central neural system [34]. One important role of GABA receptors is to modulate different forms of anxiety, fears, phobias or depression. Besides, they also play a key role in cognitive processes, including memory formation and consolidation [35].

We found that two synaptic proteins, synaptojanin (XM_395173.4), syntaxin binding protein 5 (XM_391820.4) and a neuropeptide, TWKSPDIVIRFa-containing neuropeptide (XM_001120453.2), are differentially expressed during learning. Synaptojanin is a polyphosphoinositide phosphatase that mediates phosphatidylinositol (4,5)-bisphosphate [PI(4,5)P2] dephosphorlation [36, 37]. Recent research showed that it regulates the postsynaptic AMPA responses by triggering internalization of AMPA receptors [38]. AMPA receptors are responsible for most of the fast excitatory synaptic transmission, alterations in AMPA receptor number and/or function at the synapse are likely to play an important role in synaptic plasticity and in learning and memory [39]. In mouse models of Down's syndrome, over expression of synaptojanin perturb PtdIns(4,5)P2 homeostasis at the synapse and result in brain dysfunction and cognitive disabilities [40]. Syntaxin binding protein 5 (tomosyn) is an important component in the neurotransmitter release process where it stimulates the formation of soluble N-ethylmaleimide-sensitive factor attachment receptor complex in nerve terminals [41]. In drosophila, Syntaxin binding protein 5 is required for cAMP-dependent associative odor learning [42]. Neuropeptides are by far the largest group of messenger molecules in the brain [43]. It has increasingly been recognized as regulators of 'cognitive' pathways in the brain [44, 45]. In mammalian, a number of neuropeptides have been implicated in learning and memory processing via a direct or indirect modulation of excitatory/inhibitory systems in the hippocampal formation [46].

In conclusion, through DGE analysis, we obtained a total of 259 differentially expressed genes after olfactory learning, some of them were reported to be important genes involved in the olfactory learning and memory, such as octopamine receptor. These genes provide important clues for future olfactory learning and memory study in honeybee. As far as we know, this is the first research about the learning and memory related genes in honeybee at the genome wide level, but it still requires much work to fully understand the molecular mechanism of learning and memory in honeybee.

Acknowledgments We thank Prof. Shao-Wu Zhang for invaluable guidance and assistance in PER experiment and thank Dr. Zachary Huang for reviewing this manuscript. This work was supported by the Earmarked Fund for China Agriculture Research System (No.CARS-45-KXJ12) and the National Natural Science Foundation of China (No. 31060327).

References

1. von Frisch K (1914) Der Farbensinn und Formensinn der Biene. Fischer, Jena
2. Horridge GA (1996) The honeybee (*Apis mellifera*) detects bilateral symmetry and discriminates its axis. J Insect Physiol 42(8):755–764. doi:10.1016/0022-1910(96)00026-1
3. Menzel R, Hammer M, Müller U, Rosenboom H (1996) Behavioral, neural and cellular components underlying olfactory learning in the honeybee. J Physiol Paris 90(5–6):395–398. doi:10.1016/S0928-4257(97)87928-4
4. Srinivasan MV, Zhang SW, Zhu H (1998) Honeybees link sights to smells. Nature 396:637–638. doi:10.1038/25272
5. Collett TS, Cartwright BA (1983) Eidetic images in insects: their role in navigation. Trends Neurosci 6:101–105. doi:10.1016/0166-2236(83)90048-6
6. Giurfa M, Zhang SW, Jenett A et al (2001) The concepts of "sameness" and "difference" in an insect. Nature 410:930–933. doi:10.1038/35073582
7. Zhang SW, Bartsch K, Srinivasan MV (1996) Maze learning by honeybee. Neurobiol Learn Mem 66(3):267–282. doi:10.1006/nlme.1996.0069
8. Zhang SW, Sebastian S, Pahl M et al (2006) Honeybee memory: a honeybee knows what to do and when. J Exp Biol 209(Pt 22):4420–4428. doi:10.1242/jeb.02522
9. Laska M, Galizia C, Giurfa M et al (1999) Olfactory discrimination ability and odor structure–activity relationships in honeybees. Chem Senses 24(4):429–438. doi:10.1093/chemse/24.4.429
10. Takeda K (1961) Classical conditioned response in the honey bee. J Inseet Physiol 6(3):168–179. doi:10.1016/0022-1910(61)90060-9
11. Smith BH, Abramson CI, Nadel LI (1992) Insect learning: case studies in comparative psychology. In: Squire LR (ed) Encyclopedia of learning and memory. Macmillan, New York, pp 276–283
12. Smith BH, Cobey S (1994) The olfactory memory of the honey bee, *Apis mellifera*. II. Blocking between odorants in binary mixtures. J Exp Biol 195:91–108
13. Gerber B, Ullrieh J (1999) No evidence for olfactory blocking in honey bee classical conditioning. J Exp Biol 202(Pt 13):1839–1854
14. Fiala A, Müller U, Menzel R (1999) Reversible downregulation of protein kinase A during olfactory learning using antisense technique impairs long-term memory formation in the honeybee *Apis mellifera*. J Neurosci 19(22):10125–10134
15. Dacher M, Gauthier M (2008) Involvement of NO-synthase and nicotinic receptors in learning in the honey bee. Physiol Behav 95(1–2):200–207. doi:10.1016/j.physbeh.2008.05.019
16. Wachten S, Schlenstedt J, Gauss R et al (2006) Molecular identification and functional characterization of an adenylyl cyclase from the honeybee. J Neurochem 96(6):1580–1590. doi:10.1111/j.1471-4159.2006.03666.x
17. Eisenhardt D, Friedrich A, Stollhoff N et al (2003) The AmCREB gene is an ortholog of the mammalian CREB/CREM family of transcription factors and encodes several splice variants in the honeybee brain. Insect Mol Biol 12(4):373–382. doi:10.1046/j.1365-2583.2003.00421.x
18. Kucharski R, Mitri C, Grau Y et al (2007) Characterization of a metabotropic glutamate receptor in the honeybee (*Apis mellifera*): implications for memory formation. Invert Neurosci 7(2):99–108. doi:10.1007/s10158-007-0045-3
19. Blenau W, Balfanz S, Baumann A (2000) Amtyr1: characterization of a gene from honeybee (*Apis mellifera*) brain encoding a functional tyramine receptor. J Neurochem 74(3):900–908. doi:10.1046/j.1471-4159.2000.0740900.x
20. Eisenhardt D, Fiala A, Braun P et al (2001) Cloning of a catalytic subunit of cAMP-dependent protein kinase from the honeybee (Apis mellifera) and its localization in the brain. Insect Mol Biol 10(2):173–181. doi:10.1046/j.1365-2583.2001.00252.x
21. Markovets AA, Herman D (2011) Analysis of cancer metabolism with high-throughput technologies. BMC Bioinformatics 12(Suppl 10):S8. doi:10.1186/1471-2105-12-S10-S8
22. Trapnell C, Williams BA, Pertea G et al (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol 28(5):511–515. doi:10.1038/nbt.1621
23. Mu Y, Ding F, Cui P et al (2010) Transcriptome and expression profiling analysis revealed changes of multiple signaling pathways involved in immunity in the large yellow croaker during Aeromonas hydrophila infection. BMC Genomics 11:506. doi:10.1186/1471-2164-11-506
24. Letzkus P, Ribi WA, Wood JT et al (2006) Lateralization of olfaction in the honeybee *Apis mellifera*. Curr Biol 16(14):1471–1476. doi:10.1016/j.cub.2006.05.060
25. Audic S, Claverie JM (1997) The significance of digital gene expression profiles. Genome Res 7(10):986–995. doi:10.1101/gr.7.10.986
26. Pelosi P, Maida R (1995) Odorant-binding proteins in insects. Comp Biochem Physiol B 111(3):503–514. doi:10.1016/0305-0491(95)00019-5
27. Vogt RG (2005) Molecular basis of pheromone detection in insects. In: Gilbert LI, Iatro K, Gill S (eds) Comprehensive insect physiology, biochemistry, pharmacology and molecular biology, endocrinology, vol 3. Elsevier, London, pp 753–804
28. Farooqui T, Robinson K, Vaessin H et al (2003) Modulation of early olfactory processing by an octopaminergic reinforcement pathway in the honeybee. J Neurosci 23(12):5370–5380
29. Purves RD (1976) Function of muscarinic and nicotinic acetylcholine receptors. Nature 261(5556):149–151. doi:10.1038/261149a0
30. Gauthier M (2010) State of the art on insect nicotinic acetylcholine receptor function in learning and memory. Adv Exp Med Biol 683:97–115. doi:10.1007/978-1-4419-6445-8_9
31. Hasselmo ME (2006) The role of acetylcholine in learning and memory. Curr Opin Neurobiol 16(6):710–715. doi:10.1016/j.conb.2006.09.002
32. Poulin B, Butcher A, McWilliams P et al (2010) The M3-muscarinic receptor regulates learning and memory in a receptor phosphorylation/arrestin-dependent manner. Proc Natl Acad Sci USA 107(20):9440–9445. doi:10.1073/pnas.0914801107
33. Rezayof A, Khajehpour L, Zarrindast MR (2009) The amygdala modulates morphine-induced state-dependent memory retrieval via muscarinic acetylcholine receptors. Neuroscience 160(2):255–263. doi:10.1016/j.neuroscience.2009.02.069
34. Sivilotti L, Nistri A (1991) GABA receptor mechanisms in the central nervous system. Prog Neurobiol 36(1):35–92. doi:10.1016/0301-0082(91)90036-Z
35. Kalueff A, Nutt DJ (1996/1997) Role of GABA in memory and anxiety. Depress Anxiety 4(3):100–110. doi:10.1002/(SICI)1520-6394(1996)
36. Cremona O, Di Paolo G, Wenk MR et al (1999) Essential role of phosphoinositide metabolism in synaptic vesicle recycling. Cell 99(2):179–188. doi:10.1016/S0092-8674(00)81649-9
37. McPherson PS, Garcia EP, Slepnev VI et al (1996) A presynaptic inositol-5-phosphatase. Nature 379(6563):353–357. doi:10.1038/379353a0

38. Gong LW, De Camilli P (2008) Regulation of postsynaptic AMPA responses by synaptojanin 1. Proc Natl Acad Sci USA 105(45):17561–17566. doi:10.1073/pnas.0809221105
39. Song I, Huganir RL (2002) Regulation of AMPA receptors during synaptic plasticity. Trends Neurosci 25(11):578–588. doi:10.1016/S0166-2236(02)02270-1
40. Voronov SV, Frere SG, Giovedi S et al (2008) Synaptojanin 1-linked phosphoinositide dyshomeostasis and cognitive deficits in mouse models of Down's syndrome. Proc Natl Acad Sci USA 105(27):9415–9420. doi:10.1073/pnas.0803756105
41. Fujita Y, Shirataki H, Sakisaka T et al (1998) Tomosyn: a syntaxin-1-binding protein that forms a novel complex in the neurotransmitter release process. Neuron 20(5):905–915. doi:10.1016/S0896-6273(00)80472-9
42. Chen K, Richlitzki A, Featherstone DE et al (2011) Tomosyn-dependent regulation of synaptic transmission is required for a late phase of associative odor memory. Proc Natl Acad Sci USA 108(45):18482–18487. doi:10.1073/pnas.1110184108
43. De Wied D, Burbach JPH (1999) Neuropeptides and behavior. In: Adelman G, Smith BH (eds) Encyclopedia of neuroscience, vol 2. Elsevier, Amsterdam, pp 1431–1436
44. Ogren SO, Kuteeva E, Elvander-Tottie E et al (2010) Neuropeptides in learning and memory processes with focus on galanin. Eur J Pharmacol 626(1):9–17. doi:10.1016/j.ejphar.2009.09.070
45. Feany MB (1996) Neuropeptide modulation of learning and memory processes. Rev Neurosci 7(2):151–164. doi:10.1515/REVNEURO.1996.7.2.151
46. Shen Y, Li R (1995) The role of neuropeptides in learning and memory: possible mechanisms. Med Hypotheses 45(6):529–538. doi:10.1016/0306-9877(95)90235-X