Molecular Characterization and Differential Expression of Olfactory Genes in the Antennae of the Black Cutworm Moth *Agrotis ipsilon*

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**Abstract**

Insects use their sensitive and selective olfactory system to detect outside chemical odorants, such as female sex pheromones and host plant volatiles. Several groups of olfactory proteins participate in the odorant detection process, including odorant binding proteins (OBPs), chemosensory proteins (CSPs), odorant receptors (ORs), ionotropic receptors (IRs) and sensory neuron membrane proteins (SNMPs). The identification and functional characterization of these olfactory proteins will enhance our knowledge of the molecular basis of insect chemoreception. In this study, we report the identification and differential expression profiles of these olfactory genes in the black cutworm moth *Agrotis ipsilon*. In total, 33 OBPs, 12 CSPs, 42 ORs, 24 IRs, 2 SNMPs and 1 gustatory receptor (GR) were annotated from the *A. ipsilon* antennal transcriptomes, and further RT-PCR and RT-qPCR revealed that 22 OBPs, 3 CSPs, 35 ORs, 14 IRs and the 2 SNMPs are uniquely or primarily expressed in the male and female antennae. Furthermore, one OBP (AipsOBP6) and one CSP (AipsCSP2) were exclusively expressed in the female sex pheromone gland. These antennae-enriched OBPs, CSPs, ORs, IRs and SNMPs were suggested to be responsible for pheromone and general odorant detection and thus could be meaningful target genes for us to study their biological functions in vivo and in vitro.

**Citation:** Gu S-H, Sun L, Yang R-N, Wu K-M, Guo Y-Y, et al. (2014) Molecular Characterization and Differential Expression of Olfactory Genes in the Antennae of the Black Cutworm Moth *Agrotis ipsilon*. PLOS ONE 9(8): e103420. doi:10.1371/journal.pone.0103420

**Editor:** Richard David Newcomb, Plant and Food Research, New Zealand

**Received** March 20, 2014; **Accepted** June 28, 2014; **Published** August 1, 2014

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**Data Availability:** The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

**Funding:** This work was supported by the China National 973 Basic Research Program (Grant No. 2012CB114104) the National Natural Science Foundation of China (Grant No. 31271694, 31171858 and 31272048) and the Foundation for Innovative Research Groups of the National Natural Science Foundation of China (Grant No. 31321004). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors declare that they have no competing interests.

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**Introduction**

Insects use their sensitive and selective antennae, which express various olfactory proteins, to detect air borne odorant molecules, such as sex pheromones and plant volatiles. Species-specific pheromone molecules and general plant volatiles enter the sensillum lymph of the different types of antennae sensilla via the multipores of the insect cuticle [1,2]. During the last 30 years, our knowledge of the molecular and cellular basis of insect chemoreception has greatly expanded. It is commonly accepted that several different groups of antennae-enriched olfactory proteins participate in the first stage of the detection of olfactory signals, including odorant binding proteins (OBPs), chemosensory proteins (CSPs), odorant receptors (ORs), ionotropic receptors (IRs) and sensory neuron membrane proteins (SNMPs) [3].

Insect OBPs are small water-soluble olfactory proteins that are presumed to be synthesized by non-neuronal auxiliary cells (trichogen and tormogen cells) of the sensory neurons and secreted into the sensillum lymph in high concentrations (up to 10 mM) [4–7]. The insect OBPs are commonly believed act as carrier proteins to transport odorants to the olfactory receptors. Functional studies of insect OBPs at both molecular and behavior levels have proven that insect OBPs are indispensable in insect chemoreception. For example, *Drosophila* OBP LUSH is required for the activation of pheromone-sensitive chemosensory neurons by the pheromone 11- cis vaccenyl acetate (cVA) [8,9]. Additionally, in the fire ant *Solenopsis invicta*, the pheromone binding protein gene *Gp-9* regulates the colony social organization between the monogyne social form (with a single queen) and the polygyne form (with multiple queens) [10].

Insect CSPs, which were also called OS-D like proteins [11] or sensory appendage proteins (SAPs) [12], represent one novel group of olfactory proteins that are involved in insect olfaction. These proteins have shown broad expression profiles in chemosensory tissues, including antennae [13–17], maxillary palps [18], labial palps [19,18] and proboscis [20]. However, these proteins are also found in non-chemosensory organs, such as legs [21,22], wings [23,24] and pheromone glands [15]. Functional studies of insect CSPs revealed that these proteins have multiple-functions in insect chemoreception, growth and development. For example, in the tsetse fly *Glossina morsitans morsitans*, the female antennae-
enriched CSP transcripts were showed remarkable expression levels after a blood meal, which suggested that these proteins participate in the female host-seeking behavior [14]. In the American cockroach Periplaneta americana, one CSP homologous gene named P10 was expressed 30 times higher in regenerating legs than in normal legs, which indicated that the P10 gene had a putative function in the regeneration of insect legs [21,22]. In the migratory locust Locusta migratoria, the antennae-expressed CSP gene has been proposed to regulate the rapid switch between attraction and repulsion behaviors [25].

The insect odorant receptors (ORs) are odorant-gated ion channels which compose of one odorant-binding subunit and the olfactory coreceptor Orco [26,27]. The functional study of insect ORs, particularly the pheromone receptors (PRs), revealed their essential role in insect olfaction [28,29]. The classical method to identify and annotate insect OR genes is through bioinformatic screenings of genomic sequences. At present, using this method, insect OR genes have been identified and annotated from various insect species, including Drosophila melanogaster [30–32], Anopheles gambiae [33], Aedes aegypti [34],Apis mellifera [35], Nasiona vitripennis [36], Bombyx mori [37], Tribolium castaneum [38], and Acrystosiphon pison [39].

Recently, a novel chemosensory receptor family called ionotropic receptors (IRs) was discovered in D. melanogaster [40]. In total, 66 IRs, which included two putative conserved coreceptors, IR25a and IR8a, were identified by screening D. melanogaster genomic data [41]. The expression analysis revealed that 15 DmelIR genes were specially expressed in the antennae [40]. The genomic data [41]. The expression analysis revealed that 15 DmelIR genes were specially expressed in the antennae [40]. The expression analysis revealed that 15 DmelIR genes were specially expressed in the antennae [40]. The expression analysis revealed that 15 DmelIR genes were specially expressed in the antennae [40]. The expression analysis revealed that 15 DmelIR genes were specially expressed in the antennae [40]. The expression analysis revealed that 15 DmelIR genes were specially expressed in the antennae [40]. The expression analysis revealed that 15 DmelIR genes were specially expressed in the antennae [40]. The expression analysis revealed that 15 DmelIR genes were specially expressed in the antennae [40]. The expression analysis revealed that 15 DmelIR genes were specially expressed in the antennae [40]. The expression analysis revealed that 15 DmelIR genes were specially expressed in the antennae [40]. The expression analysis revealed that 15 DmelIR genes were specially expressed in the antennae [40]. The expression analysis revealed that 15 DmelIR genes were specially expressed in the antennae [40]. The expression analysis revealed that 15 DmelIR genes were specially expressed in the antennae [40]. The expression analysis revealed that 15 DmelIR genes were specially expressed in the antennae [40].

Previously, functional studies of insect olfactory genes primarily focused on model species, such as D. melanogaster and B. mori, whose genomic data are available. However, the functional studies of olfactory genes of other insect species have been restricted due to the deficiency of the genomic data for these species. Recently, the high-throughput sequencing of antennae and other tissues have proved to be an efficient strategy for identifying and annotating different types of olfactory genes in various insect species, including A. gambiae [43], M. sexta [44], C. pomonella [45], H. armigera [46], Cotesia vestalis [47], Agrilus planipennis [48], Aphis gossypii [49], S. littoralis [50], I. typographus [51], and Dendroctonus ponderosae [51].

In the present study, using a next-generation sequencing (NGS) 454 GS FLX platform, we have identified and annotated several families of chemosensory genes (including OBs, CSPs, ORs, IRs and SNMPs) from the antennae of the black cutworm moth Agrotis ipsilon (Hufnagel) (Lepidoptera: Noctuidae), which is known as a destructive pest of many crops [52–53]. Using semi-quantitative RT-PCR and real-time quantitative-PCR (RT-qPCR), we have screened a number of antennae-specific or enriched olfactory genes from the A. ipsilon antennal transcriptomes, which may play important functions in the chemosensation of A. ipsilon.

Results and Discussion

454 sequencing and de novo assembly

Two non-normalized cDNA libraries of the male and female A. ipsilon antennae were constructed. After a single sequencing run using the 454 GS FLX platform, a total of 551388 (mean length 539 bp) and 537572 raw reads (mean length 548 bp) were produced from the male and female antennae samples, respectively. After trimming adaptor sequences, contaminating sequences and low quality sequences, 550436 (mean length 531 bp) and 536474 clean reads (mean length 540 bp) from male and female antennae, respectively, remained for the following assembly.

All clean reads from male and female antennae were assembled and produced 40126 (mean length 1072 bp) and 41358 (mean length 1054 bp) unigenes, respectively. Furthermore, we assembled all clean reads from male and female antennae together and finally generated 40795 unigenes. Among these unigenes, 41173 are contigs (84.4%) and 7622 are singletones (15.6%). The assembled unigene lengths ranged from 100 bp to 15432 bp, with an average length of 967 bp. The size distribution of the assembled unigenes is shown in Figure 1. An overview of the sequencing and assembly process is presented in Table 1.

Homology searching of A. ipsilon antennal unigenes with other insect species

We search for homologs in other insect species using the BLASTx and BLASTn programs with the e-value cutoff of 1e-5 [54]. The results indicated that 23180 of the 48795 unigenes (51.6%) had BLASTx hits in the non-redundant protein (nr) databases and that 17947 unigenes (36.8%) had BLASTn hits in the non-redundant nucleotide sequence (nt) databases. Some unigenes are homologous to more than one species. Most annotated A. ipsilon antennal unigenes have the best hits with Lepidoptera insect genes (8542 of the 17947 nt-hit unigenes); the highest hits included 2818 unigenes that were homologous to B. mori genes, 1820 unigenes that were homologous to H. armigera genes. The second highest hits are with Dipteran species genes, with 276 hits of D. melanogaster genes, and 392 and 383 hits that were homologous to genes of the mosquitoes A. gambiae and A. aegypti, respectively. The other unigenes were found to be homologous to genes from the wasp N. vitripennis (348 hits), the beetle T. castaneum (244 hits) and from the western honey bee A. mellifera (261 hits) (Figure 2).

Functional annotation of the A. ipsilon antennal unigenes

Similar to those genes that were found in the antennal transcriptomes of M. sexta [44], S. littoralis [55] and H. armigera [46], most A. ipsilon antennal unigenes (approximately 72%) could not be assigned to a Gene Ontology (GO) category. In total, 11987 male antennal unigenes and 12240 female antennal unigenes were annotated into different functional groups (biological process, cellular components and molecular functions) according to GO analysis [56] (Figure 3). Some transcripts were annotated into more than one GO category. The numbers of each GO category were similar between the male and female antennal transcriptomes (Figure 3). The cellular process (6301 male antennal unigenes and 6425 female antennal unigenes) and metabolic process (5243 male antennal unigenes and 5349 female antennal unigenes) GO categories were most abundantely represented within the biological process GO ontology. In the cellular components GO ontology, the transcripts were primarily distributed in the cell (7148 male antennal unigenes and 7308 female antennal unigenes) and in cell part (6619 male antennal unigenes and 6752 female antennal unigenes). The GO analysis also showed that the binding (4705 male antennal unigenes and 4787 female antennal unigenes) and catalytic activity (5133 male antennal unigenes and 5210 female antennal unigenes) were most abundant in the molecular function ontology (Figure 3).
Chemosensory genes are highly abundant in the *A. ipsilon* antennae

Because a non-normalized cDNA library was used for 454 sequencing in this study, the number of reads of per unigene can represent the relative mRNA abundance in the *A. ipsilon* antennal transcriptomes. Among the top 500 most highly abundant transcripts, 89 transcripts are annotated as olfactory genes, which suggests their involvement in insect chemosensory reception, including olfactory receptors, odorant-binding proteins, chemosensory proteins, antennal cytochrome P450s, antennal-enriched UDP-glycosyltransferases, antennal oxidoreductases, antennal aldehyde oxidases, sensory neuron membrane proteins and takeout-like proteins (Table S1).

Candidate odorant binding proteins in the *A. ipsilon* antennae

OBPs are believed to be involved in the initial biochemical recognition steps in insect odorant perception by capturing and transporting odorant molecules to the olfactory receptors (ORs) [57–59]. In the *A. ipsilon* antennal transcriptomes, a total of 33 OBP genes were annotated (Table 2) based on the tBLASTn results. The number of *A. ipsilon* OBP identified in present study is a little fewer than the number identified from the genome of *B. mori* (44) [60], *A. gambiae* (57) [61] and *D. melanogaster* (51) [62], so there may still some OBP genes are not identified from the *A. ipsilon* antennae due to their low expression level. Among the identified 33 OBP genes, 28 have intact ORFs with lengths ranging from 402 bp to 759 bp. The RPKM value analysis

**Table 1.** An overview of the sequencing and assembly process.

|                | Male   | Female  | Total   |
|----------------|--------|---------|---------|
| Raw reads      | 551388 | 537572  | 1088960 |
| Clean read     | 550456 | 536474  | 1086930 |
| Clean read mean length | 531 bp | 540 bp  | 535.5 bp |
| Singletons     | 3583   | 4039    | 7622    |
| Contigs        | 36543  | 36543   | 41173   |
| Unigenes       | 4022   | 41358   | 48795   |
| Unigene mean length | 1072 bp | 1054 bp | 967 bp  |

Figure 1. The size distribution of the clean reads and assembled unigenes from *A. ipsilon* male and female antennal transcriptomes. doi:10.1371/journal.pone.0103420.g001
revealed that 9 OBP genes (PBP1, PBP2, PBP3, GOBP1, GOBP2, OBP4, OBP11, OBP18 and OBP24) are highly abundant in the male and female antennal transcriptomes (RPKM > 1000) (Table 2). The RT-PCR results indicated that 22 OBP genes (PBP1, PBP2, PBP3, GOBP1, GOBP2, OBP1, OBP2, OBP4, OBP5, OBP9, OBP11, OBP12, OBP13, OBP15, OBP16, OBP17, OBP19, OBP20, OBP21, OBP22, OBP24 and OBP26) are uniquely or primarily expressed in the male and female antennae (Figure 4). Based on the different expression profiles of these OBPs in male and female antennae, we suggest these male antennae-enriched expressed OBPs are involved in sex pheromone detection, whereas female antennae-enriched expressed OBPs play important roles in locating suitable host plants and oviposition sites.

Furthermore, real-time quantitative PCR (RT-qPCR) analysis was performed to compare the accurate quantitative expression
levels of these OBP genes among different tissues between sexes, and the results suggested that the three PBP genes (PBP1, PBP2 and PBP3) are expressed higher in the male antennae than in the female antennae (p<0.01) (Figure 5). However, the RT-qPCR results lack concordance with the RPKM values, this reason may be the sequencing depth of 454 is not good enough. PBP1 and PBP2 showed high binding affinities with the two main sex pheromones of A. ipsilon, whereas PBP3 specifically binds to the minor amount sex pheromone Z11-16: Ac with a high binding ability [63]. In contrast, the expression levels of GOBP1, GOBP2 and OBP17 were much higher in the female antennae than in the male antennae (Figure 5). Interestingly, one OBP (OBP6) was primarily expressed in the pheromone gland (PG) (Figure 4 and Figure 5); this result was also reported in another study [64]. Unlike the common antennae-enriched OBPs, this PG-expressed OBP may play a different role in odorant and pheromone detection and transportation.

Candidate chemosensory proteins in the A. ipsilon antennae

Chemosensory proteins (CSPs) represent a new class of soluble carrier proteins in the lymph of insect antennal chemosensilla and they are proposed to play similar functions as OBPs in insect chemoreception [65]. In this study, we have identified 12 novel CSP genes in the A. ipsilon antennae (Table 3). Based on the extensive expression profiles of CSPs, the remaining CSPs which expressed in other tissues such as legs and wings may not be identified in present study. In total, 11 of the novel genes had intact ORFs, and the protein sequences had the typical four conserved cysteines, which are recognized as the signature feature of insect CSPs [65]. The RPKM value analysis revealed that 4 CSP genes (CSP4, CSP7, CSP9 and CSP10) are highly abundant in the male and female antennal transcriptomes (RPKM>1000) (Table 3). The RT-PCR and RT-qPCR results indicated that 3 CSP genes (CSP8, CSP9 and CSP10) are highly expressed in the male and female antennae (Figure 4 and Figure 6). This result suggested that these three antennae-enriched CSPs might play essential roles in the chemical communication process in insects. Interestingly, one CSP gene (CSP2) was not expressed in the antennae but was specifically expressed in the female pheromone gland (PG) (Figure 4 and Figure 6). CSPs that are expressed in the pheromone gland of the cabbage armyworm M. brassicae can bind sex pheromone analogs, which suggests that these CSPs may play a role in pheromone capture [15]. In Heliothis virescens and B. mori, CSPs are all detected in the pheromone gland [66–68]. This observation suggests the possible involvement of these proteins in carrying and releasing sex pheromones, as demonstrated for the antennal OBPs and CSPs. The insect may use these female PG-enriched OBPs and CSPs to auto-detect and monitor the sex pheromones released by themselves [69–70].

Candidate olfactory receptors in the A. ipsilon antennae

Insect olfactory receptors (ORs) are the most important players in sex pheromone and general odorant detection. In the present study, we have identified 42 OR genes (41 typical ORs and one atypical coreceptor) from the A. ipsilon antennal transcriptomes (Table 4). In insect, the axons from the sensory neurons converge into glomeruli in the antennal lobe. There are 66 glomeruli in the antennal lobe of the male A. ipsilon moth [71], based on the hypothesis that the number of the glomeruli equals the number of olfactory receptors [72,73], we predict there are about 24 OR
Table 2. List of OBP genes in *A. ipsilon* antennae.

| Gene      | Length (bp) | ORF (bp) | BLASTx annotation | Score | E-value | % Identify | RPKM value | Male | Female |
|-----------|-------------|----------|--------------------|-------|---------|------------|------------|------|--------|
| Unigene_5952 | 1182       | 513      | gb|AFM36756.1| pheromone-binding protein 1 (Agrotis ipsilon) | 353 | 2e-122 | 100% | 5829 | 18523 |
| Unigene_10109 | 968       | 498      | gb|AFM36757.1| pheromone-binding protein 2 (Agrotis ipsilon) | 338 | 1e-116 | 100% | 9759 | 3136 |
| Unigene_14658 | 2153      | 495      | gb|AFM36758.1| pheromone-binding protein 3 (Agrotis ipsilon) | 278 | 6e-53 | 100% | 995 | 18 |
| Unigene_33505 | 1087      | 489      | gb|AFM36759.1| general odorant-binding protein 1 (Agrotis ipsilon) | 248 | 6e-30 | 100% | 624 | |
| Unigene_7317 | 765       | 470      | gb|ACX53761.1| odorant binding protein (Heliothis virescens) | 120 | 3e-22 | 53% | 83 | |
| Unigene_6175 | 579       | 447      | gb|AAL66739.1| AF461143_1 pheromone binding protein 4 (Mamestra brassicae) | 109 | 6e-12 | 80% | 83 | |
| Unigene_3156 | 743       | 378      | gb|AFM93773.1| odorant-binding protein 19 (Helicoverpa armigera) | 226 | 1e-75 | 86% | 1 | |
| Unigene_9245 | 1025      | 453      | gb|AEB54591.1| OBP7 (Helicoverpa armigera) | 169 | 3e-38 | 52% | 14 | |
| Unigene_5755 | 1124      | 414      | gb|ACX53795.1| odorant binding protein (Heliothis virescens) | 169 | 4e-51 | 71% | 18 | |
| Unigene_8140 | 378       | ---      | gb|ACX53743.1| odorant binding protein (Heliothis virescens) | 231 | 4e-75 | 86% | 1 | |
| Unigene_31090 | 896      | 438      | gb|AEB54587.1| OBP6 (Helicoverpa armigera) | 99.4 | 4e-22 | 43% | 14 | |
| Unigene_5992 | 1474      | 726      | gb|AFG72998.1| odorant-binding protein 1 (Cnaphalocrocis medinalis) | 183 | 4e-56 | 57% | 19 | |
| Unigene_6859 | 1051      | 420      | gb|AEB54589.1| OBP8 (Helicoverpa armigera) | 215 | 4e-69 | 83% | 36 | |
| Unigene_6275 | 1126      | 764      | gb|ADD71058.1| odorant-binding protein (Chilo suppressalis) | 192 | 1e-90 | 63% | 37 | |
| Unigene_29151 | 1635      | 429      | gb|AEB54586.1| OBP2 (Helicoverpa armigera) | 254 | 1e-78 | 82% | 31 | |
| Unigene_36163 | 1694      | 516      | gb|EFA09155.1| odorant binding protein 22 ( Tribolium castaneum) | 248 | 3e-80 | 86% | 19 | |
| Unigene_43247 | 569       | 292      | gb|AEB54581.1| OBP13 (Helicoverpa armigera) | 238 | 1e-73 | 87% | 34 | |
| Unigene_8227 | 791       | 402      | gb|AFI57166.1| odorant-binding protein 17 (Helicoverpa armigera) | 248 | 1e-79 | 82% | 35 | |
| Unigene_6670 | 886       | 423      | gb|EHJ65654.1| antennal binding protein 4 (Danaus plexippus) | 176 | 4e-54 | 67% | 20 | |
| Unigene_8505 | 1351      | 426      | gb|AEB54588.1| OBP13 (Helicoverpa armigera) | 238 | 1e-73 | 87% | 34 | |
| Unigene_33871 | 1206      | 438      | gb|AEB54580.1| OBP19 (Helicoverpa armigera) | 202 | 4e-43 | 53% | 101 | |
| Unigene_82912 | 569       | 450      | gb|EFA09155.1| odorant-binding protein 22 (Tribolium castaneum) | 125 | 4e-31 | 44% | 30 | |

--- represents that gene is partial and has not intact ORF. The nucleotide sequences of all 33 OBP genes are listed in Table S2.

**Table 2.** List of OBP genes in *A. ipsilon* antennae.
Figure 5. *A. ipsilon* OBP transcript levels in different tissues as measured by RT-qPCR. MA: male antennae; FA: female antennae; Bo: body. Pheromone gland rather than body was used in the analysis of *AipsOBP6*. The internal controls β-actin and ribosomal protein S3 were used to normalize transcript levels in each sample. This figure was presented using β-actin as the reference gene to normalize the target gene expression and to correct sample-to-sample variation; similar results were obtained with ribosomal protein S3 as the reference gene. The standard error is represented by the error bar, and the different letters (a, b, c) above each bar denote significant differences (p<0.05).
doi:10.1371/journal.pone.0103420.g005
genes still need to be identified. In total, 12 of the 42 ORs have intact ORFs. The RPKM value analysis revealed that the ORco had the highest expression level among the 42 ORs, with RPKM value of 741 and 997 in the male and female antennae, respectively. The other 41 typical ORs, however, showed a relative low expression level (RPKM ranged from 0 to 367) compared with the ORco, OBP and CSP genes. Three ORs (OR1, OR3 and OR4) showed a higher RPKM in the male antennae than in the female antennae (more than 20 times) (Table 4). The RT-PCR and RT-qPCR results indicated that 35 ORs were exclusively or primarily expressed in the antennae. Among these ORs, 4 ORs (OR1, OR3, OR4 and OR14) have male antennae-specific expression (Figure 7 and Figure 8), which suggests that these ORs may play essential roles in the detection of sex pheromones. In total, 4 ORs (OR6, OR7, OR8 and OR23) have female antennae-enriched expression (Figure 7 and Figure 8), which suggests that these ORs may play important roles in the detection of general odorants, such as host plant volatiles. The OR tree from three Lepidoptera insects is extremely divergent; however, the olfactory coreceptor family and the pheromone receptor family are highly conserved (Figure 9).

Figure 6. A. ipsilon CSP transcript levels in different tissues as measured by RT-qPCR. MA: male antennae; FA: female antennae; Bo: body. Pheromone gland rather than body was used in the analysis of AipsCSP2. The internal controls β-actin and ribosomal protein S3 were used to normalize transcript levels in each sample. This figure was presented using β-actin as reference gene to normalize the target gene expression and correct sample-to-sample variation; similar results were obtained with ribosomal protein S3 as the reference gene. The standard error is represented by the error bar, and the different letters (a, b) above each bar denote significant differences (p<0.05).

doi:10.1371/journal.pone.0103420.g006

Candidate ionotropic receptors in the A. ipsilon antennae

Insect chemosensory ionotropic receptors (IRs) belong to an ancient chemosensory receptor family, that was first discovered in D. melanogaster and are expressed in sensory neurons that respond to different odorants but that do not express either ORs or gustatory receptors (GRs) [40]. The misexpression of D. melanogaster IRs conferred ectopic odorant responsiveness [40]. At present, 66 IRs in D. melanogaster [41], 12 IRs in the noctuid S. littoralis [42], 15 IRs in C. pomonella [45] and 12 IRs in H. armigera [46] have been identified. In the present study, we have identified 24 IRs, including two highly conserved coreceptors, IR8a and IR25a, from the A. ipsilon antennal transcriptomes (Table 5). Five of the IR genes, including coreceptors IR8a and IR25a, had intact ORFs. Eighteen of these 24 IRs showed high amino acid identity (52%–90%) with three Lepidoptera insects, C. pomonella, S. littoralis and B. mori. Similar to the ORs, the RPKM value analysis revealed that all the 24 IRs showed a relative low expression level (RPKM value ranged from 0 to 69) compared with the OBPs and CSPs. The antennae-enriched IRs may play important roles in odorant detection; 15 D. melanogaster IRs [40], 10 H. armigera IRs [46] and 7 S. littoralis IRs [42] were expressed exclusively in the antennae. Our RT-PCR and RT-qPCR results indicated that 14 A. ipsilon IRs (IR8a, IR25a, IR21a, IR41a, IR75q.1, IR75q.2, IR76b, IR87a, IR1, IR3, IR4, IR8, IR12 and IR13) are highly expressed in the antennae; in particular, one IR IR12 was specifically expressed in the male antennae (Figure 7 and Figure 10), which suggested that this IR may be devoted to the response to the female sex pheromones. IRs from different insect species are extremely divergent; however, the two coreceptors IR8a and IR25a are highly conserved among different insect species (Figure 11).

Candidate sensory neuron membrane proteins and gustatory receptors in the A. ipsilon antennae

Insect SNMPs are two trans-membrane domain-containing proteins that are suggested to play significant roles in insect chemoreception [74–76]. Two SNMP subfamilies, SNMP1 and SNMP2, were identified in insects; however, these subfamilies showed different expression profiles in the antennae sensilla: SNMP1 proteins are detected in pheromone-sensitive olfactory receptor neurons (ORNs) [77–79]; however, the SNMP2 proteins are expressed in the supporting cells [78,79]. In the present study, we have identified two SNMP genes, SNMP1 and SNMP2, in the A. ipsilon antennal transcriptomes (Table 5). Both have intact
Table 3. List of CSP genes in *A. ipsilon* antennae.

| Unigene      | Gene | Length (bp) | ORF (bp) | BLASTx annotation                                      | Score | E-value | % Identify | RPKM value | Male | Female |
|--------------|------|-------------|----------|--------------------------------------------------------|-------|----------|------------|------------|------|--------|
| Unigene_32747| CSP1 | 479         | 375      | gb|ACK53825.1| chemosensory protein [Heliothis virescens]            | 125   | 1e-33    | 47%        | 171        | 155  |
| Unigene_10019| CSP2 | 585         | 360      | dbj|BAF91716.1| chemosensory protein [Papilio xuthus]                 | 159   | 8e-48    | 66%        | 6          | 9    |
| Unigene_32521| CSP3 | 927         | 387      | gb|AAF71290.2|AF255919_1 chemosensory protein [Mamestra brassicae] | 224   | 6e-70    | 82%        | 66         | 74   |
| Unigene_5484 | CSP4 | 653         | 363      | gb|AEQ27265.1| CSP2 [Helioverpa armigera]                            | 221   | 2e-70    | 86%        | 2246       | 2979 |
| Unigene_4019 | CSP5 | 823         | 324      | gb|EHJ67380.1| chemosensory protein [Danaus plexippus]              | 185   | 3e-57    | 84%        | 25         | 31   |
| Unigene_6911 | CSP6 | 1762        | 384      | gb|AAM77040.1| chemosensory protein 2 [Heliothis virescens]         | 225   | 3e-67    | 87%        | 279        | 571  |
| Unigene_33786| CSP7 | 980         | 387      | gb|AAP7460.1| chemosensory protein [Agrotis ipsilon]               | 218   | 1e-67    | 98%        | 1658       | 1327 |
| Unigene_4517 | CSP8 | 702         | 372      | gb|ACK53806.1| chemosensory protein [Heliothis virescens]           | 210   | 7e-66    | 76%        | 159        | 72   |
| Unigene_33739| CSP9 | 1617        | 447      | gb|ABM67686.1| chemosensory protein CSP1 [Plutella xylostella]      | 173   | 3e-52    | 65%        | 1756       | 1371 |
| Unigene_37440| CSP10| 799         | 366      | gb|ACK53813.1| chemosensory protein [Heliothis virescens]           | 197   | 2e-59    | 85%        | 1821       | 1921 |
| Unigene_7374 | CSP11| 1186        | 897      | ref|NP_001037069.1| chemosensory protein 9 precursor [Bombyx mori] | 236   | 1e-70    | 76%        | 539        | 414  |
| Unigene_16782| CSP12| 360         | 897      | gb|AFR92094.1| chemosensory protein 10 [Helioverpa armigera]        | 202   | 1e-63    | 80%        | 4          | 10   |

"---" represent that gene is partial and has not intact ORF. The nucleotide sequences of all 12 CSP genes are listed in Table S2.

doi:10.1371/journal.pone.0103420.t003
ORFs with lengths of 1569 bp and 1563 bp for SNMP1 and SNMP2, respectively, in agreement with our previous analyses [79]. The RT-PCR and RT-qPCR results revealed that both SNMP1 and SNMP2 were primarily expressed in the antennae of both sexes (Figure 7 and Figure 10). Furthermore, one gustatory receptor (AipsGR63) was identified in the A. ipsilon antennal transcriptomes (Table 5); AipsGR63 showed 46% amino acid identity with the B. mori gustatory receptor 63. The RT-PCR and RT-qPCR analyses showed that AipsGR63 was expressed in both the antennae and body part (Figure 7 and Figure 10).

Conclusions

Olfaction is an important sensory modality in insect. In present study we have successfully identified and annotated several groups of olfactory genes in the antennae of the noctuid moth A. ipsilon. The expression profile analysis revealed that 22 OBPs, 3 CSPs, 35 ORs, 14 IRs and the 2 SNMPs are uniquely or primarily expressed in the male and female antennae. These antennae-enriched OBPs, CSPs, ORs, IRs and SNMPs may play important physiological function in the pheromone and general odorant detection; thus, these genes could be meaningful targets for the study their biological functions, both in vivo and in vitro. An important direction of our future research will be the functional study of these olfactory genes.

Materials and Methods

Ethics statement

The black cutworm moth Agrotis ipsilon is common agricultural insect pests and are not included in the “List of Endangered and Protected Animals in China”. All operations were performed according to ethical guidelines in order to minimize pain and discomfort to the insects.

Insect rearing and tissue collection

The A. ipsilon colony was established in our laboratory in 2006. The larvae were reared with an artificial diet that was composed of...
Table 4. List of OR genes in *A. ipsilon* antennae.

| Unigene   | Gene | Length (bp) | ORF (bp) | BLASTx annotation                                      | Score | E-value  | % Identify | RPKM value |
|-----------|------|-------------|----------|--------------------------------------------------------|-------|----------|------------|------------|
| Unigene_225 | OR1  | 1550        | 1308     | *emb*|CAG38117.1| putative chemosensory receptor 16 [Heliothis virescens] | 396   | 3e-131   | 54%        | 126        |
| Unigene_6374 | OR2  | 2121        | 1308     | *gb*|ACF32965.1| olfactory receptor 11 [Helicoverpa armigera]           | 724   | 0.0       | 80%        | 34         |
| Unigene_30282 | OR3  | 1464        | 1272     | *dbj*|BAG71423.2| olfactory receptor [Mythimna separata]                | 585   | 0.0       | 72%        | 567        |
| Unigene_1457 | OR4  | 1589        | 1299     | *gb*|AC54306.1| candidate odorant receptor 3 [Helicoverpa armigera]    | 606   | 0.0       | 69%        | 178        |
| Unigene_13891 | OR5  | 1314        | 1209     | *emb*|CAG81122.1| putative chemosensory receptor 21 [Heliothis virescens] | 561   | 0.0       | 74%        | 13         |
| Unigene_14810 | OR6  | 581         | ---      | *emb*|CAG81122.1| putative chemosensory receptor 21 [Heliothis virescens] | 157   | 1e-42     | 39%        | 26         |
| Unigene_7733 | OR7  | 1558        | 1179     | *gb*|ACC63240.1| olfactory receptor 20, partial [Helicoverpa armigera]  | 617   | 0.0       | 74%        | 49         |
| Unigene_10999 | OR8  | 1594        | 1185     | *emb*|CAD31949.1| putative chemosensory receptor 8 [Heliothis virescens] | 503   | 2e-171    | 61%        | 27         |
| Unigene_10668 | OR9  | 1759        | 1251     | *ref*|NP_001103476.1| olfactory receptor 35 [Bombyx mori] | 436   | 2e-147    | 53%        | 19         |
| Unigene_10397 | OR10 | 1483        | 1290     | *gb*|AFC91732.1| putative odorant receptor OR24 [Cydia pomonella]       | 459   | 4e-156    | 54%        | 14         |
| Unigene_12603 | OR11 | 480         | ---      | *ref*|NP_001116817.1| olfactory receptor-like [Bombyx mori] | 182   | 1e-73     | 84%        | 5          |
| Unigene_14326 | OR12 | 861         | ---      | *emb*|CAG8113.1| putative chemosensory receptor 12 [Heliothis virescens] | 512   | 1e-177    | 85%        | 13         |
| Unigene_16749 | OR13 | 495         | ---      | *ref*|NP_001166603.1| olfactory receptor 13 [Bombyx mori] | 165   | 1e-45     | 59%        | 6          |
| Unigene_16622 | OR14 | 1453        | 1248     | *dbj*|BAG71414.1| olfactory receptor-1 [Mythimna separata]             | 624   | 0.0       | 71%        | 0          |
| Unigene_24590 | OR15 | 183         | ---      | *emb*|CAG8111.1| putative chemosensory receptor 10 [Heliothis virescens] | 127   | 8e-30     | 98%        | 14         |
| Unigene_15088 | OR16 | 693         | ---      | *gb*|EHU70341.1| olfactory receptor 16 [Danaus plexippus]             | 343   | 9e-115    | 73%        | 7          |
| Unigene_12606 | OR17 | 673         | ---      | *gb*|AFFL07813.1| odorant receptor 50, partial [Manduca sexta]            | 243   | 4e-75     | 60%        | 11         |
| Unigene_2007 | OR18  | 1080        | ---      | *gb*|ACL81185.1| putative olfactory receptor 18 [Agrotis segetum]       | 660   | 0.0       | 98%        | 74         |
| Unigene_18000 | OR19 | 923         | ---      | *ref*|NP_001166621.1| olfactory receptor 64 [Bombyx mori] | 281   | 2e-89     | 56%        | 4          |
| Unigene_28103 | OR20 | 291         | ---      | *ref*|NP_001166605.1| olfactory receptor 20 [Bombyx mori] | 115   | 9e-28     | 62%        | 7          |
| Unigene_15599 | OR21 | 532         | ---      | *emb*|CAG81122.1| putative chemosensory receptor 21 [Heliothis virescens] | 168   | 4e-47     | 47%        | 10         |
| Unigene_11593 | OR22 | 138         | ---      | *gb*|AF197121.1| putative odorant receptor OR12 [Cydia pomonella]       | 80.5  | 4e-14     | 82%        | 12         |
| Unigene_14448 | OR23 | 915         | ---      | *gb*|EHU75410.1| olfactory receptor [Danaus plexippus]                   | 173   | 1e-48     | 65%        | 62         |
| Unigene_17502 | OR24 | 675         | ---      | *ref*|NP_001166617.1| olfactory receptor 56 [Bombyx mori] | 424   | 4e-144    | 67%        | 9          |
| Unigene_13261 | OR25 | 744         | ---      | *gb*|AC65323.1| olfactory receptor 9 [Helicoverpa armigera]            | 89.0  | 5e-18     | 26%        | 12         |
| Unigene_10818 | OR26 | 648         | ---      | *emb*|CAD31950.1| putative chemosensory receptor 9 [Heliothis virescens] | 311   | 6e-130    | 79%        | 13         |
| Unigene_21855 | OR27 | 220         | ---      | *emb*|CAG81181.1| putative chemosensory receptor 17 [Heliothis virescens] | 85.5  | 1e-24     | 82%        | 5          |
| Unigene_11342 | OR28 | 411         | ---      | *ref*|NP_001166621.1| olfactory receptor 64 [Bombyx mori] | 137   | 2e-56     | 53%        | 6          |
| Unigene_13860 | OR29 | 1177        | ---      | *ref*|NP_001166894.1| olfactory receptor 29 [Bombyx mori] | 552   | 0.0       | 70%        | 13         |
| Unigene_14601 | OR30 | 645         | ---      | *gb*|EEZ9413.1| odorant receptor 50 [Tribolium castaneum]               | 51.6  | 2e-04     | 25%        | 34         |
| Unigene_21353 | OR31 | 239         | ---      | *gb*|ACF32961.1| olfactory receptor 3 [Helicoverpa armigera]            | 120   | 9e-37     | 79%        | 11         |
| Unigene_28909 | OR32 | 451         | ---      | *gb*|AF197124.1| putative odorant receptor OR16 [Cydia pomonella]       | 176   | 2e-50     | 60%        | 4          |
| Unigene_12299 | OR33 | 537         | ---      | *ref*|NP_001155301.1| olfactory receptor 60 [Bombyx mori] | 199   | 8e-104    | 71%        | 24         |
| Unigene_13820 | OR34 | 603         | ---      | *gb*|AFFL07813.1| odorant receptor 50, partial [Manduca sexta]           | 210   | 7e-63     | 58%        | 25         |
wheat germ, casein and sucrose as the main components [63,64,79]. The laboratory colony was kept at 24°C with 75% relative humidity and a 16:8 light:dark cycle. Pupae were sexed and maintained separately in hyaline plastic cups before emergence. Adult moths were given a 20% honey solution after emergence. Antennae were excised from 3-day-old male and female moths and immediately frozen and stored in liquid nitrogen until use.

RNA extraction and cDNA library construction

400 antennae from each sex were pooled for total RNA extraction using TRIzol reagent using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. The quantity of RNA samples was determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and 1.1% agarose electrophoresis. Approximately 500 ng messenger RNA was further purified from 50 μg total RNA using a PolyATtract mRNA Isolation System III (Promega, Madison, WI, USA). The mRNAs were then sheared into approximately 800 nucleotides via RNA Fragmentation Solution (Autolab, Beijing, China) at 70°C for 30 sec, then cleaned and condensed using an RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA, USA). The first-strand cDNA was synthesized using N6 random primers and MMLV reverse transcriptase (TaKaRa, Dalian, China). Then, the second strand cDNAs were synthesized using secondary strand cDNA synthesis enzyme mixtures (Autolab, Beijing, China). The cDNAs with the desired length were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and eluted with 10 μl elution buffer. After blunted and appended with a poly-A tail at the 3' end according to Roche’s Rapid Library Preparing protocols (Roche, USA), the purified cDNAs were linked to GS-FLX Sequencing Adaptors (Roche, USA). Finally, the cDNAs that were shorter than 500 bp were removed using AMPure Beads according to the manufacturer’s instructions (Beckman, USA) before the preparation of the cDNA library for next generation sequencing.

454 sequencing

Pyrosequencing of the cDNA library was performed by the Beijing Autolab Biotechnology Company using a 454 GS-FLX sequencer (Roche, IN, USA) according to the manufacturer’s instructions. All sequencing reads were deposited into the Short Read Archive (SRA) of the National Center for Biotechnology Information (NCBI), and can be accessed under the accession numbers SRR838973 and SRR838974 for the male and female antennal transcriptomes, respectively.

Sequence analysis and assembly

Base calling of the raw 454 reads in SFF files were performed using the python script sff_extract.py that was developed by COMAV (http://bioinf.comav.upv.es). All the raw reads were then processed to remove low quality and adaptor sequences using the programs TagDust [80], LUCY [81] and SeqClean [82] with default parameters. The resulting sequences were then screened against the NCBI UniVec database (http://www.ncbi.nlm.nih.gov/VecScreen/UniVec.html) to remove possible vector sequence contamination. The cleaned reads that were shorter than 60 bases were discarded based on the assumption that these reads might represent sequencing artifacts [83].

Two steps were taken to assemble the clean reads. First, the sequence assembler MIRA3 [84] was used with the assembly settings of a minimum sequence overlap of 30 bp and a minimum percentage overlap identity of 80%. Then, CAP3 was used with the assembly parameters of an overlap length cutoff >30 and an
Figure 8. *A. ipsilon* OR transcript levels in different tissues as measured by RT-qPCR. MA: male antennae; FA: female antennae; Bo: body. The internal controls *β*-actin and ribosomal protein S3 were used to normalize transcript levels in each sample. This figure was presented using *β*-actin as the reference gene to normalize the target gene expression and to correct sample-to-sample variation; similar results were obtained with ribosomal protein S3 as the reference gene. The standard error is represented by the error bar, and the different letters (a, b, c) above each bar denote significant differences (p < 0.05). doi:10.1371/journal.pone.0103420.g008
Table 5. List of IR, GR and SNMP genes in *A. ipsilon* antennae.

| Unigene       | Gene     | Length (bp) | ORF (bp) | BLASTx annotation | Score | E-value | % Identify | RPKM value |
|---------------|----------|-------------|----------|-------------------|-------|---------|------------|------------|
| **Male**      |          |             |          |                   |       |         |            |            |
| Unigene_13688 | IR8a     | 3301        | 2442     | gb|AFC91764.1|putative ionotropic receptor IR8a, partial [Cydia pomonella] | 1047  | 0.0       | 82%        | 68 28      |
| Unigene_18779 | IR25a    | 2960        | 2772     | gb|AFC91757.1|putative ionotropic receptor IR25a [Cydia pomonella] | 1562  | 0.0       | 87%        | 60 35      |
| Unigene_13062 | IR21a    | 2175        | ---      | gb|ADR64678.1|chemosensory ionotropic receptor IR21a [Spodoptera littoralis] | 1204  | 0.0       | 83%        | 24 19      |
| Unigene_149   | IR41a    | 975         | ---      | gb|ADR64681.1|chemosensory ionotropic receptor IR41a [Spodoptera littoralis] | 608   | 0.0       | 73%        | 12 17      |
| Unigene_368   | IR7q.1   | 1464        | ---      | gb|ADR64686.1|chemosensory ionotropic receptor IR7q.1 [Spodoptera littoralis] | 524   | 2e-173    | 58%        | 15 25      |
| Unigene_302   | IR7q.2   | 2168        | 1881     | gb|AFC91752.1|putative ionotropic receptor IR7q.2 [Cydia pomonella] | 811   | 0.0       | 69%        | 30 23      |
| Unigene_11960 | IR75p    | 492         | ---      | gb|ADR64684.1|chemosensory ionotropic receptor IR75p [Spodoptera littoralis] | 208   | 2e-60     | 65%        | 40 14      |
| Unigene_21404 | IR76b    | 2236        | 1629     | gb|ADR64687.1|chemosensory ionotropic receptor IR76b [Spodoptera littoralis] | 917   | 0.0       | 85%        | 37 18      |
| Unigene_31214 | IR87a    | 534         | ---      | gb|ADR64689.1|chemosensory ionotropic receptor IR87a [Spodoptera littoralis] | 330   | 3e-109    | 90%        | 21 9       |
| Unigene_16111 | IR93a    | 228         | ---      | gb|AFC91753.1|putative ionotropic receptor IR93a, partial [Cydia pomonella] | 117   | 3e-28     | 69%        | 15 0       |
| Unigene_14129 | IR1      | 897         | ---      | gb|ADR64688.1|putative chemosensory ionotropic receptor IR1 [Spodoptera littoralis] | 333   | 9e-106    | 74%        | 10 5       |
| Unigene_1452  | IR2      | 1651        | 1452     | ref|XP_001655464.1|glutamate receptor [Aedes aegypti] | 477   | 1e-155    | 55%        | 3 0        |
| Unigene_11336 | IR3      | 876         | ---      | gb|EHJ72198.1/ionotropic glutamate receptor-invertebrate [Danaus plexippus] | 187   | 6e-51     | 39%        | 19 4       |
| Unigene_13871 | IR4      | 1317        | ---      | gb|AFC91763.1|putative ionotropic receptor IR4, partial [Cydia pomonella] | 166   | 5e-45     | 52%        | 15 7       |
| Unigene_18619 | IR5      | 342         | ---      | ref|XP_002431269.1|glutamate receptor [Pediculus humanus corporis] | 74.7  | 3e-13     | 41%        | 5 11       |
| Unigene_11730 | IR6      | 972         | ---      | gb|AB036124.1|glutamate receptor Gr1 [Bombyx mori] | 329   | 2e-100    | 80%        | 67 69      |
| Unigene_23096 | IR7      | 615         | ---      | gb|ADR64688.1|chemosensory ionotropic receptor IR7 [Spodoptera littoralis] | 332   | 7e-108    | 76%        | 9 18       |
| Unigene_22453 | IR8      | 330         | ---      | gb|ADR64681.1|chemosensory ionotropic receptor IR41a [Spodoptera littoralis] | 156   | 8e-43     | 68%        | 11 0       |
| Unigene_19516 | IR9      | 307         | ---      | ref|XP_002431270.1|glutamate receptor [Pediculus humanus corporis] | 201   | 7e-59     | 89%        | 6 12       |
| Unigene_8307  | IR10     | 714         | ---      | gb|EHJ70235.1/ionotropic glutamate receptor-invertebrate [Danaus plexippus] | 99    | 4e-22     | 46%        | 3 2        |
| Unigene_19025 | IR11     | 255         | ---      | gb|ADR64683.1|chemosensory ionotropic receptor IR75d [Spodoptera littoralis] | 152   | 8e-42     | 86%        | 15 0       |
| Unigene_11460 | IR12     | 723         | ---      | ref|XP_001845244.1/ionotropic glutamate receptor [Culex quinquefasciatus] | 59.7  | 5e-07     | 31%        | 5 8        |
| Unigene_945   | IR13     | 1134        | ---      | gb|ADR64689.1|chemosensory ionotropic receptor IR87a [Spodoptera littoralis] | 172   | 6e-78     | 84%        | 13 7       |
| Unigene_17908 | IR14     | 287         | ---      | gb|ADR64684.1|chemosensory ionotropic receptor IR75p [Spodoptera littoralis] | 138   | 7e-36     | 56%        | 9 4        |
| **Female**    |          |             |          |                   |       |         |            |            |

--- represent that gene is partial and has not intact ORF. The nucleotide sequences of all 24 IR, 2 SNMP and 1 GR genes are listed in Table S2.

doi:10.1371/journal.pone.0103420.t005
Homology searches and functional classification

Following the assembly, homology searches of all unigenes were performed using the BLASTx and BLASTn programs against the GenBank non-redundant protein (nr) and nucleotide sequence (nt) databases at NCBI [86]. Matches with an E-value that was less than 1.0E-5 were considered significant [54]. Gene names were assigned to each unigene based on the best BLASTx hit with the highest score value.

Gene Ontology terms were assigned by the tool Blast2GO [87] through the BLASTx program with an E-value less than 1.0E-5. Then, the WEGO [88] software was used for the assignment of each GO ID to the related ontology entries. The longest open reading frame (ORF) of each unigene was determined by an ORF finder tool [http://www.ncbi.nlm.nih.gov/gorf/gorf.html].

Identification of *A. ipsilon* chemosensory genes

The tBLASTn program was performed, with available sequences of OBP, CSP, OR, GR, IR and SNMP proteins from Lepidoptera species as “query” to identify candidate unigenes encoding putative OBPs, CSPs, ORs, GRS, IRs and SNMPs in the unigenome.

Figure 9. Neighbor-joining tree of candidate odorant receptor proteins from *A. ipsilon* (red), *B. mori* (green) and *H. virescens* (blue). The protein names and sequences of ORs that were used in this analysis are listed in Table S5. doi:10.1371/journal.pone.0103420.g009

overlap percent identity cutoff >90% [85]. The resulting contigs and singletons that were more than 100 bases were retained as unigenes and annotated as described below.
A. ipsilon. All candidate OBPs, CSPs, ORs, GRs, IRs and SNMPs were manually checked by the BLASTx program at the National Center for Biotechnology Information (NCBI). The nucleotide sequences of all chemosensory genes that were identified from the A. ipsilon antennal transcriptomes are listed in Table S2.

Figure 10. A. ipsilon IR, SNMP and GR transcript levels in different tissues as measured by RT-qPCR. MA: male antennae; FA: female antennae; Bo: body. The internal controls β-actin and ribosomal protein S3 were used to normalize transcript levels in each sample. This figure was presented using β-actin as the reference gene to normalize the target gene expression and to correct sample-to-sample variation; similar results were obtained with ribosomal protein S3 as the reference gene. The standard error is represented by the error bar, and the different letters (a, b, c) above each bar denote significant differences (p < 0.05).

doi:10.1371/journal.pone.0103420.g010

Comparative analysis of chemosensory genes in the A. ipsilon male and female antennae

To compare the differential expression of chemosensory genes in the A. ipsilon male and female antennal transcriptomes, the
read number for each chemosensory gene between male and female antennae was converted to RPKM (Reads Per Kilobase per Million mapped reads) [89], using the formula: RPKM (A) = (1,000,000\times C \times 1,000)/(N \times L), where RPKM (A) is the expression of chemosensory gene A, C is the number of reads that are uniquely aligned to chemosensory gene A, N is the total number of reads that are uniquely aligned to all unigenes, and L is the number of bases in chemosensory gene A. The FDR (false discovery rate) was used to determine the threshold of the P-value for multiple testing. FDR <0.001 and absolute values of the log2ratio >1 were used as the threshold to determine significant differences in gene expression. The RPKM method eliminates the influence of gene length and sequencing depth on the calculation of gene expression. Thus, the calculated gene expression can be directly used to compare gene expression between samples.

Sequence and phylogenetic analysis
The putative N-terminal signal peptides and the most likely cleavage site were predicted using the SignalP V3.0 program [90] (http://www.cbs.dtu.dk/services/SignalP/). Sequence alignments were performed using the program ClustalX 2.1 [91] with default gap penalty parameters of gap opening 10 and extension 0.2, and were edited using the GeneDoc 2.7.0 software. A neighbor-joining tree [92] was constructed using the program MEGA 5.0 [93] with a p-distance model and a pairwise deletion of gaps. The bootstrap

Figure 11. Neighbor-joining tree of candidate ionotrophic receptor proteins from different insect species. The protein names and sequences of IRs that were used in this analysis are listed in Table S6. doi:10.1371/journal.pone.0103420.g011
support of tree branches was assessed by re-sampling amino acid positions 1000 times.

**RT-PCR and RT-qPCR analysis**

Two biological samples each with 150 male antennae, 150 female antennae and two moth body part (mixture of heads, thoraxes, abdomens, legs, wings) were used for RNA extraction using TRIZol reagent. Before transcription, total RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, USA) to remove residual genomic DNA. cDNAs from male antennae, female antennae and the body part were synthesized using a GoScript Reverse Transcription System (Promega, Madison, USA). An equal amount of cDNA (200 ng) was used as RT-PCR and RT-qPCR templates. Specific primer pairs that were used for RT-PCR were designed with the program Primer 3 (http://frodo.wi.mit.edu/) (see Table S3). The β-actin (GenBank Acc. JQ822245) of A. ipsilon was used as the control gene to test the integrity of the cDNAs. The PCR was performed under following conditions: 95°C for 2 min, followed by 25–35 cycles (depending on the expression level of each gene) of 95°C for 30 sec, 56°C for 30 sec, 72°C for 1 min, and a final extension for 10 min at 72°C. PCR products were analyzed on 1.2% agarose gel and visualized after staining with ethidium bromide. To reach reproducibility, each sample was performed at least six times with two biological samples.

RT-qPCR analysis was conducted using an ABI 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The primers that were used for RT-qPCR were designed using the program Beacon Designer 7.90 (PREMIER Biosoft International) (see Table S4). Two reference genes, β-actin (GenBank Acc. JQ822245) and ribosomal protein S3 (GenBank Acc. JQ822246) were used for normalizing the target gene expression and for correcting for sample-to-sample variation. Each RT-qPCR reaction was conducted in a 25 µl reaction mixture containing 12.5 µl of SuperReal PreMix Plus (TianGen, Beijing, China), 0.75 µl of each primer (10 µM), 0.5 µl of Rox Reference Dye, 1 µl of sample cDNA, and 9.5 µl of sterilized H2O. The RT-qPCR cycling parameters were as follows: 95°C for 15 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 32 sec. Then, the PCR products were heated to 95°C for 15 sec, cooled to 60°C for 1 min, heated to 95°C for 30 sec and cooled to 60°C for 15 sec to measure the dissociation curves. Negative controls without either template or transcriptase were included in each experiment. To check reproducibility, each RT-qPCR reaction for each sample was performed in three technical replicates and two biological replicates. The comparative 2^(-ΔΔCT) method [94] was used to calculate the relative quantification between tissues. The comparative analyses of each target gene among various tissues were determined using a one-way nested analysis of variance (ANOVA), followed by Tukey’s honestly significance difference (HSD) test using the software SPSS Statistics 18.0 (SPSS Inc., Chicago, IL, USA). When applicable, values were presented as the mean±SE.

**Supporting Information**

Table S1 The 500 most highly abundant unigenes in the A. ipsilon antennal transcriptome.

(XLSX)

Table S2 The nucleotide sequences of 33 OBPs, 12 CSPs, 42 ORs, 24 I Rs, 2 SNMPs and 1 GRs identified in present study.

(DOCX)

Table S3 Primers used for RT-PCR analysis of olfactory genes of the A. ipsilon moth.

(DOCX)

Table S4 Primers used for RT-qPCR analysis of olfactory genes of the A. ipsilon moth.

(DOCX)

Table S5 Protein names and sequences of ORs used in Figure 9.

(TXT)

Table S6 Protein names and sequences of I Rs used in Figure 11.

(TXT)

**Acknowledgments**

The authors thank Ms. Xiaoyang Qi and Wei Wang for their contributions in the insect rearing. The authors gratefully acknowledge the language help from Elsevier Language Editing Services.

**Author Contributions**

Conceived and designed the experiments: SHG YJZ JJZ. Analyzed the data: SHG KMW YG XCL. Contributed reagents/materials/analysis tools: SHG. Contributed to the writing of the manuscript: SHG YJZ JJZ. Performed the sample collection, library construction, data processing, bioinformatics analysis and qRT-PCR: SHG. Performed the RT-PCR: LS RNY.

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