Identification and Expression Analyses of Olfactory Gene Families in the Rice Grasshopper, *Oxya chinensis*, From Antennal Transcriptomes

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The rice grasshopper *Oxya chinensis* is an important agricultural pest of rice and other gramineous plants. Chemosensory genes are crucial factors in direct interactions with odorants in the olfactory process. Here we identified genes encoding 18 odorant-binding proteins (OBPs), 13 chemosensory proteins (CSPs), 94 olfactory receptors (ORs), 12 ionotropic receptors (IRs), and two sensory neuron membrane proteins (SNMPs) from *O. chinensis* using a transcriptomic approach. Semi-quantitative RT-PCR assays revealed that six OBP-encoding genes (*OchiOBP4*, 5, 8, 9, 10, and 14), one CSP gene (*OchiOBP10*) and two IR genes (*OchiIR28* and 29) were exclusively expressed in antennae, suggesting their roles in olfaction. Real-time quantitative PCR analyses revealed that genes expressed exclusively or predominantly in antennae also displayed significant differences in expression levels between males and females. Among the differentially expressed genes, 17 OR-encoding genes, one CSP- and one SNMP-gene showed female-biased expression, suggesting that they may be involved in some female-specific behaviors such as seeking oviposition site; whereas the three remaining OR-encoding genes showed male-biased expression, indicating their possible roles in sensing female sex pheromones. Our results laid a solid foundation for future studies to reveal olfactory mechanisms as well as designing strategies for controlling this rice pest.

Keywords: antennal transcriptome, olfactory gene, identification, expression analysis, *Oxya chinensis*

INTRODUCTION

Olfaction plays an important role in regulating various physiological behaviors, such as foraging, mating, oviposition and avoiding predators (Leal, 2013). The process of olfactory perception is mediated by a series of peripheral olfactory proteins involving odorant-binding proteins (OBPs), chemosensory proteins (CSPs), sensory neuron membrane proteins (SNMPs), olfactory receptors (ORs), gustatory receptors (GRs), and ionotropic receptors (IRs) (Clyne et al., 1999, 2000; Vosshall et al., 1999; Galindo and Smith, 2001; Benton et al., 2007, 2009; Vieira and Rozas, 2011). OBPs and CSPs are suggested to be involved in binding and trafficking of hydrophobic odorant molecules across the sensillum lymph surrounding the olfactory sensory neurons (OSNs) on the sensilla of antennae and
other chemosensory organs (Vogt et al., 1985; Sandler et al., 2000; Xu et al., 2005; Gomez-Diaz et al., 2013). ORs and GRs are the principle receptor proteins responsible for the detection of odorants and tastants, whereas IRs are involved in the detection of chemosensory, thermo- and hygro-sensory stimuli (Ai et al., 2010, 2013; Grosjean et al., 2011; Silbering et al., 2011; Kain et al., 2013; Su and Carlson, 2013; Koh et al., 2014; Chen et al., 2015; Stewart et al., 2015; Gorter et al., 2016; Hussain et al., 2016; Knecht et al., 2016, 2017; Ni et al., 2016). SNMPs play important roles in pheromone detection, which are expressed on the dendritic membranes of pheromone sensitive neurons (Rogers et al., 1997, 2001; Li et al., 2014; Jiang et al., 2016).

Gene families involved in olfactory perception have been extensively reported in many species in Lepidoptera, Coleoptera, Diptera, Hemiptera, and Hymenoptera during recent decades (Grosse-Wilde et al., 2011; Andersson et al., 2013, 2014; Brand et al., 2015; Paula et al., 2016). However, in the Orthoptera order, ORs have been identified from only three species: the genome of Locusta migratoria (142 ORs) (Wang et al., 2015), the antennal transcriptome of Schistocerca gregaria (119 ORs) (Pregitzer et al., 2017), and Oedaleus asiaticus (60 ORs) (Zhou et al., 2019). Members of the IR families have been identified in L. migratoria (32 IRs) (Wang et al., 2015) and O. asiaticus (6 IRs) (Zhou et al., 2019), the OBPF families in S. gregaria (14 OBPs) (Jiang et al., 2017), Oedaleus infernalis (18 OBPs) (Zhang et al., 2018) and O. asiaticus (15 OBPs) (Zhou et al., 2019), the CSP families in L. migratoria (58 CSPs) and S. gregaria (42 CSPs) (Martin-Blazquez et al., 2017), and SNMP families in S. gregaria (2 SNMPs) (Jiang et al., 2016) and O. asiaticus (3 SNMPs) (Zhou et al., 2019). Consequently, additional Orthoptera species need to be investigated to reach a better understanding on olfactory receptive mechanisms for insect olfactory system.

Oxya chinensis is an oligophagous grasshopper pest and primarily feeds on graminaceous grasses. Electroantennogram (EAG) and behavioral bioassay have showed that O. chinensis adults have significantly higher olfactory sensitivity to geraniol compared to other host volatiles (Lu et al., 2008). Identification of target genes that are involved in olfactory perception may lead to environmentally-friendly approaches for controlling this pest (Venthur and Zhou, 2018). The objectives of this study were to generate antennal transcriptomes to identify major olfactory-related gene families (OBPs, CSPs, ORs, IRs, and SNMPs) from O. chinensis, examine expression profiles of the genes in various tissues to identify the antenna-specific genes, and compare their sex-specific expression patterns to identify female- and male-specific olfactory-related genes.

MATERIALS AND METHODS

Insects, Tissue Collections, and RNA Extraction

The colony of O. chinensis used in this study was derived from insects originally collected from rice fields in the environs of Leizhou, Guangdong, China (110°05'E, 20°34′N). The colony was maintained in the laboratory on bristle grass in climate-controlled chamber under 30°C ± 1°C with 60% relative humidity and a photoperiod of 16 h light versus 8 h.

For transcriptomic analyses, 100 pairs of antennae were dissected separately from O. chinensis adult females and males. For semi-quantitative RT-PCR analysis, antennae, maxillary palps, foreleg tarsus, wings, and genitals were separately collected from adults (male: female = 1:1, n = 25 each). Two replicates were included for each tissue. For RT-qPCR analyses, 50 pairs of antennae were obtained from both females and males, separately along with other body parts including 20 pairs of maxillary palps, 20 pairs of foreleg tarsus, 20 pairs of wings, and 10 genitals from both males and females. Three replicates were included for each tissue sample. Tissues were homogenized to powder immediately in liquid nitrogen and stored at −80°C for further analyses.

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, United States) and treated with RNase-free DNase I (Takara, Dalian, China) to remove potential genomic DNA contamination. RNA concentration, quality and quantity were analyzed on a NanoDrop ND-2000 Spectrophotometer (Nanodrop Technologies, United States) and a Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies, United States). RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, United States).

cDNA Library Construction, Illumina Sequencing and de novo Assembly

Transcriptomes were generated through a commercial contract with Novogene Bioinformatics Technology, Co., Ltd. (Beijing, China). Libraries for sequencing were generated from 1.5 μg purified RNA per sample using a TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA, United States) according to Illumina instructions and sequenced on an Illumina HiSeq 2500 platform (San Diego, CA, United States). Approximately 150 bp paired-end reads were generated.

Raw reads were firstly processed through in-house perl scripts. High-quality clean reads were obtained by removing reads containing adapter and unknown (poly-N) and low-quality reads. Clean reads were combined from both female and male antennae and were assembled into unigenes using Trinity (Version: r2013-11-10) with min_kmer_cov set to 2 under default settings (Grabherr et al., 2011). The clean reads from the O. chinensis female antennae and male antennae were deposited in the NCBI Sequence Read Archive (Female antennae: SAMN11484273; Male antennae: SAMN11484274).

Functional Annotation

Unigenes were searched against databases including NCBI non-redundant protein (nr), NCBI non-redundant nucleotide (nt), Swiss-Prot1, the Kyoto Encyclopedia of Genes and Genomes (KEGG)2, using BLASTx with a cut-of E-value of 10−5. Gene orthology (GO) and cluster of orthologous groups of
proteins (COG) using Blast2GO program (Conesa et al., 2005; Gotz et al., 2008).

Gene Identification
To identify genes coding for OBPs, CSPs, ORs, IRs, and SNMPs, known protein sequences (OBPs, CSPs, ORs, IRs, and SNMPs) from other Orthopteran species were selected as queries to search the *O. chinensis* antennal transcriptomes. *L. migratoria*, *S. gregaria*, *O. asiaticus*, *O. infernalis*, and *Ceracris kiangsu* were used for OBPs; *L. migratoria*, *S. gregaria*, and *O. asiaticus* for CSPs; *L. migratoria*, *S. gregaria*, and *O. asiaticus* for ORs; *L. migratoria* and *O. asiaticus* for IRs; *S. gregaria* and *O. asiaticus* for SNMPs (Supplementary Table S1). tBLASTn was used to search and identify candidate chemosensory genes against the *O. chinensis* antennal transcriptomes, with a cut-off E-value of $10^{-5}$. Putative *O. chinensis* chemosensory genes were in turn used as queries to identify additional genes (tBLASTx and BLASTp). Repetitions were completed until no new candidates were identified. Candidate unigenes from the initial search were further manually examined using BLASTx against Genbank. Open reading frames (ORFs) were predicted with the ORF Finder in NCBI. Amino acid sequences were aligned with MAFFT (version 7.308) (E-INS-I parameter set) (Katoh and Standley, 2013) and visualized with Geneious (version 9.1.3) (Kearse et al., 2012). Transmembrane domains, signal peptides, conserved cysteine locations in candidates were analyzed using the InterProScan tool plug-in in Geneious (Version: 9.1.3.) (Quevillon et al., 2005). Candidate genes coding for OBPs, CSPs, ORs, IRs, and SNMPs were listed in Supplementary Table S2, together with genetic characteristics, best matches in NCBI-nr database, protein domains and estimated expression levels.

Phylogenetic Analyses
Multiple amino acid sequence alignments were made with MAFFT (E-INS-I parameter) (Katoh and Standley, 2013). Phylogenetic trees were constructed using maximum likelihood analyses as implemented in FastTree2 [Jones-Taylor-Thornton (JTT) amino acid substitution model, 1,000 bootstrap replications] (Price et al., 2009, 2010). Dendrograms were created and colored in FigTree. Sequences used for phylogenetic analysis are listed in Supplementary Table S3.

Analyses of Expression Levels and Differentially Expressed Genes
Gene expression levels were estimated using RSEM (Version: 1.2.15) (Li and Dewey, 2011) for each sample. The clean reads were mapped back to the assembled transcriptome. The read counts normalized using the Trimmed Mean of M-value normalization method (Robinson and Oshlack, 2010) for each mapped gene was used to calculate gene expression levels following the FPKM (fragments per kilobase per million read) method (Cock et al., 2010; Trapnell et al., 2010), and were used to identify differentially expressed genes between females and males using DEGseq edgeR package (Version: 3.4.2) (Robinson et al., 2010). Criteria for estimating significantly differentially expressed genes were set as q-value < 0.005 and the absolute value of log2 Fold Change > 1.

Semi-Quantitative RT-PCR and Real-Time Quantitative PCR
Semi-quantitative RT-PCR was carried out to compare expression levels of genes coding for OBPs, CSPs, and IRs in various tissues including antennae, maxillary palps, foreleg tarsus, wings and genitals. cDNA template was synthesized from total RNA using a PrimeScript RT reagent Kit (Takara, China). Two housekeeping genes, β-actin (*Ochirp-actin*) and ribosomal protein 49 (*Ochirp49*) were used as controls. PCR reactions were conducted in a thermal cycler from Bio-Rad (CA, United States). PCR conditions were as follows: 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 1 min, and a final extension for 10 min at 72°C. PCR products were separated on 1.5% agarose gels. Individual PCR reactions were repeated twice with independently isolated RNA samples.

Expression profiles of the antenna-predominant candidate genes for OBPs, CSPs, and IRs as well as all ORs and SNMPs were analyzed on a LightCycler 480 system (Roche Applied Science). RT-qPCR cycling parameters were set at 95°C for 5 min, followed by 45 cycles of 95°C for 10 s, and 60°C for 20 s. A melting curve analysis was then performed at 95°C for 20 s, 60°C for 30 s, and 95°C for 30 s in order to determine the specificity of primers. Negative controls without cDNA template (nuclease-free water) were included for each reaction. Three independent biological replications were performed with each biological replication. Relative quantification was calculated following the 2−ΔΔCT method (Livak and Schmittgen, 2001), and normalized against the two reference genes (*Ochirp-actin* and *Ochirp49*). Gene-specific primers were designed using Primer3 and listed in Supplementary Table S4.

Statistical Analysis
RT-qPCR data were analyzed and plotted using Prism 6.0 (GraphPad Software, CA, United States). The comparative analyses of each target gene among various tissues were determined using a one-way nested analysis of variance (ANOVA) followed by Duncan's new multiple range test ($\alpha = 0.05$) using SPSS 22.0 (SPSS Inc., Chicago, IL, United States). Values are presented as mean ± SE.

RESULTS
Illumina Sequencing and de novo Assembly
A total of 53.4 and 44.8 million raw reads were obtained from male and female antennae cDNA libraries of *O. chinensis*,

1http://clov.org/docs/clusters-of-orthologous-groups-cogs/
2https://www.ncbi.nlm.nih.gov/orffinder/
3http://tree.bio.ed.ac.uk/software/figtree

4http://primer3.ut.ee/
respectively. After removing adaptor sequences, low quality reads and contaminant sequences, 52.2 and 44.7 million of clean reads were generated. The reads were assembled into 120,803 unigenes with a mean length of 1,213 bp, and an N50 of 2,458 bp. The number of unigenes longer than 500 bp was 33,079, which accounted for 35.21% of all unigenes (Supplementary Table S5).

**Functional Annotation**
There were 26,923 (45.5%), 15,092 (25.5%), 20,546 (34.7%), 5,662 (9.57%), 10,911 (18.4%), and 20,542 (34.7%) unigenes that had homologous sequences in NCBI-nr, NCBI-nt, Swiss-Prot, GO, COG, and KEGG databases, respectively. Based on the homologous sequences, a total of 29,067 (49.1%) unigenes were annotated and the remaining unigenes were unmappable at present (Supplementary Figure S1A). GO analyses categorized annotated genes into three functional categories, including “biological process,” “cellular component,” and “molecular function” (Supplementary Figure S1B). In “biological process,” major subcategories included “cellular,” “metabolic” and “single-organism.” In “cellular component,” the subcategories “cell” and “cell part” and “membrane” were major subcategories. In “molecular function,” the subcategories “binding” and “catalytic activity” contained the largest numbers of unigenes.

**Candidate Genes Coding for Odorant Binding Proteins**
Eighteen OBP unigenes were identified from the *O. chinensis* antennal transcriptomes (Supplementary Table S2: Sheet 1). All identified unigenes except *OchiOBP18* had a full-length ORF with sizes ranged from 128 to 266 amino acid residues. Except three OBP genes (*OchiOBP12, 14, and 17*), all predicted proteins had a predicted signal peptide. Sequence identities of
predicted OBPs with those from other Orthopteran insects in the NCBI-nr database ranged from 45.9 to 88.6%, with an average of 73.8%. Consistent with previous Orthopteran OBP classification by Jiang et al. (2017), twelve OBPs (OchiOBP1, 3-8, 10, 12-14, 16) were classic OBPs, with the typical six conserved C-residues (Supplementary Figure S2). Two (OchiOBP9 and 17) were atypical OBPs, with extraordinary long stretches between conserved C1 and C2 (Supplementary Figure S3). Four OBPs were plus-C OBPs, which were further divided into plus-C OBP type-A (OchiOBP2, 11, and 15) (with the extra C-residue both in C4-C5 and C5-C6) (Supplementary Figure S4) and plus-C OBP type-B (OchiOBP18) (with the extra C-residue in front of C1 and behind C6) (Supplementary Figure S5). Phylogenetic analyses of OBPs from O. chinensis and other five locust species (O. infernalis, O. asiaticus, L. migratoria, S. gregaria, and C. kiangsu) showed that all the locust OBPs formed distinct clades based on the number and position of cysteine residues, and segregated into the classic OBP, atypical OBP, and plus-C OBP sub-families as well as other locust OBPs (Figure 1).

**Candidate Genes Coding for Chemosensory Proteins**

Thirteen unigenes encoding CSPs were identified from the O. chinensis antennal transcriptomes (Supplementary Table S2: Sheet 2). All but two (OchiCSP11 and 13) had full-length ORFs encoding 120–297 amino acid residues. All predicted

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**FIGURE 2** | Phylogenetic tree of putative chemosensory proteins (CSPs). Branch support (circles at the branch nodes) was estimated using an approximate likelihood ratio test based on the scale indicated at the top left. Bars indicate branch lengths in proportion to amino acid substitutions per site.
OchiCSPs contained four highly conserved four-cysteine profiles (Supplementary Figure S6). Except OchiCSP1, all predicted proteins possessed a signal peptide. A phylogenetic tree was built using all predicted OchiCSPs together with those from other locust species. Most OchiCSPs had orthologs with CSPs from other locust species, and no O. chinensis-specific CSP lineage was evident (Figure 2).

**Candidate Genes Coding for Odorant Receptors**

Ninety-four OR-encoding unigenes were identified, including one Orco (OchiOR1) and 93 conventional OR genes (OchiOR2-94), which were classified to the 7-transmembrane receptor superfamily (Supplementary Table S2: Sheet 1). Among these OR unigenes, 54 had full-length ORFs encoding proteins with 367 to 487 amino acid residues. The highly conserved co-receptor OchiOR1 shared 94.25% identity to a co-receptor from L. migratoria (ALD51504.1), while other OchiORs shared 33.56 to 91.13% identity with average 68.7% with the respective L. migratoria ORs. A phylogenetic tree was generated using our identified ORs along with a data set containing representative ORs from L. migratoria and S. gregaria (Figure 3). The locust ORs formed three distinct clades, with the O. chinensis OR co-receptor (OchiOR1) formed a clade with the protein Orco from two other related species. The vast majority of OchiORs were clustered with orthologs from other locust species. Only a few species-specific clades and sister pairs were observed. Three OchiORs (OchiOR27, 53 and 56) were segregated into unique clades, while OchiOR36/60 and OchiOR28/76 formed the sister pairs.

**FIGURE 3** | Phylogenetic tree of putative odorant receptors (ORs). The distance tree was rooted by the conservative ORco gene orthologs (red). The species-specific clades and sister pairs are labeled with red dots. Branch support (circles at the branch nodes) was estimated using an approximate likelihood ratio test based on the scale indicated at the top left. Bars indicate branch lengths in proportion to amino acid substitutions per site. The clade I is in orange; clade II in green; and clade III in blue.
Candidate Genes Coding for Ionotropic Receptors (iGluRs/IRs)

Twelve putative iGluR/IR unigenes were identified, which were predicted to encode ligand-binding domain (S1 and S2) with three transmembrane domains (M1, M2, and M3) or portions of domains (Supplementary Table S2: Sheet 2). Of these iGluR/IR unigenes, eight had complete ORFs encoding at least 535 amino acid residues. Distinct clades were observed in a phylogenetic tree generated with our identified sequences and paralogs from other species including Drosophila melanogaster and L. migratoria (Figure 4). All identified iGluRs/IRs from O. chinensis were assigned to two phylogenetic groups, including N-Methyl-D-aspartic acid (NMDA) iGluRs (OchiGluR8, 9, and 12) and antennal IRs (OchiIR25a, 8a, 76b, 22, 6, 17, 20, 28, and 29). No candidates were assigned to non-NMDA iGluRs and divergent IRs (Figure 4). A set of “antennal IR” conserved among other species were absent from O. chinensis and L. migratoria, and only three “IR co-receptor” orthologs (OchiIR25a, 8a, and 76b) were clustered with D. melanogaster orthologs.

Candidate Genes Coding for Sensory Neuron Membrane Proteins

Two SNMP unigenes were identified, which matched the CD36 family. The genes contained complete ORFs that encoded proteins with two transmembrane domains (Supplementary Table S2: Sheet 3). Phylogenetic analyses revealed that all SNMPs were classified into two distinct subgroups, SNMP1 and SNMP2 (Figure 5). In the SNMP1 clade, OchiSNMP1 and OchiSNMP2 were clustered into two subclades along with those from other locust species.

Transcript Abundance Based on FPKM

In this study, genes with FPKM values ≥ 1,000 were defined as highly expressed genes, those with values 300–1,000 were defined as moderately expressed genes, and those with values ≤ 300 were defined as weakly expressed genes. Based on these criteria, OchiOBP1-5 and OchiCSP1-4 were highly expressed in antennae (Supplementary Table S2: Sheets 1,2);
OchiOR1 (Orco) was moderately expressed (Male FPKM: 318.52, Female FPKM: 391.73); other conventional ORs (OchiOR2-94), all iGluRs/IRs, and the two SNMPs were weakly expressed (RPKM ≤ 30) (Supplementary Table S2: Sheet 3). DEG analyses showed that OchiCSP10 and OchiOR2, 6, 8, 9, 11, 15, 33, 38, 39, 41, 43, 44, 48, 50, 53, 56, and 62 were female-predominant, whereas OchiCSP9, OchiOR34, 46 and 86, and OchiGluR8 and 12 were male-biased (Supplementary Table S2: Sheets 1–5).

Tissue- and Sex-Specific Expression

Based on semi-quantitative RT-PCR analyses, OchiOBP4, 5, 8, 9, 10, and 14 were almost exclusively expressed in antennae, while OchiOBP3 and 17 were abundant in antennae and maxillary palps (Figure 6A). The remaining OBP-encoding genes were abundant in multiple body parts. For CSP-encoding genes, OchiCSP10 was exclusively expressed in antennae, while other OchiCSPs were present multiple body parts (Figure 6B). For iGluR/IR-encoding genes, OchiIR28 and 29 were expressed predominately in antennae (Figure 6C).

All antenna-predominant genes were further analyzed using RT-qPCR (Figure 7). OchiOR2, 6, 8, 9, 11, 15, 33, 38, 39, 41, 43, 44, 48, 50, 53, 56, and 62 were significantly expressed at high levels in female antennae and OchiOR34, 46 and 86 were significantly expressed at higher levels in male antennae. The remaining OR-encoding unigenes were equally expressed in the antennae of both males and females (Figure 7A). For CSP-encoding genes, OchiCSP10 was expressed at significantly higher levels in female antennae. Additionally, the SNMP-encoding gene OchiSNMP1 were significantly upregulated in female antennae (Figure 7B). Other OBP- and IR-encoding genes were expressed largely equally in both males and females (Figure 7B).

DISCUSSION

Identification and characterization of chemosensory genes are important steps toward understanding the evolution and primary functions of the insect olfactory system. In this study, we identified a set of candidate chemosensory genes from O. chinensis via analyzing antennal transcriptomes. Genetic and phylogenetic analyses of candidate chemosensory genes in O. chinensis was carried out to examine the similarities and differences of molecular components in chemosensory pathways. We further analyzed the expression profiles of chemosensory genes to identify olfaction-specific genes for future functional studies.

Odorant-binding proteins play vital roles in carrying odorants through the hemolymph to OR neurons, and transducing the resultant signals to downstream effectors in the olfactory system (Hallem et al., 2006). The number of OBPs identified in our antennal transcriptome was comparable to its related Locust species, namely 18 from O. infernalis, 16 from L. migratoria, 15 from O. asiaticus, and 14 from S. gregaria) (Ban et al., 2003; Xu et al., 2009;
Yu et al., 2009; Zhang et al., 2015, 2018; Jiang et al., 2017). This may reflect physiological and evolutionary consistency between O. chinensis and other Locust species. OBPs from O. chinensis can be classified into four types: classic, atypical, plus-C (type-A and B), as well as Locust-specific OBPs (Jiang et al., 2017). Expression analysis revealed that five classic OBP (OchiOBP4, 5, 8, 10, and 14) and one atypical OBP (OchiOBP9) were expressed exclusively in antennae, suggesting that these genes are likely to have a role in antennal chemical-recognition processes. CSPs and OBPs can act as carriers for odorant molecules (Liu et al., 2012; Yi et al., 2013; Zhang T. et al., 2013; Zhang et al., 2014). However, in our RT-PCR analysis, only OchiCSP10 showed significantly antennae-biased expression, suggesting that this CSP could be involved in olfactory function.

Olfactory receptors, which connect binding proteins and OSNs to transduce olfactory signals, are the best known group of insect chemoreceptors. Like other locust species, O. chinensis possesses an expanded OR family (94) compared with Dipterans, Lepidopterans, and Hemipterans (Vieira and Rozas, 2011). A large number of OR-encoding genes may be associated with its ability for diverse host-odor perception to feed on a wider range of host plants. Great variation in the number of OR genes was found in different Locust species. Specifically, 94 OR genes were identified in O. chinensis, less than 120 from S. gregaria (Pregitzer et al., 2017), and 141 from L. migratoria (Wang et al., 2015), but significantly more than 60 from O. asiaticus (Zhou et al., 2019). The variation in gene numbers may reflect that O. chinensis inhabits a different ecological niche than other locust species. Generally, sexually dimorphic expression of ORs in antennae indicates possible pheromone receptors contributing sexual behaviors. Typically, Lepidopteran sex pheromones are released via females to attract males. Several moth sex pheromone for ORs have been functionally characterized, and most of them are expressed at higher levels in the male antennae (Krieger et al., 2004; Wanner et al., 2010; Zhang and Löfstedt, 2013). ORs expressed predominantly in female antennae are predicted to function in the detection of egg-laying-related odorant (Pelletier et al., 2010) or male released pheromones (or signals) (Anderson et al., 2009); ORs expressed evenly in the male and female antennae are predicted to function in general odorant perception (Yan et al., 2015). Therefore, we hypothesize that some or all of female-predominant
Olfactory Genes in O. chinensis Antennae

Figure 7 | Relative expression levels of the olfactory-related genes in the female antennae, male antennae, and other body parts from Oxya chinensis. (A) ORs. (B) SNMPs and the antenna-predominant candidates (OBPs, CSPs, and IRs). FA, female antennae; MA, male antennae; Bo, other body parts. The expression levels were estimated using the $2^{-\Delta \Delta CT}$ method. Bars represent standard error of three independent biological replicates with three technical duplicates for each replicate. Different small letters indicate statistically significant difference between tissues ($p < 0.05$, ANOVA, HSD).

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FIGURE 7 | Relative expression levels of the olfactory-related genes in the female antennae, male antennae, and other body parts from Oxya chinensis. (A) ORs. (B) SNMPs and the antenna-predominant candidates (OBPs, CSPs, and IRs). FA, female antennae; MA, male antennae; Bo, other body parts. The expression levels were estimated using the $2^{-\Delta \Delta CT}$ method. Bars represent standard error of three independent biological replicates with three technical duplicates for each replicate. Different small letters indicate statistically significant difference between tissues ($p < 0.05$, ANOVA, HSD).

OchiORs, including OchiOR2, 6, 8, 9, 11, 15, 33, 38, 39, 41, 43, 44, 48, 50, 53, 56, and 62, are likely involved in female specific behaviors such as finding plant hosts for oviposition. Male-predominant ORs (OchiOR34, 46, and 86) may be associated with detecting female sex pheromones. The remaining ORs with roughly equal expression levels in female and male antennae might be dedicated to general odorant detection.

Ionotropic receptors are conserved proteins and play key roles in the synaptic ligand-gated ion channels involved in olfaction, gustation, thermosensation and hygrosensation (Min et al., 2013; Zhang Y.V. et al., 2013; Koh et al., 2014; Chen et al., 2015; Stewart et al., 2015; Croset et al., 2016; Gorter et al., 2016; Hussain et al., 2016; Knecht et al., 2016, 2017; Ni et al., 2016; Prieto-Godino et al., 2016; Ganguly et al., 2017; Tauber et al., 2017). We identified nine antennal IR genes (OchiIR25a, 8a, 76b, 22, 6, 17, 20, 28, and 29) and three iGluRs (OchiGluR8, 9, and 12) from O. chinensis. In general, most antennal IRs were conserved across orders Diptera, Lepidoptera and Coleoptera, but only three IR co-receptor orthologs (OchiIR25a, 8a, and 76b) were found in O. chinensis, and there were no antennal IRs orthologs with D. melanogaster (Benton et al., 2009). In D. melanogaster, antennal IRs function as chemoreceptors (Benton et al., 2009) and are expressed in the peripheral olfactory neurons associated with the detection of amines, acids, general odors and sex pheromones (Ai et al., 2010; Silbering et al., 2011; Kain et al., 2013; Koh et al., 2014; Hussain et al., 2016). Notably, we found that two antennal IR genes (OchiIR28 and 29) were exclusively expressed in antennae, suggesting they potentially involved in odorant reception.

We also identified two SNMP unigenes. SNMPs are conserved throughout holometabolous insects and the SNMP1 subfamily are usually expressed in pheromone-sensitive OSNs, and mediate responses to lipid pheromones (Jin et al., 2008; Nichols and Vogt, 2008; Vogt et al., 2009; Gomez-Diaz et al., 2016). All SNMPs from locust species were clustered into the SNMP1 subclade. Both SNMPs were predominantly expressed in antennae in O. chinensis, supporting that these two SNMPs may perform functions in pheromone perception. Interestingly, OchiSNMP1 was predominantly expressed in female antennae (Figure 7B), suggesting that OchiSNMP1 may participate in sex pheromone reception.

**DATA AVAILABILITY STATEMENT**

The datasets generated for this study can be found in the clean reads from the O. chinensis female antennae and male antennae were deposited in the NCBI Sequence Read Archive (Female antennae: SAMN11484273; Male antennae: SAMN11484274).

**AUTHOR CONTRIBUTIONS**

YC and CK performed the experiments. ZW analyzed the data. ZW and JL wrote and revised the manuscript.
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SUPPLEMENTARY MATERIAL
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FIGURE S2 | The amino acid alignment of the predicted classic OBPs.
FIGURE S3 | The amino acid alignment of the predicted atypical OBPs.
FIGURE S4 | The amino acid alignment of the predicted plus-C OBPs type-A.
FIGURE S5 | The amino acid sequences used for phylogenetic analyses.
TABLE S1 | List of OBPs, CSPs, ORs, IRs, and SNMPs in Orthopetra species.
TABLE S2 | A list of the identified OBPs, CSPs, ORs, IRs and SNMPs in O. chinensis, and detailed genetic characteristics, best matches in NCBI-nr database, protein domains and estimated expression levels found in each petal module.
TABLE S3 | The amino acid sequences for semi-quantitative RT-PCR and RT-qPCR.
FIGURE S5 | Overview of the sequencing and assembly process.
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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