Molecular Characterization of Odorant Receptor Gene *GmolOR7* in *Grapholita molesta* (Lepidoptera: Tortricidae)[1]

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Abstract *Grapholita molesta* Busck (Lepidoptera: Tortricidae) is a major fruit pest of a wide range of Rosaceae trees. Olfaction plays a crucial role in insect behavioral activities, and insects primarily rely on odorant receptors (ORs) to detect odorant molecules. Several olfactory-related genes in *G. molesta* have been studied; however, the ORs of *G. molesta* are unclear. Here, *GmolOR7*, a general OR gene, was cloned and characterized. Sequence alignment and phylogenetic tree analysis showed that *GmolOR7* is closely related to *Cydia pomonella* OR54, with the amino acid sequence identity of 80.21%. Furthermore, the different developmental stages and tissues of *GmolOR7* in *G. molesta* were analyzed by real-time quantitative polymerase chain reaction. The results demonstrated that *GmolOR7* was expressed at higher levels in adults, and particularly in the antennae of females. *GmolOR7* expression was the highest in 3-d-old adults. These results will provide an indication for further functional study of the *GmolOR7* in *G. molesta* and its role in the detection of host-plant volatiles by insects in general.

Key Words *Grapholita molesta*, olfactory gene, odorant receptor, expression profile

Olfaction plays a major role in insects where it assists the insect in locating host and food plants, find mates, identify oviposition sites, and avoid predators (Hallem et al. 2006, Wu et al. 2017). The antennae of insects possess thousands of sensillae with different morphological and functional types, including trichodea, chaetica, basiconica, coeloconica, styloconica, auricillica, and squamiformia. Sensilla trichodea are the most abundant olfactory sensillae on the antennae (Gómez et al. 2003, Zacharuk and Shields 1991). Odorant molecules enter olfactory sensillae through pore tubules in the plasma membranes, passing into the lymph around the olfactory receptor neurons (Carey and Carlson 2011). The olfactory process of insects has been suggested to contain several major peripheral olfactory

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proteins, including odorant receptors (ORs), odorant-binding proteins (OBPs), chemosensory proteins (CSPs), pheromone-binding proteins (PBPs), ionotropic receptors, sensory neuron membrane proteins, and odorant-degrading enzymes (Bian et al. 2018, Jacquin-Joly and Merlin 2004, Leal 2013, Wang et al. 2021). Insects mainly depend on ORs for the detection of odorant molecules (An et al. 2016).

Compared with mammalian ORs, insect ORs have evolved an opposite membrane topology from that of G-protein coupled receptors where the N- and C-terminus are intracellular and extracellular, respectively (Benton et al. 2006, Hansson and Stensmyr 2011). A functional OR is a heterodimeric complex composed of a poorly conserved ligand-binding receptor (OR) and a conserved co-receptor (Orco, formerly Or83b) (Benton 2009). The ligand-binding ORs are classified into pheromone receptors, gustatory receptors, and general ORs (Fleischer et al. 2018). General ORs are used for the detection of plant volatile compounds (Tanaka et al. 2009).

Grapholita molesta Busck (Lepidoptera: Tortricidae), commonly known as the oriental fruit moth, causes severe damage worldwide to pome and stone fruits (Barros-Parada et al. 2018, Wei et al. 2015). The first- and second-generation larvae damage extremely young shoots of peach (*Prunus persica* [L.] Batsch), nectarine (*P. persica* var. *nectarina*), and plum (*Prunus salicina* Lindl) trees, while the third- and fourth-generation larvae mainly feed on the pulp, including that of apple (*Malus pumila* Borkhausen) and pear (*Pyrus* sp,) (Duarte et al. 2015). The adults can switch to a different host by detecting and following changes in host-plant volatiles (Hughes and Dorn 2002, Siegwart et al. 2015). To control *G. molesta*, it may be useful to target host-plant volatile reception by targeting OR genes. The *G. molesta* antennal transcriptome is available, and many olfaction genes have been identified, including 48 ORs, 28 OBPs, and 17 CSPs (Li et al. 2015). Several OBPs, PBPs, and CSPs have been studied (Chen et al. 2018; Li et al. 2016, 2019; Song et al. 2014; Zhang et al. 2012). The ORs of *G. molesta*, however, are poorly understood. In this study, *GmolOR7* was identified and evaluated to determine its expression patterns in *G. molesta*. The results will provide an indication for further functional study of the *GmolOR7* in *G. molesta*.

Materials and Methods

Insects. *Grapholita molesta* were supplied by the College of Plant Protection, Northwest A&F University (Yangling, Shaanxi, China). Larvae and adults were fed on an artificial diet and with a 5% honey solution, respectively. All insects were reared at 25 ± 1°C, 70 ± 10% relative humidity, and a 15:9 h (L:D) photoperiod (Du et al. 2010).

Samples, RNA extraction, and cDNA synthesis. *Grapholita molesta* samples were obtained from various stages of development (eggs; 1st- to 5th-instar larvae; pupae; and 1-d-old adults, both male and female), antennae of 1-, 3-, 5-, and 7-d-old adult females, and different tissues of adults, including antennae, heads without antennae, thoraxes, abdomens, wings, and legs. Tissues were frozen in liquid nitrogen and subsequently stored at −80°C. RNAiso Plus was used for extraction of total RNA (TaKaRa, Dalian, China), and concentration and quality were evaluated by a SimpliNano spectrophotometer (GE Healthcare, London, U.K.) and 1%

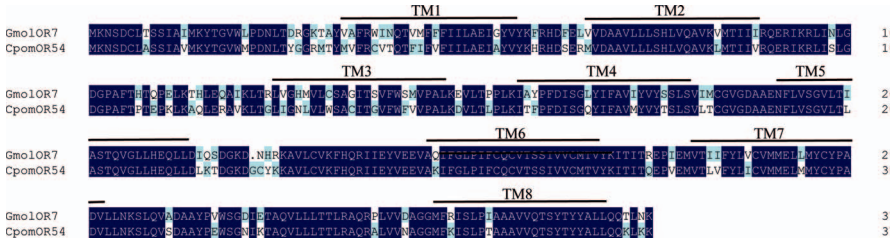

Fig. 1. Alignment of amino acid sequences of *GmolOR7*. Eight transmembrane domains (TM1–TM8) are indicated by solid lines. *GmolOR7* (*Grapholita molesta*, MH844555); *CpomOR54* (*Cydia pomonella*, AFC91717.2).

agarose gel electrophoresis. The RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA) was used for first-strand cDNA synthesis from 1 μg of total RNA following the kit instructions and stored at −20°C.

Molecular cloning. Referring to antennal transcriptome of adult female of *G. molesta* (Li et al. 2015), the *GmolOR7* ORF (open reading frame) was cloned using 3′ RACE (rapid amplification of cDNA ends) polymerase chain reaction (PCR) and ordinary PCR. For 3′ RACE, the 3′ region of *GmolOR7* was amplified as previously described. Synthesis of primers was done by Sangon Biotech (Shanghai, China). The RACE primers were 3′Outer-F: 5′-TTAGTATGCGTGATGATGGA-3′, 3′Outer-R: 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′; 3′Inner-F: 5′-TGCGTGATGATGGAGTTG-3′, 3′Inner-R: 5′-CTAATACGACTCACTATAGGGC-3′. The 20-μL reaction contained 10 μL Premix Taq™ (TaKaRa), 1 μL each primer (10 μM), 1 μL cDNA, and 7 μL RNase-free water. Conditions for the first amplification step were: four cycles at 98°C for 30 s, 64°C for 1 min, 72°C for 2 min; after which the annealing temperature was reduced by 2°C for four cycles. A further 25 cycles at 98°C for 30 s, 56°C for 1 min, 72°C for 2 min, and 72°C for 10 min were performed. The second step was the same as the first step. A Universal DNA Purification Kit (TianGen, Beijing, China) was used to purify the PCR products which were then ligated into the pMD™-19T cloning vector (TaKaRa) and transformed into DH5α competent cells (TianGen). Sequencing of positive clones was done by Sangon Biotech. The primers OR7-F: 5′-ACAGATACTACCATGAAAAA-3′, OR7-R: 5′-TTATCCTTTGTTCAGTGTC-3′ containing the start and stop codons were used for *GmolOR7* ORF amplification.

Quantitative real-time PCR (qPCR). *GmolOR7* expression in the different tissues and stages was evaluated by qPCR with *β-actin* as reference (GenBank number: KF022227.1). The specific primers were as follows: qOR7-F: 5′-GCAGCCTACCCAGTATGGTC-3′, qOR7-R: 5′-TGGGAAGTGAGATCCGAAAC-3′; qActin-F: 5′-TGCGTGACATCAAGGAGAAG-3′, qActin-R: 5′-TACCGATGGTGATGACCTGA-3′. The reaction system contained 10 μL of SYBR® Premix Ex Taq™ II (TaKaRa), 0.8 μL of each primer (10 μM), 2 μL of cDNA, and 6.4 μL of RNase-free water in 20 μL total volume. Triplicate amplifications were conducted on the Bio-Rad CFX96 Real-Time System (Bio-Rad, Hercules, California, USA) as following: 95°C for 30 s, 40 cycles of 95°C for 10 s, 59°C for 30 s, and 72°C for 30 s. Measurements every 0.5°C during the 59–95°C cycle were used to generate melting curves to verify

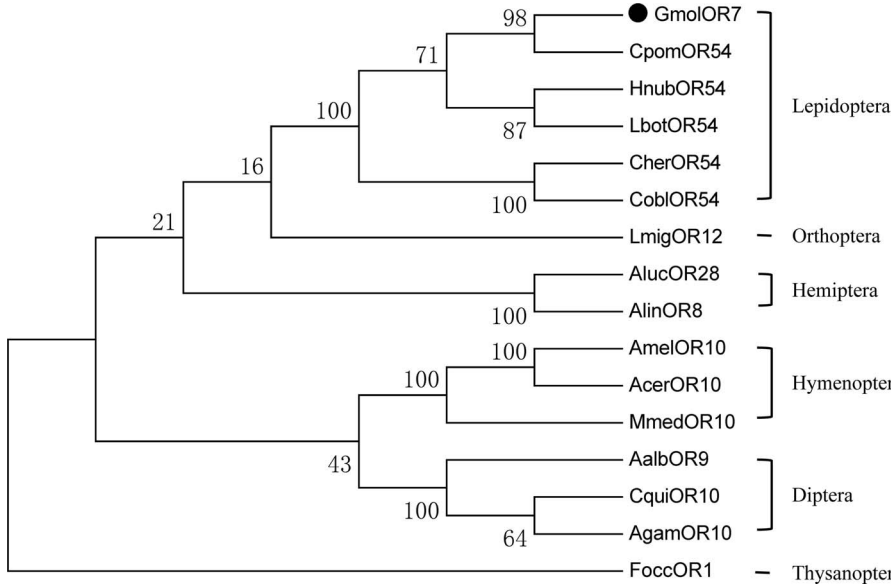

Fig. 2. Phylogenetic tree of *GmolOR7* of *G. molesta* and general odorant receptors (ORs) of several insect species from six orders including Lepidoptera, Diptera, Hymenoptera, Orthoptera, Hemiptera, and Thysanoptera: *GmolOR7* (*Grapholita molesta*, MH844555); *CpomOR54* (*Cydia pomonella*, AFC91717.2); *HnubOR54* (*Hedya nubiferana*, AST36277.1); *LbotOR54* (*Lobesia botrana*, AXF48796.1); *CherOR54* (*Ctenopseustis herana*, AIT69900.1); *CoblOR54* (*Ctenopseustis obliquana*, AIT72010.1); *LmigOR12* (*Locusta migratoria*, ALD51478.1); *AlucOR28* (*Apolygus lucorum*, AKS44362.1); *AlinOR8* (*Adelphocoris lineolatus*, APZ81430.1); *AmelOR10* (*Apis mellifera*, NP_001229890.2); *AcerOR10* (*Apis cerana cerana*, AVE17572.1); *MmedOR10* (*Microplitis mediator*, AGG17942.1); *AalbOR9* (*Aedes albopictus*, JAC10728.1); *CquiOR10* (*Culex quinquefasciatus*, ADF42902.1); *AgamOR10* (*Anopheles gambiae*, ACH95385.1); *FoccOR1* (*Frankliniella occidentalis*, AKF17721.1).

primers. The standard curve was used to evaluate the efficiency of amplification using a 5-fold dilution series of cDNA. Three biological replicates from each sample were used to calculate the relative sample expressions of *GmolOR7* were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Sequence and data analysis. *GmolOR7* orthologs were identified by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The TMHMM Server v 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) was used for transmembrane domain prediction, and the Compute pI/Mw tool (https://web.expasy.org/compute_pi/) was used for the prediction of molecular weight and isoelectric point. Primers were designed by Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/#PRIMER_SEQUENCE_INPUT). Sequence alignment and comparisons were done using DNAMAN 6.0 and ClustalX

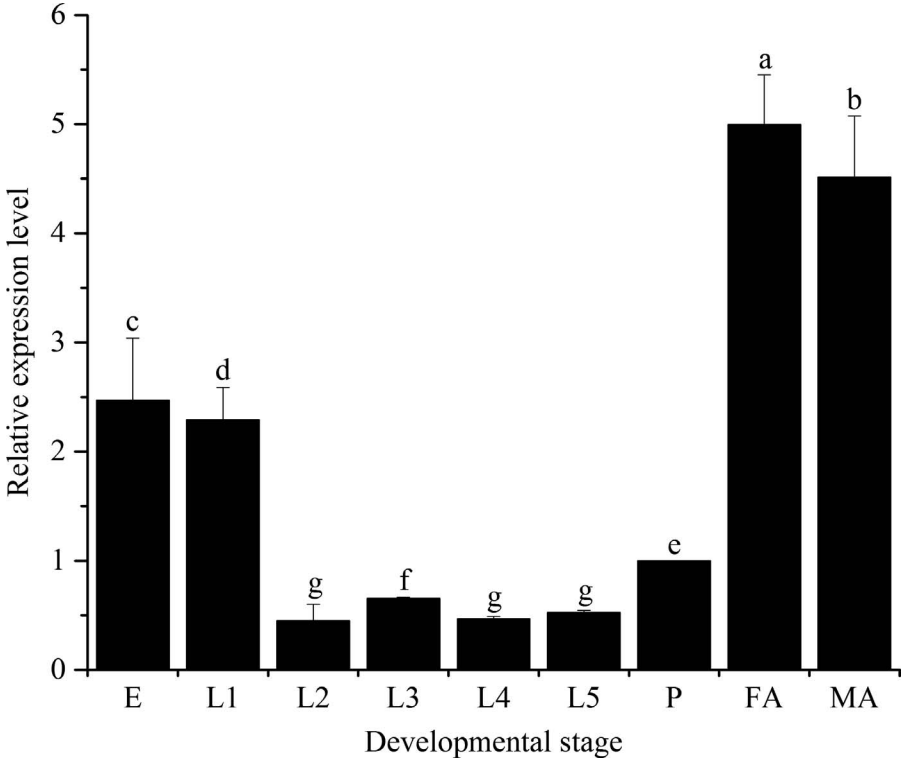

Fig. 3. Relative expression level of *GmolOR7* in different developmental stages of *G. molesta*. E: eggs; L1–L5: 1st- to 5th-instar larvae, respectively; P: pupae; FA: female adults; MA: male adults. Different lowercase letters indicate significant differences among different developmental stages and antennae of adults of different age ($P < 0.05$, Tukey's test).

2.1 software. Phylogeny was evaluated by MEGA 6 software using the neighbor joining method with bootstrap values based on 1,000 replicates at the nodes. Data were analyzed using SPSS 22, and figures were plotted using Origin 2021.

Results

Identification of G. molesta GmolOR7. The *GmolOR7* (GenBank accession number: MH844555) cDNA contained an ORF of 1,125 base pairs, which encoded a protein of 374 amino acids. The molecular weight of the protein was 41.86 kDa and its theoretical isoelectric point of 6.70. Eight transmembrane domains were predicted by sequence analysis with the TMHMM Server v 2.0; the N-terminus was predicted to be intracellular and the C-terminus extracellular (Fig. 1).

Sequence analysis of G. molesta GmolOR7. Sequence alignment showed that the ORF regions in *GmolOR7* and *Cydia pomonella* OR54 (*CpomOR54*)

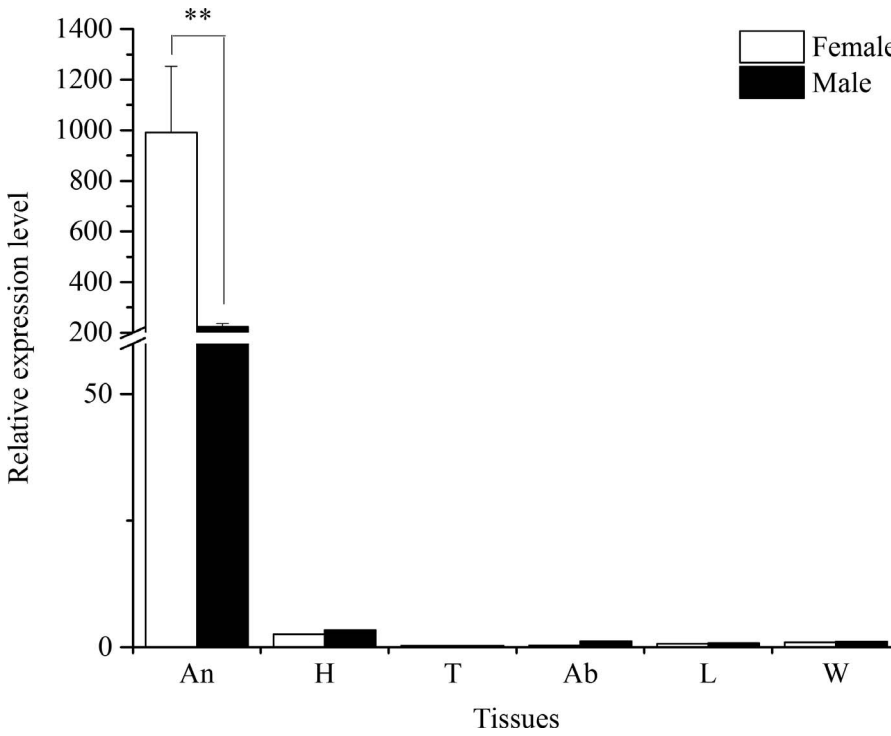

Fig. 4. Relative expression level of *GmolOR7* in different tissues of adult *G. molesta*. An: antennae; H: heads (with antennae removed); T: thoraxes; Ab: abdomens; L: legs; W: wings. The double asterisk indicates extremely significant difference between female and male ($P < 0.01$, independent samples *t*-test).

shared 80.21% sequence identity (Fig. 1). As expected, *GmolOR7* and *CpomOR54* were in the same phylogenetic clade and were closely related (Fig. 2).

Expression profiles of G. molesta GmolOR7. *GmolOR7* was expressed in all developmental stages from eggs to adults, with especially high expression in adults where females showed markedly higher levels than males ($P < 0.05$) (Fig. 3). Expression was predominant in the antennae in both sexes, in comparison with other tissues (heads, thoraxes, abdomens, legs, and wings). Moreover, *GmolOR7* expression in female antennae was significantly higher than that in males ($P < 0.01$). *GmolOR7* also expressed in other tissues, although the levels were very low (Fig. 4). Adult females of all ages showed high levels of expression in antennae, with the highest expression seen in 3-d-old female adults ($P < 0.05$) (Fig. 5).

Discussion

ORs are responsible for detecting host-plant volatiles, indicative of suitable oviposition sites and sources of food (Jordan et al. 2009). Here, a general OR gene

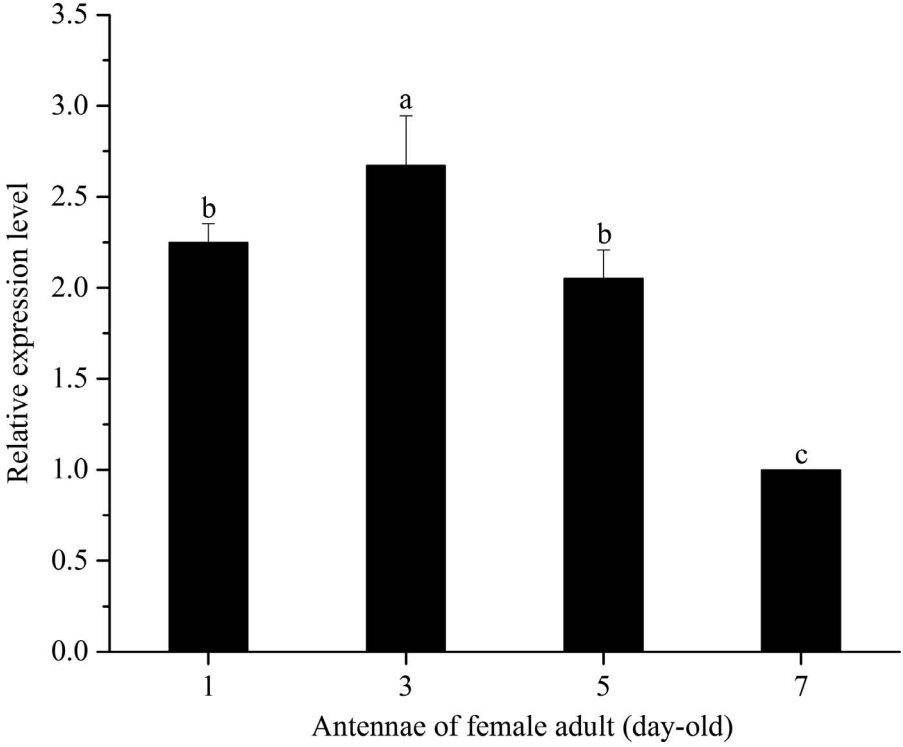

Fig. 5. Relative expression level of *GmolOR7* in antennae of female adults of different ages (day-old). Data were mean ± standard error (SE). Different lowercase letters indicate significant differences among different developmental stages and antennae of adults of different ages ($P < 0.05$, Tukey's test).

GmolOR7 was cloned from the oriental fruit moth. *GmolOR7* was found to have eight transmembrane domains (Fig. 1), which is consistent with the typical characteristics of insect ORs (An et al. 2016, Bengtsson et al. 2012). *GmolOR7* was presented a low homology across several insect species, which was consistent with the previous studies (Hallem et al. 2006, Robertson et al. 2003). Previously, the different ORs were clustered into diverse branches (Chen et al. 2020b, Zhang et al. 2013). In our phylogenetic tree, *GmolOR7* was classified into Lepidoptera (Fig. 2), confirming that providing a foundation for study of the correspondence between different insect species and OR functions.

GmolOR7 was found to be highly expressed in adults (Fig. 3), particularly in the antennae with transcript levels higher in female antennae than male (Fig. 4). The expression profiles of *GmolOR7* were very similar to those of other insect ORs (Sun et al. 2019, Yan et al. 2015, Zhang et al. 2019), suggesting the role of this gene in detecting plant volatiles. Three-day-old females showed the highest expression of *GmolOR7* in their antennae (Fig. 5). Previous studies showed that peak flight, mating, and oviposition activities are maximal in individuals 3 d after eclosion

(Hughes and Dorn 2002, Zhang and Wu 2012). This indicates the vital role of *GmolOR7* in controlling female moth behaviors, such as host-plant location and oviposition site selection.

Generally, diverse OR genes display different functions in the same insect species. For example, *BmorOR56* of *Bombyx mori* L., *AlucOR30* of *Apolygus lucorum* (Meyer-Dur), and *AlepOR3* of *Athetis lepigone* (Möschler) specifically respond to *cis*-jasmone, (1*S*)-(-)-verbenone, and *Z*7-12:AC, respectively. Nevertheless, *BmorOR24* of *B. mori* and *AlucOR28* of *Apolygus lucorum* broadly tune to several volatiles, and *AlepOR4* of *Athetis lepigone* responds to five sex pheromones (Tanaka et al. 2009, Yan et al. 2015, Zhang et al. 2019). In *G. molesta*, *GmolOR9* and *GmolOR12* responded to eight and five host-plant volatiles, respectively. *GmolOR20* and *GmolOR21* had no responses to any of the odorant compounds tested (Chen et al. 2020a, 2020b). Consequently, *GmolOR7* may be involved in the recognition of host-plant volatiles, and *GmolOR*s and *GmolOR7* have different functions.

In summary, *GmolOR7* shows similar functional properties to general ORs. These results improve the understanding of OR characteristics and provide an indication for further functional study of the *GmolOR7* in *G. molesta*.

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