Journal of Integrative Agriculture 2022, 21(7): 2042–2054

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RESEARCH ARTICLE

A conserved odorant receptor identified from antennal transcriptome of *Megoura crassicauda* that specifically responds to *cis*-jasmone

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Abstract

Herbivore-induced plant volatiles (HIPVs) play a key role in the interactions between plants and herbivorous insects, as HIPVs can promote or deter herbivorous insects' behavior. While aphids are common and serious phloem-feeding pests in farmland ecosystems, little is known about how aphids use their sensitive olfactory system to detect HIPVs. In this study, the antennal transcriptomes of the aphid species *Megoura crassicauda* were sequenced, and expression level analyses of *M. crassicauda* odorant receptors (ORs) were carried out. To investigate the chemoreception mechanisms that *M. crassicauda* uses to detect HIPVs, we performed *in vitro* functional studies of the ORs using 11 HIPVs reported to be released by aphid-infested plants. In total, 54 candidate chemosensory genes were identified, among which 20 genes were ORs. *McraOR20* and *McraOR43* were selected for further functional characterization because their homologs in aphids were quite conserved and their expression levels in antennae of *M. crassicauda* were relatively high. The results showed that McraOR20 specifically detected *cis*-jasmone, as did its ortholog ApisOR20 from the pea aphid *Acyrthosiphon pisum*, while McraOR43 did not respond to any of the HIPV chemicals that were tested. This study characterized the ability of the homologous OR20 receptors in the two aphid species to detect HIPV *cis*-jasmone, and provides a candidate olfactory target for mediating aphid behaviors.

Keywords: *Megoura crassicauda*, transcriptome, chemosensory genes, odorant receptors, *cis*-jasmone

1. Introduction

Insects rely on their highly sensitive sensory system to distinguish various chemical signals in a complex natural environment and trigger relevant behavioral responses, such as host plant location, mate selection and predator avoidance (Bruce *et al.* 2005; Fleischer and Krieger 2018). The accurate detection of chemical signals

Received 15 January, 2021 Accepted 16 April, 2021
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doi: 10.1016/S2095-3119(21)63712-7

by insects is mainly accomplished by the products of several gene families, including odorant receptors (ORs), gustatory receptors (GRs), ionotropic receptors (IRs), odorant-binding proteins (OBPs), and chemosensory proteins (CSPs) (Clyne *et al.* 1999, 2000; Galindo and Smith 2001; Pelosi *et al.* 2006; Benton *et al.* 2009).

During olfactory reception, OBPs and CSPs are responsible for binding hydrophobic odorants, surrounding olfactory sensory neurons (OSNs) and transporting the odorants to chemosensory receptors located on the dendrites of OSNs (Sandler *et al.* 2000; Sun *et al.* 2018). The OR, GR and IR gene families are the key receptors in the chemosensory system (Jacquin-Joly and Merlin 2004). OR genes usually co-express with odorant receptor co-receptor (Orco), forming a ligand-gated channel that plays a role in the transduction of chemical signals (Joseph and Carlson 2015). They are widely tuned to various chemicals that exist in the environment, such as alcohols, esters, and ketones (Cao *et al.* 2020; Liu *et al.* 2020b). GRs are mainly expressed in gustatory organs such as the mouthparts and are involved in taste and contact stimuli (Chyb 2004). They are responsible for the detection of non-volatile compounds such as sugars, salts, and bitter compounds, and even for the detection of carbon dioxide (CO_2) (Jones *et al.* 2006; Dahanukar *et al.* 2007; Jiao *et al.* 2007; Sung *et al.* 2017). IRs constitute a relatively newly described chemosensory receptor gene family (Benton *et al.* 2009). Recent functional studies of IRs have revealed that they are capable of perceiving a wide range of environmental factors, including odorants, humidity and temperature (Hassan *et al.* 2016; Chen and Amrein 2017; Knecht *et al.* 2017; Budelli *et al.* 2019).

Aphids are phytophagous hemipteran insects, with around 5 000 species that are globally distributed. Some of them are primary crop pests in many regions, causing major economic losses (Pickett *et al.* 2003). Like other insects, aphids use their chemosensory system to accurately locate host plants and detect their intraspecific alarm signals (Fan *et al.* 2017; Li *et al.* 2018). With the development of sequencing technology, numerous aphid genomes and transcriptomes have been sequenced, providing essential resources for mining chemosensory genes. OBPs and CSPs have been identified by genome and transcriptome analyses in many aphid species, including *Aphis gossypii* (Gu *et al.* 2013), *Acyrthosiphon pisum* (Zhou *et al.* 2010), *Myzus persicae* (Wang *et al.* 2019), *Daktulosphaira vitifoliae* (Zhao *et al.* 2017), *Megoura viciae* (Bruno *et al.* 2018), and *Sitobion avenae* (Xue *et al.* 2016). Most functional studies focus on investigating the detection of plant volatiles and aphid alarm pheromones such as (*E*)-β-farnesene (EBF) and EBF derivatives (Qiao *et al.* 2009; Zhong *et al.* 2012; Northey *et al.* 2016; Fan *et al.* 2017; Qin *et al.* 2020).

ORs, GRs, and IRs have been identified from the genomes of *A. gossypii*, *A. pisum*, and *Aphis glycines*, each showing significant differences in the numbers of genes. The OR gene family of *A. pisum* (87 ORs) is almost twice as large as those of *A. glycines* and *A. gossypii* (47 and 45 ORs, respectively), resulting from the recent expansions of particular gene lineages in *A. pisum*. IRs are presented as simple orthologs between different aphid species. For example, 14 IRs are found in *A. gossypii*, while 19 IRs are found in both *A. pisum* and *A. glycines* (Smadja *et al.* 2009; Cao *et al.* 2014; Robertson *et al.* 2019). Although chemosensory receptors have been reported extensively, only a few have been functionally characterized. Previous studies have shown that ApisOR5 is responsible for EBF detection (Zhang *et al.* 2017) and ApisOR4 could respond to various plant volatiles (Zhang *et al.* 2019). The *SaveOrco* gene is not only involved in the olfactory response to plant volatiles and EBF, but also in wing differentiation triggered by EBF (Fan *et al.* 2015).

When aphids feed on plants, plant defense responses are triggered by the systemic release of various secondary metabolites, some of which are volatile compounds commonly referred to as herbivore-induced plant volatiles (HIPVs) (Turlings and Tumlinson 1992). HIPVs usually consist of green leaf volatiles (GLVs), which are six-carbon chemical compounds, and terpene volatiles, as well as a few other commonly released volatiles (e.g., methyl salicylate) (Abuin *et al.* 2011; Kroes *et al.* 2017; Turlings and Erb 2018). Studies have shown that a series of volatiles, including ocimene, α-pinene, *trans*-2-hexen-1-ol, *cis*-3-hexen-1-ol, linalool, and (–)-*trans*-caryophyllene, could be released from the broad bean plant *Vicia faba* after infestation with *A. pisum* and *Aphis craccivora* (Schwartzberg *et al.* 2011; Takemoto and Takabayashi 2015); and potatoes infested by *Macrosiphum euphorbiae* produced *cis*-jasmone, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), *trans*-2-hexenal and methyl salicylate (Sobhy *et al.* 2017). Numerous HIPV compounds are also reported to be associated with aphid repellence (Birkett *et al.* 2000; Hegde *et al.* 2012), illustrating the important role of HIPVs in mediating plant–aphid interactions. However, the olfactory coding of aphids to candidate HIPVs in the peripheral nervous system remains unknown.

In this study, we selected the legume specialist aphid species *Megoura crassicauda*, a close relative of *M. viciae* (Kim and Lee 2008), to perform antennal transcriptome

sequencing. Chemosensory-related genes including ORs, GRs, IRs, OBPs, and CSPs were identified in the transcriptomes. Expression level analyses were carried out for all identified OR genes. Assuming that the interactions of ORs with structurally related HIPVs are conserved across aphid species, the homologous OR sequences from different aphid species were selected for functional testing. We therefore selected the full-length *McraOR20* and *McraOR43* genes, which share high sequence homology with those of *A. pisum* and/or exhibited high expression levels, for further analysis. To verify our hypothesis, we cloned the full-length OR20 and OR43 genes, based on sequences from the *M. crassicauda* transcriptome, and characterized the functions of both McraOR20 and McraOR43 with 11 HIPVs using a two-electrode voltage clamp technique. This study preliminarily reveals the molecular mechanism of HIPV detection in aphids.

2. Materials and methods

2.1. Insect rearing and tissue collection

Megoura crassicauda aphids were fed on broad bean (*Vicia faba* L.) plants and maintained at (21±2)°C with (70±5)% relative humidity and a photoperiod of 16 h light:8 h dark. Clonal populations were reared at the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing. Antennae and legs were dissected from apteral parthenogenetic adults, then instantly frozen and stored in liquid nitrogen.

2.2. cDNA library construction and Illumina sequencing

Total RNA was extracted using Quick-RNA Microprep Kit (Beijing Tianmo Biotech Company Limited, China). Total RNA was dissolved in RNase-free water, and the integrity of the RNA was determined by gel electrophoresis. RNA purity and concentration were determined on a Nanodrop ND-2000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA). Three replicates containing tissues excised from 250 and 150 parthenogenetic adult females were generated for antennae and legs, respectively. A total of 2 μg total RNA from each sample (antenna (A1–A3), leg (L1–L3)) were used to construct the cDNA libraries. Library construction and Illumina sequencing were carried out by the Beijing Genome Institute (Shenzhen, China). The libraries were sequenced on the Illumina NoveSeq 6000 (Illumina, San Diego, CA, USA) platform, generating 200-bp long, paired-end reads.

2.3. Assembly and annotation of chemosensory-related genes

The clean reads were generated by removing low-quality reads and adaptors, and are available in the NCBI SRA database (Project number PRJNA674404). The clean reads were *de novo* assembled using Trinity v2.4.0 (Grabherr *et al.* 2011). TGICL v2.1 (Pertea *et al.* 2003) was used to filter duplicate and highly similar sequences from each sample (A1–A3 and L1–L3) to obtain the final assembly. For functional annotation, all transcripts were selected as queries for BLASTX searches against a pooled database of non-redundant (NR) and SwissProt protein sequences with an E-value cutoff set at 1E–5 (Wang *et al.* 2017; Liu *et al.* 2020a). To identify chemosensory-related genes, we integrated the traditional BLAST-based method with a domain-based search approach. First, we selected transcripts of chemosensory-related genes from the functional annotation results according to specific keywords. For example, when screening for ORs, we extracted transcripts that were annotated as 'odorant receptor' or 'olfactory receptor'. Next, we compiled the chemosensory-related genes of other aphid species from previous studies (Robertson *et al.* 2019; Wang *et al.* 2019), used these genes as queries for BLASTN searches against all transcripts with E-values<1E–10, and selected the transcripts with the most hits. Furthermore, we used the HMMER v3.1 Software (Mistry *et al.* 2013) to search for genes containing functional domains of ORs or GRs from the transcript datasets. Characteristic domains of ORs (ID no. PF02949) and GRs (ID no. PF08395) were downloaded from the Pfam protein families database (El-Gebali *et al.* 2018). Finally, candidate chemosensory genes identified using the above methods were merged and redundancies were removed, and the remaining transcripts were selected for analyses in the next step.

2.4. Sequence and phylogenetic analysis

The SignalP v4.0 server was used for predicting putative N-terminal signal peptides of OBPs and CSPs (Petersen *et al.* 2011). Amino acid sequence alignment was executed using MAFFT v7 (Katoh and Standley 2013) with default parameters. The phylogenetic trees of *M. crassicauda* chemosensory genes were constructed by RAxML v8 using the Jones-Taylor-Thornton (JTT) amino acid substitution model (Stamatakis 2014). Branch support was assessed by a bootstrap method based on 1 000 replicates. The dataset submitted for phylogenetic analysis consisted of the annotated

M. crassicauda chemosensory genes, as well as previously reported sequences of ORs and GRs from *A. pisum* and *A. glycines* (Robertson *et al.* 2019); IRs from *Drosophila melanogaster* (Benton *et al.* 2009; Croset *et al.* 2010), *A. pisum* and *A. glycines* (Robertson *et al.* 2019); and OBPs and CSPs from *Myzus persicae* (Wang *et al.* 2019), *Sitobion avenae* (Xue *et al.* 2016), *A. pisum* (Zhou *et al.* 2010), *A. glycines* (Robertson *et al.* 2019) and *A. gossypii* (Gu *et al.* 2013). DNAMAN v8 (Lynnon LLC, San Ramon, CA, USA) was used to obtain amino acid sequence alignments of the orthologous ORs between *A. pisum* and *M. crassicauda*. Putative chemosensory-related genes of *M. crassicauda* were named based on their homologs in the pea aphid *A. pisum*.

2.5. Tissue-specific expression analysis

Bowtie2 v2.2.5 was used to map the clean reads to the chemosensory-related transcripts (Langmead and Salzberg 2012). RSEM v1.2.12 was applied to count the fragments per kilobase per million fragments (FPKM) values of each transcript (Li and Dewey 2011). Semi-quantitative reverse transcription PCR (RT-PCR) was also employed to analyze the expression level of putative OR genes. Tissue samples from three biological replicates were collected from the antennae and legs of asexual females. Total RNA was extracted using the Quick-RNA Microprep Kit (Beijing Tianmo Biotech Company Limited, China). The cDNA was synthesized using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) based on the manufacturer's protocol. Gene-specific primers were designed by Primer 3 (Untergasser *et al.* 2012) and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) (Appendix A). Easy*Taq* PCR SuperMix was used for PCR reactions, using cycling conditions of 33 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The nicotinamide adenine dinucleotide (NADH) dehydrogenase gene of *A. pisum* (GenBank accession no. NM_001162436) (Yang *et al.* 2014) was used as the query for identifying its ortholog in *M. crassicauda*, and the identified NADH gene was named as *McraNADH*, which was then used as a control gene.

2.6. Molecular cloning

The open reading frames (ORFs) of *McraOR20*, *McraOR43*, *McraOrco*, *ApisOrco* and *ApisOR20* were used for cloning. The 50-μL PCR reaction system consisted of 0.25 μL TaKaRa *EX Taq* (5 U μL^{-1}), 2 μL cDNA template, 5 μL 10× *EX Taq* buffer (Mg^{2+} plus) (20 mmol L^{-1}), 4 μL dNTP mixture (2.5 mmol L^{-1} of each dNTP), and 10 μmol L^{-1} of each primer (Appendix A). PCR conditions were as follows: 94°C for 3 min; 35 cycles of 98°C for 10 s, 55°C for 30 s, 72°C for 90 s, and 72°C for 5 min. PCR products were ligated into the pEASY-T3 vector (TransGen Biotech, Beijing, China), then sequenced by the Beijing Genome Institute (Shenzhen, China).

2.7. Chemical compounds

The 11 representative compounds used in this study are listed in Appendix B. All compounds are HIPVs released by *V. faba*, which was used as the food resource for *M. crassicauda* in this study (Schwartzberg *et al.* 2011; Takemoto and Takabayashi 2015). These HIPVs consist of three types of chemical compounds, including GLVs (*trans*-2-hexenal, *cis*-3-hexen-1-ol, *trans*-2-hexen-1-ol), terpenes (ocimene, (–)-*trans*-caryophyllene, linalool, α-pinene, DMNT, TMTT), and aliphatic compounds (*cis*-jasmone and methyl salicylate).

2.8. Receptor expression in *Xenopus* oocytes and electrophysiological recordings

The ORFs of *McraOR20*, *McraOR43*, *McraOrco*, *ApisOR20*, and *ApisOrco* were subcloned into the pT7TS vector using the ClonExpressII One Step Cloning Kit (Vazyme Biotech Co., Ltd., China). Specific primers with Kozak consensus sequences are listed in Appendix A. cRNAs were synthesized using the mMESSAGE mMACHINE T7 Kit (Ambion, Austin, TX, USA). Mature, healthy *Xenopus* oocytes were pre-treated in line with a previous study (Zhang *et al.* 2017). A mixture of 27.6 ng of OR cRNA and 27.6 ng of Orco cRNA was microinjected into the oocytes, which were then cultured for 4–7 days at 18°C in Ringer's solution (Liu *et al.* 2020a). Currents triggered by the odorants were recorded by a two-electrode voltage clamp (TEVC). The data generated from TEVC were collected and analyzed using Digidata 1440A and pCLAMP v10.2 Software (Axon Instruments Inc., Union City, CA, USA).

Each odorant used for recording was prepared as a 1 mol L^{-1} stock solution in dimethyl sulphoxide (DMSO) and stored at −20°C. The stock solutions were diluted in 1× Ringer's buffer to a final concentration of 1×10^{-4} mol L^{-1} for response profile experiments with six replicates. For dose-response recording, serial dilutions of odorant stock solution were made at 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, 3×10^{-6}, 1×10^{-5} and 3×10^{-5} mol L^{-1}, and six replicates were recorded. Data were analyzed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Antennal transcriptome sequencing and assembly

The three libraries of antennae of *M. crassicauda* (Mcra_Al_1, Mcra_Al_2 and Mcra_Al_3) were sequenced on the Illumina NoveSeq 6000 Platform. After filtering out low-quality and adaptor-polluted reads, 37.95, 40.33, and 39.82 million clean reads of each library were generated, respectively. The datasets generated for this study can be found in NCBI BioProject PRJNA674404. *De novo* assemblies led to the generation of 15 566, 16 765, and 18 047 transcripts in each replicate, respectively. Sequences from the three libraries were then merged and clustered into one set containing 15 984 transcripts. The final dataset had a total length of 47 860 781, average length of 2 994, N50 of 3 711 bp, and GC content of 34.44% (Appendix C).

3.2. Identification of candidate chemosensory genes, phylogenetic analysis, and homology analysis

A total of 54 candidate chemosensory genes were identified from the transcriptomes of *M. crassicauda*. We identified 20 candidate ORs in the transcriptomes, among which six ORs and one Orco contained complete ORFs, ranging from 369 to 463 amino acids (aa) (Appendix D). Phylogenetic analysis revealed that some clades which consisted of the ORs from *M. crassicauda*, *A. pisum* and *Aphis glycines* were highly conserved, such as the Orco, OR2, OR4, OR5, and OR20 clades. The species-specific expansion was observed in the OR subfamilies of *A. pisum* and *Aphis glycines*. However, we did not find any OR expansion in *M. crassicauda* (Fig. 1). To investigate the homology between the ORs of *M. crassicauda* and *A. pisum*, seven full-length McraORs were selected for performing amino acid alignment with their *A. pisum* orthologous genes. Besides Orco, four of the six ORs showed significantly high identities (greater than 80%) with their *A. pisum* orthologs, including OR43 (90.05%), OR20 (89.29%), OR37 (88.94%) and OR5 (86.65%) (Table 1).

Four GRs were annotated from the transcriptomes, but only McraGR1 produced a full-length ORF of 419 aa, while McraGR5 and McraGR21 lacked the 5′ end of the ORF, and McraGR20 lacked both ends of the ORF (Appendix E). Phylogenetic analysis using the GRs of *A. glycines*, *A. pisum* and *M. crassicauda* showed that McraGR1 and McraGR5 were clustered with putative sugar receptors (Robertson 2015) (Fig. 2).

We identified nine putative IRs, and the complete

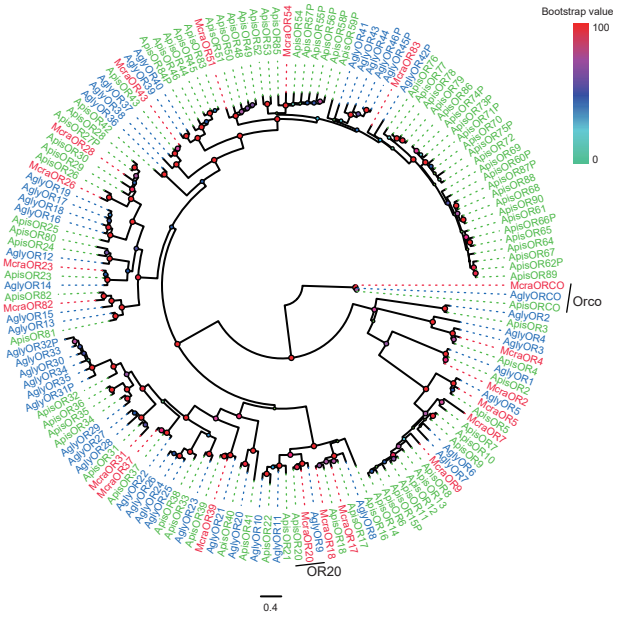

Fig. 1 Phylogenetic tree of candidate odorant receptors (ORs) in *Aphis glycines* (Agly), *Acyrthosiphon pisum* (Apis) and *Megoura crassicauda* (Mcra). The distance tree was rooted by the conserved lineage of Orco.

Table 1 Homology analysis of odorant receptors between *Megoura crassicauda* and *Acyrthosiphon pisum*

M. crassicauda	*A. pisum*	Identity (%)
McraOrco	ApisOrco	95.68
McraOR43	ApisOR43	90.05
McraOR20	ApisOR20	89.29
McraOR37	ApisOR37	88.94
McraOR5	ApisOR5	86.65
McraOR51	ApisOR51	68.51
McraOR7	ApisOR7	49.34

ORFs of three IRs were predicted (Appendix F). Based on a phylogenetic analysis of the IRs with the fly species *D. melanogaster* and other aphid species, we annotated two putative co-receptors, McraIR8a and McraIR25a, the conserved IR clades McraIR40a, McraIR75d, McraIR75j, and McraIR93a; as well as divergent IRs in the aphid, McraIR323 and McraIR325. We failed to identify any IRs from the IR21a, IR68a, IR76b, IR 100a, IR322 and IR324 subsets (Fig. 3).

A total of 12 OBPs were identified in *M. crassicauda*, all of which contained full-length ORFs. Eight putative OBPs in *M. crassicauda* (McraOBP2–10) showed high identities with the OBPs of *M. viciae*. A signal peptide was predicted in every OBP, except for McraOBP6 (Appendix G). The phylogenetic tree showed that 10 of the 12 McraOBPs had clear orthologues in other species as the amino acid identities of OBPs among

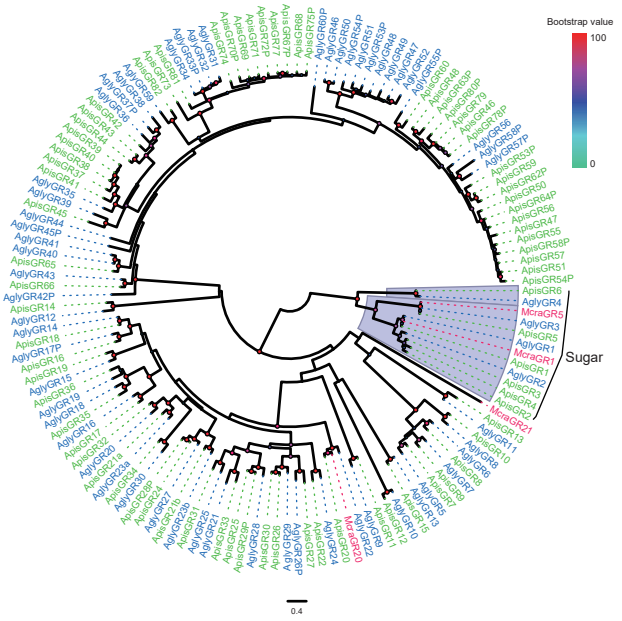

Fig. 2 Phylogenetic tree of candidate gustatory receptors (GRs) in *Aphis glycines* (Agly), *Acyrthosiphon pisum* (Apis) and *Megoura crassicauda* (Mcra). The distance tree was rooted by the lineage of putative sugar receptors.

aphids ranged from 70.95 to 96.71%, while McraOBP14 and McraOBP15 did not display any homologs (Fig. 4). Ten identified OBPs belonged to the classic OBP subfamily that typically contains six conserved cysteine residues, while McraOBP5 and McraOBP6 belonged to the plus-C OBP subfamily containing one additional cysteine as well as a conserved proline next to the sixth cysteine (Appendix H). Nine candidate CSP genes containing full-length ORFs were identified. Among all the CSPs in *M. crassicauda*, only McraCSP1 lacked a signal peptide (Appendix I). A phylogenetic tree was constructed using the amino acid sequences of CSPs from six aphid species, and the results showed that all the McraCSPs were clustered with CSPs from other aphids and did not show any expansion in this subfamily (Fig. 5). The sequence alignment results showed that all the identified CSPs possess four conserved cysteines (Appendix J).

3.3. Tissue-specific expression patterns of candidate OR genes from *M. crassicauda*

Expression profiles of the candidate OR genes in the

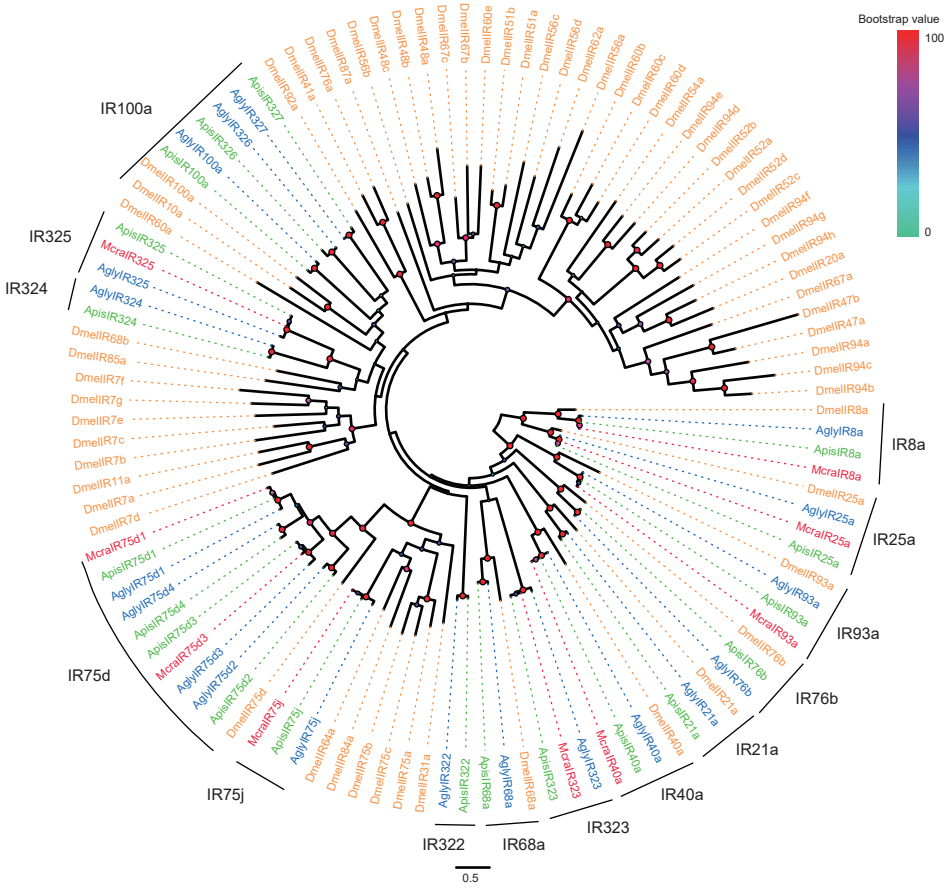

Fig. 3 Phylogenetic tree of candidate ionotropic receptors (IRs) in *Aphis glycines* (Agly), *Acyrthosiphon pisum* (Apis), *Megoura crassicauda* (Mcra) and *Drosophila melanogaster* (Dmel). The distance tree was rooted by the IR8a and IR25a clade.

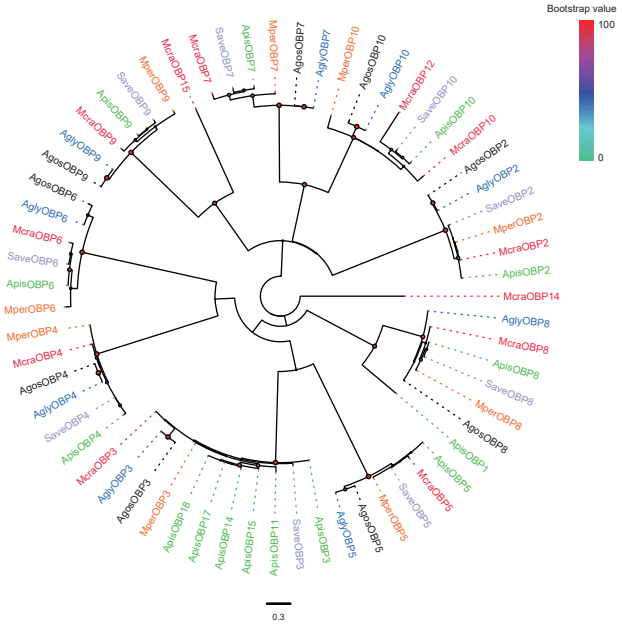

Fig. 4 Phylogenetic tree of candidate odorant binding proteins (OBPs) in *Aphis glycines* (Agly), *Aphis gossypii* (Agos), *Acyrthosiphon pisum* (Apis), *Megoura crassicauda* (Mcra), *Myzus persicae* (Mper) and *Sitobion avenae* (Save).

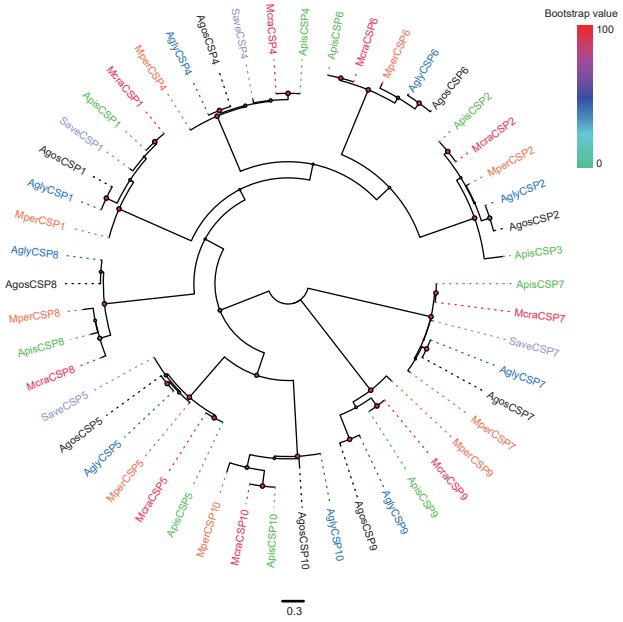

Fig. 5 Phylogenetic tree of candidate chemosensory proteins (CSPs) in *Aphis glycines* (Agly), *Aphis gossypii* (Agos), *Acyrthosiphon pisum* (Apis), *Megoura crassicauda* (Mcra), *Myzus persicae* (Mper) and *Sitobion avenae* (Save).

transcriptomes of antennae and legs were assessed using FPKM values. All the ORs presented significantly high expression levels in antennae, with *McraOrco*, *McraOR4*, *McraOR7*, *McraOR20*, *McraOR26* and *McraOR28* possessing relatively high transcript abundances. We also found that some of the ORs were expressed at significantly lower levels in legs, including *McraOR4*, *McraOR18*, *McraOR26* and *McraOR54* (Appendices K and L). The tissue expression patterns of ORs were further investigated by RT-PCR. A total of 20 putative OR genes, as well as the control gene *McraNADH,* were examined. High expression level of *McraOrco*, *McraOR7*, *McraOR20* and *McraOR26* was observed, which is generally matched with those generated by FPKM (Fig. 6).

3.4. Functional characterization of McraOR20/Orco and McraOR43/Orco in the *Xenopus* oocyte expression system

We cloned McraOR20 and McraOR43 for functional analysis, as they produced full-length ORFs, displayed strong homology with their orthologs in *A. pisum*, and also showed relatively high expression levels in the antennae. These characteristics indicated that McraOR20 and McraOR43 may undertake important functions in chemoreception by *M. crassicauda*. In total, eleven HIPV compounds (Appendix B), including DMNT, TMTT, ocimene, etc., were used to test the functions of McraOR20 and McraOR43 using TEVC technology. The results demonstrated that oocytes co-expressing McraOR20/Orco gave strong responses to 1×10^{-4} mol L^{-1} *cis*-jasmone, with an average current of (280.5±49.54) nA, but none of the other tested chemicals evoked responses of McraOR20/Orco at the same concentration (Fig. 7-A and B). Moreover, the McraOR20/Orco response to *cis*-jasmone was concentration-dependent, from a threshold concentration of 1×10^{-8} mol L^{-1} to a final concentration of 3×10^{-5} mol L^{-1}, and with an EC_{50} value of 1.116×10^{-6} mol L^{-1} (Fig. 7-C and D). We also recorded responses of ApisOR20, the ortholog of McraOR20 in *A. pisum*. As expected, among the tested chemicals, only *cis*-jasmone elicited a response from ApisOR20/Orco ((410.6±96.24) nA; Fig. 7-B). A TEVC test for McraOR43/Orco was carried out in parallel. Surprisingly, McraOR43/Orco did not respond to any of the tested HIPV chemicals, suggesting that McraOR43 may not be involved in HIPV-detection (Appendix M).

4. Discussion

In the present study, we identified a total of 33 putative chemosensory receptor genes, including 20 ORs, four GRs, and nine IRs, in the antennal transcriptomes of *M. crassicauda*. However, a previous study showed that more chemosensory receptors were annotated from the

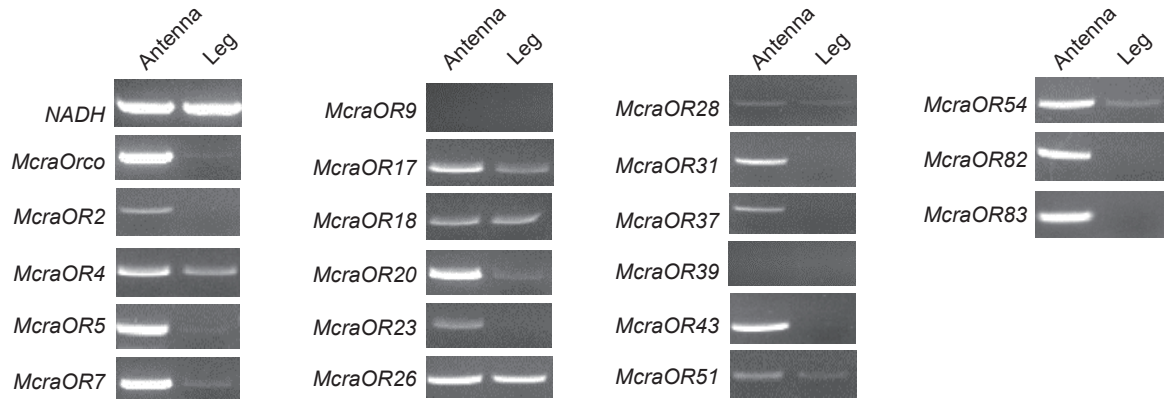

Fig. 6 Tissue-specific expression levels of odorant receptor genes in *Megoura crassicauda* (Mcra).

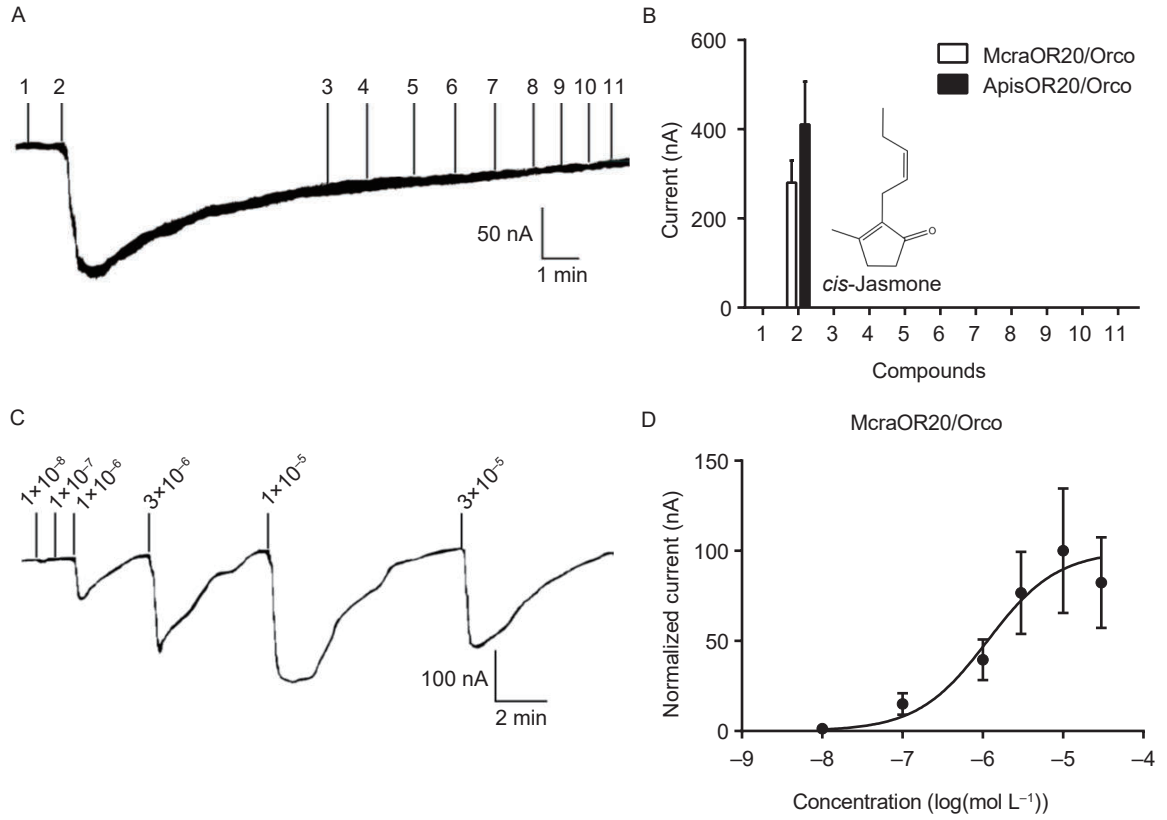

Fig. 7 Functional characterization of McraOR20/Orco and ApisOR20/Orco co-expressed in *Xenopus* oocytes to 11 herbivore-induced plant volatile (HIPV) compounds. A, inward current responses of McraOR20/Orco to the tested HIPVs (1×10^{-4} mol L^{-1}). B, response profiles of McraOR20/Orco and ApisOR20/Orco co-expressed in *Xenopus* oocytes in response to the tested compounds. C, inward current dose-responses of McraOR20/Orco activated by *cis*-jasmone. D, dose-response curve of McraOR20/Orco to *cis*-jasmone. The tested HIPV compounds are as follows: 1, (–)-*trans*-caryophyllene; 2, *cis*-jasmone; 3, *trans*-2-hexenal; 4, ocimene; 5, linalool; 6, *cis*-3-hexen-1-ol; 7, *trans*-2-hexen-1-ol; 8, α-pinene; 9, (*E*)-4,8-dimethyl-1,3,7-nonatriene; 10, methyl salicylate; 11, (*E*, *E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. Error bars indicate mean±SE (*n*=6).

genomes of *A. glycines* and *A. pisum* (Robertson *et al.* 2019). There are two possible reasons for the reduced number of chemosensory receptor genes obtained from the transcriptomes used in this study. First, transcripts with low expression levels are often only partially assembled, or even lost from the assembly. Studies on the sensilla morphology of aphids have presented a limited number of sensilla, also indicating that the types

of sensilla in aphids are simple (De Biasio *et al.* 2015; Bruno *et al.* 2018), suggesting a relatively low abundance of these receptor genes in the aphid antennae. Second, chemosensory receptor genes exhibit tissue expression specificity. The paucity of GRs in antennal transcriptomes may be attributed to the specific expression of GRs in mouthparts (Chen *et al.* 2016).

Phylogenetic analysis of the OR family shows that McraOR5 is orthologous to ApisOR5, which is reportedly responsible for alarm pheromone detection in *A. pisum* (Zhang *et al.* 2017). Amino acid sequence alignment also revealed a relatively high sequence identity (86.65%) between these two ORs, indicating that McraOR5 may also respond to EBF. We also found that McraOR4 clusters phylogenetically with another functionally investigated OR, ApisOR4 (Zhang *et al.* 2019), suggesting that they may share a similar odorant detection profile. GRs are responsible for detecting non-volatile compounds, such as sugars and caffeine (Moon *et al.* 2006; Dus *et al.* 2011). Two GRs (McraGR1 and McraGR5) clustered in the putative sugar receptor lineage inferred from a large-scale phylogenetic analysis of different insects (Robertson 2015), which suggests that GR1 and GR5 may be responsible for sugar detection in *M. crassicauda*. Several studies in *Drosophila* show that IR25a, IR40a, and IR93a are involved in the perception of humidity (Knecht *et al.* 2016), and the IRs in the IR75 clade are reportedly responsible for the detection of acids (Prieto-Godino *et al.* 2017). Hence, the functions of aphid IRs can be inferred from their homologies with *Drosophila* IRs.

Twelve OBPs and nine CSPs were identified in the present transcriptomic analysis, which is comparable to the numbers of OBPs and CSPs found in other aphids, such as *A. glycines*, *A. gossypii* and *S. avenae* (Gu *et al.* 2013; Xue *et al.* 2016; Robertson *et al.* 2019). The number of OBPs in *M. crassicauda* was less than that of *A. pisum* (Robertson *et al.* 2019). Full-length assemblies were obtained for all OBP and CSP transcripts, suggesting they may be abundantly expressed in the antennae. OBPs have been reported to be essential for olfactory reception of the alarm pheromone (Pelosi *et al.* 2018). A series of studies also revealed that OBP3, OBP7, and OBP9 possess a high affinity to EBF (Qiao *et al.* 2009; Zhong *et al.* 2012; Northey *et al.* 2016; Fan *et al.* 2017; Qin *et al.* 2020). Phylogenetic analysis revealed that the OBP3, OBP7, and OBP9 clades are considerably conserved among six aphid species. Therefore, we hypothesize that McraOBP3, McraOBP7, and McraOBP9 may also be capable of binding EBF, consequently playing vital roles in EBF detection by *M. crassicauda*. Aside from the OBPs clustered in conserved clades, we also identified two OBPs, McraOBP14 and McraOBP15, which did not show orthologous relationships with other OBPs, suggesting they may be involved in the binding of volatiles that specifically exist in the niche of *M. crassicauda*.

cis-Jasmone is a natural volatile compound that is released when the plants are attacked by herbivores (Loughrin *et al.* 1995; Rose and Tumlinson 2004). It not only elicits plant defense responses (Matthes *et al.* 2010), but also serves as an attractant for predators of herbivorous insects (Powell and Pickett 2003), therefore it plays an important role in the interactions between host plants, aphids and their natural enemies. Previous studies have shown that *cis*-jasmone could be detected by an olfactory cell located on the fifth antennal segment of *Nasonovia ribisnigri* (Birkett *et al.* 2000). Behavioral studies revealed that *cis*-jasmone correlated strongly with the repellence of numerous aphid species, including *M. euphorbiae* (Sobhy *et al.* 2017), *M. persicae* (Dewhirst *et al.* 2012), *N. ribisnigri* and *Phorodon humuli* (Birkett *et al.* 2000), and *S. avenae* (Bruce *et al.* 2003). These results suggest that at least one OR is involved in the detection of *cis*-jasmone, and this chemoreception process is important for producing the repellent response. In this study, we identified McraOR20 from the antennal transcriptomes of *M. crassicauda* and screened for its best ligands among 11 HIPVs which were reported to be released by plants challenged with aphid infestation. The functional characterization results indicate that *cis*-jasmone is the best ligand linked to McraOR20, showing that McraOR20 is vital for *cis*-jasmone detection. Additionally, we found a conserved clade (with 1:1:1 orthologs in three aphid species) of OR20 in the phylogenetic analysis. ORs in conserved clades may undertake irreplaceable functions (Guo *et al.* 2020), and they may also share the same odorant binding pattern (Zhang *et al.* 2017). Our functional studies confirmed that McraOR20 and its ortholog in *A. pisum*, ApisOR20, are tuned to the same volatile, suggesting a conserved function for the OR20 clade in different aphid species. Therefore, we hypothesize that the homologous genes of McraOR20 in other aphid species may also be responsible for detecting *cis*-jasmone.

In the present study, only McraOR20 was identified as the specific OR of *cis*-jasmone. However, we cannot exclude the possibility that other ORs may also be involved in *cis*-jasmone detection, and they may affect aphid behavior through certain combinatorial coding. Future work should investigate the *in vivo* functional characterization of McraOR20 using RNA interference (RNAi). Moreover, the specific HIPVs induced by *M. crassicauda* should be identified, allowing us to

characterize the functions of species-specific ORs in *M. crassicauda*. We should also focus on studying the peripheral coding maps of aphids to *cis*-jasmone as well as other HIPVs, and combined this with behavioral experiments to reveal the mechanisms of HIPV olfactory recognition in aphids.

5. Conclusion

The putative chemosensory genes identified from the antennal transcriptomes of *M. crassicauda* will offer useful resources for future functional studies of McraORs, as well as genome and/or transcriptome annotation in other aphids and closely related species. Expression level analysis was performed by both bioinformatics and PCR-based methods, and helped in identifying some McraORs with antenna-biased expression in *M. crassicauda*. Furthermore, *in vitro* expressions of McraOR20 and ApisOR20, as well as McraOR43, in *Xenopus* oocytes were measured with the two-electrode voltage clamp technique revealing that McraOR20 and its ortholog ApisOR20 specifically respond to *cis*-jasmone, while McraOR43 did not show any response to the tested compounds. Our study not only lays the foundation for further functional studies of ORs in *M. crassicauda*, but also sheds light on the molecular mechanisms of *cis*-jasmone detection in aphids.

Acknowledgements

This work was funded by the National Natural Science Foundation of China (31572072 and 31801994), the Shenzhen Science and Technology Program, China (KQTD20180411143628272), the Natural Science Foundation of Tianjin, China (18JCYBJC96100), and the Tianjin Normal University Foundation, China (135305JF79).

Declaration of competing interest

The authors declare that they have no conflict of interest.

Appendices associated with this paper are available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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