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Design of larval chemical attractants based on odorant response spectra of odorant receptors in the cotton bollworm

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ABSTRACT

Lepidopteran caterpillars rely on olfaction and gustation to discriminate among food sources. Compared to the larval gustation, the larval olfaction has been poorly investigated. To uncover the molecular basis of olfaction in *Helicoverpa armigera* larvae, we identified 17 odorant receptor (Or) genes in larval antennae and maxillae using transcriptome sequencing, and functionally identified the response spectra of seven Ors to ecologically relevant odorants. Innate behavioural responses of larvae to active odorants were evaluated in chemotaxis assays. Several odorant blends were formulated based on the Ors tuning spectra and caterpillar chemotaxis. A four-component blend strongly attracted *H. armigera* larvae, and *cis*-jasmone and 1-pentanol were identified as essential components. Localization analyses showed that the two Ors detecting these components (Or41 and Or52) were expressed in the same sensory neurons. This is the first evidence that Ors in a polyphagous caterpillar respond to odorants in a combinatorial manner. The design of attractants to target specific olfactory pathways may promote the development of new baits for pest management.

1. Introduction

Moths are a large group of insects in the Lepidoptera with great economic importance. During their lifetime, adults are active during the night to e.g. mate and oviposit, while the larvae constitute the principal feeding stage. Olfaction plays an important role of guiding behaviour in both adult and larval stages.

Adult moths use olfactory sensory neurons (OSNs) located in antennal sensilla to detect olfactory cues. Each OSN expresses one or several odorant receptors (Ors), and OSNs expressing the same Or(s) project their axons into a single glomerulus in the antennal lobe. A further set of fibres carries olfactory information to higher brain centres. While many studies have focused on the sense of smell in adult moths, little is known about olfaction in larvae. The simplicity of the olfactory system makes caterpillars an ideal model for the study of chemoreception and processing. Moreover, because damage to crops and other plants is mainly caused by larvae, understanding the molecular basis of olfaction in caterpillars has great significance in the rational control of this group of pest insects.

Functional studies on insect Ors were initially performed in *Drosophila melanogaster* (Hallem and Carlson, 2006; Hallem et al., 2004; Kreher et al., 2005; Vosshall et al., 1999; Larsson et al., 2004), and later in mosquitoes (Carey et al., 2010; Wang et al., 2010; Xia et al., 2008; DeGennaro et al., 2013). The ligand-specific Ors are all seven-transmembrane proteins and function together with Orco, a co-receptor that is highly conserved across insect species. In moths, deorphanization of Ors is mostly confined to pheromone receptors. The function of pheromone receptors has been widely studied in e.g. *Bombyx mori*, *Heliothis virescens*, *Helicoverpa armigera*, *Ostrinia* sp., and *Spodoptera* sp. (Lin et al., 2015; Liu et al., 2014a; Miura et al., 2009; Miura et al., 2010; Montagne et al., 2012; Sakurai et al., 2004; Wang et al., 2011). Sex pheromones are typically detected by highly specific receptors, activating dedicated neural pathways. In addition, several studies have shown that several Ors in adult moths are tuned to volatile compounds produced by host plants (Jordan et al., 2009; Liu et al., 2014a; Zhang et al., 2013).

Lepidopteran larvae have a relatively simple olfactory system. Antennae and maxillae are the paired principal chemosensory organs located on the head of the caterpillar (Itagaki and Hildebrand, 1990). Each antenna bears three large sensilla basiconica. Previous studies in different species have reported that 16 OSNs innervate each antenna (Dethier, 1980; Dethier and Schoonhoven, 1969;

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Schoonhoyen, 1986), while a recent study labelled 38 OSNs in the *H. virescens* larval antenna (Zielonka et al., 2016). Several other OSNs innervate the three sensilla basiconica located on the maxillary palps (Dethier and Kuch, 1971; Hanson and Dethier, 1973; Schoonhoyen, 1986). In the tobacco hornworm *Manduca sexta,* each OSN of the antennal basiconic sensilla of larvae has only limited specificity; the specificity patterns of different OSNs overlap so that the larva can recognize a greater variety of volatile plant constituents (Dethier and Schoonhoven, 1969). However, the specific ligands for most larval candidate Ors are still unknown. One relatively comprehensive investigation comprised functional studies of Ors in the larval stage of the silkworm, *B. mori* (Tanaka et al., 2009). This study identified *cis*-jasmone in mulberry leaves as a potent attractant for the caterpillars, and indicated that a specifically tuned receptor, BmOr-56, may mediate this behavioural attraction. However, how the larvae discriminate and choose among numerous plant odorants through such a simple system remains a mystery.

We hypothesize that Ors expressed in different OSNs of lepidopteran larvae may be broadly tuned with different emphases. The superimposed tuning-spectra of the Ors would cover all important host plant odorants, and activation of set of Ors would cause an amplification cascade in the olfactory pathway. Therefore, based on the most effective ligands of Ors expressed in larval antennae and maxillae, it would be possible to design potent attractants to modify the behavioural patterns of caterpillars.

H. armigera is a worldwide agricultural pest species with a very wide host range. It damages numerous important cultivated crops such as cotton, tomato, maize, tobacco, and green pepper. The larvae are the principal feeding stage in the lifecycle. First-instar larvae often move over some distance, and then select a preferred site to hide. In cotton, this is usually on terminal leaves and in floral buds. Older larvae prefer flower buds and young fruits. Olfactory-based attraction of caterpillars plays a crucial role in this food site selection.

To understand the molecular basis of olfactory reception of this polyphagous species and to test the above hypothesis, we first identified a set of *Ors* expressed in *H. armigera* larval antennae and maxillae through transcriptome and molecular approaches. Next, we functionally analysed the response spectra of these Ors to a wide range of host plant-related odorants using the *Xenopus* oocyte expression system and a two-electrode voltage-clamp. Then, we used olfactory-based assays to investigate the behavioural responses of the first-instar larvae of *H. armigera* to a range of chemicals, according to the response spectra of the Ors. After that, based on the results of the functional studies on Ors and behavioural assays, we designed blends of crucial odorants and demonstrated that a four-component blend strongly attracted *H. armigera* larvae. *Cis*-jasmone and 1-pentanol were identified as the essential components. Finally, we performed whole-mount *in situ* hybridization and found that the two Ors detecting to *cis*-jasmone and 1-pentanol (Or41 and Or52) were expressed in the same OSN.

2. Materials and methods

2.1. Insects

H. armigera were reared at the Institute of Zoology, Chinese Academy of Sciences, Beijing. Rearing took place at a constant temperature of 27 ± 1 °C with a photoperiod of 16 h:8 h, L: D. The larvae were fed with an artificial diet, mainly constituted of wheat germ, yeast and tomato paste. Pupae were sexed, males and females were put into separate cages for eclosion. On emergence, moths were provided unlimited access to 10% honey solution. Different tissues of larvae and adults were excised and immediately put into Trizol reagent or frozen in liquid nitrogen respectively, and then stored at under −70 °C until use.

2.2. RNA isolation and transcriptome sequencing

Antennae and maxillae were hand dissected from approximately 600 5th instar larvae. The collected tissues were immediately placed in Trizol reagent (Invitrogen, Carlsbad, CA, USA) on ice prior to RNA extraction. Total RNA was extracted following the Trizol manufacturer's instructions and dissolved in H_2O. RNA quantity and integrity were confirmed using the 2100 Bioanalyzer (Agilent Technologies). Total RNA preparations were sent to The Beijing Genome Institute (Shenzhen, China) for Hiseq RNA sequencing on the Illumina sequencing platform (Hiseq2000).

2.3. De novo assembly and annotation

In order to obtain the clean data, the raw reads were initially processed for removing the adapter sequences and low-quality bases. Then, the Q30 and GC-content were used to assess the sequencing quality. The reads were assembled using Trinity and the contigs were annotated using Trinotate: an automatic functional annotation pipeline of de novo assembled transcriptomes (http://trinotate.sourceforge.net/) (Grabherr et al., 2011; Haas et al., 2013). Transcripts were next searched for sequence homologies using BLASTX analysis in GenBank to identify candidate odorant receptors. The putative protein sequences were then compared to orthologous genes from other insect species using BLASTP. Tblastx were used to match the putative receptors to the publication data.

2.4. Reverse transcription PCR

To measure the expression of candidate odorant receptors in moth and larval tissues, we performed Reverse Transcription PCR (RT- PCR). Total RNA was isolated separately from antennae of 35 male moths, antennae of 35 female moths, antennae of 600 5th instar larvae and maxilla of 600 5th instar larvae, using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and were treated with DNase I (RQ1, Promega, Madison, WI, USA). The extracted RNA was subjected to reverse transcription for first strand cDNA synthesis by M-MLV Reverse Transcriptase (Promega, Madison, WI, USA), according to the manufacturer's manual. An actin gene fragments were used as control. Primers were designed using Primer Premier 5.0 software (PREMIER Biosoft International) and the sequences were available in Table S1. All reactions were run in triplicates on ABI PCR machine. The PCR conditions were 94 °C for 2 min; 33 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s; and final extension at 72 °C for 7 min. PCR products were analysed using 1.5% agarose gels. The expected products of the genes randomly selected were sequenced to confirm the identity with their original sequence identified. At least two biological replications for each *Or* were run.

2.5. Cloning of the coding sequences of putative larval Ors

Antennae and maxillae were collected separately from 600 5th instar larvae. Total RNA was extracted and the first strand cDNA was synthesized as above. Eleven *Ors* including *Or2, Or31, Or27, Or52, Or12, Or38, Or60, Or50, Or53, Or24, Or22* were confirmed as full-length coding sequences from transcriptomic analysis. The full-length open-reading frames of three *Ors* including *Or9, Or41, Or42* were downloaded from NCBI GenBank. Gene-specific primers containing restriction enzyme recognition sites were designed and were listed in Table S2. Full-length *Ors* were amplified by PCR with LATaq polymerase (TaKaRa, Dalian, China) in 50 μl reactions containing 1 μl of cDNA template and 2 μl of each primer. The PCR

program included initial denaturation at 94 °C for 2 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C, 90 s at 72 °C; and a final extension step of 7 min at 72 °C. PCR products were purified from agarose gel with MiniBEST Agarose Gel DNA Extraction Kit (Takara, Dalian, China) and ligated into pGEM-T vector (Promega, Madison, WI, USA). TOP10 competent cells (Tiangen, Beijing, China) were transformed with the ligation products, positive clones were grown in LB medium containing ampicillin. Plasmids were purified with (Tiangen, Beijing, China) and sequenced (SinoGenoMax company, Beijing, China). Plasmids were digested with appropriate combinations of restriction enzymes (New England Biolabs, Ipswich, MA) and ligated into pre-digested pCS2+ vectors. Plasmids were then purified and digested with NotI (New England Biolabs, Ipswich, MA).

2.6. Odorants panel

To deorphanize Ors, we used a panel of 48 compounds which were previously identified by behavioural and electrophysiological experiments (Bruce et al, 2002; Bruce and Cork, 2001; Burguiere et al., 2001; Cribb et al., 2007; Deng et al., 2004; Gregg et al., 2010; Hartlieb and Rembold, 1996; Rajapakse et al., 2006; Rembold et al., 1991; Rostelien et al., 2005; Stranden et al., 2003; Sun et al., 2012; Yan et al., 2004) or known activity in other insect species (Tanaka et al., 2009) (Table 1). Two principal sex pheromone components, *cis*-11-hexadecenal and *cis*-9-hexadecenal were also included in our list. Odorants were dissolved in dimethylsulfoxide (DMSO) at a concentration of 1000 mM

Table 1
Test compounds in functional analysis of ORs of *Helicoverpa armigera* larvae.

Class	Odorant	CAS number	Company	Purity (%)	Reference*		
					Electrophysiology	Behaviour	OR-tuning
GLV	1-Hexanol	111-27-3	Aldrich	99	a, e		
	2-Hexanol	626-93-7	Aldrich	99	e		
	3-Hexanol	623-37-0	Aldrich	99	e		
	cis-3-Hexen-ol-1	928-96-1	Roth	98	e		
	trans-2-Hexen-1-ol	928-95-0	Fluka	95	e		
	trans-3-Hexen-1-ol	928-97-2	Roth	98	e		
	trans-2-Hexen-1-al	6728-26-3	Fluka	97	e, g, i		
	cis-3-Hexenyl acetate	3681-71-8	Roth	97	a, i, l	f	
	trans-2-Hexenyl acetate	2497-18-9	Aldrich	98	e		
	cis-3-Hexenyl salicylate	65405-77-8	Aldrich	97		f	
	cis-3-Hexenyl butyrate	16491-36-4	Aldrich	98		f	
	cis-3-Hexenyl 2-methyl butanoate	53398-85-9	Aldrich	97	i		
Terpenoid	(+)-Borneol	18172-67-3	Aldrich	97	b		
	(+)-3-Carene	498-15-7	Fluka	99	b, h	f, j	
	(−)-*trans*-Caryophyllene	87-44-5	Fluka	99	a, e		
	Citral	5392-40-5	Aldrich	96			n
	Farnesene	502614	Aldrich	98	k		
	Geraniol	106-24-1	Fluka	96	g	f	
	(±)-Linalool	78-70-6	Fluka	97	b, e, g, i, k		
	(S)-(−)-Limonene	5989-54-8	Fluka	98	a, e, i		
	Myrcene	123-35-3	Roth	90	e, h, i, k, l	j	
	Ocimene	13877-91-3	Aldrich	90	e, i, k, l		
	α-Phellandrene	99-86-5	Aldrich	95	e		
	(−)-α-Pinene	7785-26-4	Fluka	97	a, e, h, i, l	j	
	(−)-β-Pinene	99-83-2	Aldrich	99	l		
	α- Terpinene	99-86-5	Roth	92			
	(S)-*cis*-Verbenol	18881-04-4	Aldrich	95	b		
Aliphatic	1-Butanol	71-36-3	Sigma	99.8	e		
	Guaiene	92724-67-9	Aldrich	99	f	f	
	1-Heptanol	111-70-6	Fluka	99.5	e		
	cis-9-Hexadecenal	56219-04-6	Shin-Etsu	96			o
	cis-11-Hexadecenal	53939-28-9	Shin-Etsu	92			o
	cis-Jasmone	488-10-8	Aldrich	85			n
	2-Methyl-1-butanol	137-32-6	Aldrich	99			
	3-Methyl-1-butanol	123-51-3	Aldrich	99		f	
	Nonanal	124-19-6	Aldrich	95	m	m	
	cis-6-Nonen-1-ol	35854-86-5	Aldrich	95	g	f	
	1-Octanol	111-87-5	Aldrich	99	e		
	Octanal	124-13-0	Aldrich	99			
	1-Pentanol	71-41-0	Aldrich	99	e, h	j	
Aromatic	Anethol	4180-23-8	Aldrich	99		f	
	Benzaldehyde	100-52-7	Fluka	99	a, e, g, l		
	Benzyl alcohol	100-51-6	Aldrich	98	e, g		
	Methyl benzoate	93-58-3	Aldrich	99	b		
	Methyl salicylate	119-36-8	Aldrich	99	e, g	f	
	Phenylacetaldehyde	122-78-1	Aldrich	95	a, c, d, e, g	c, f	
	2-Phenylethanol	60-12-8	Aldrich	99	e	f	
	Salicylaldehyde	90-02-8	Aldrich	98	g	f	

Note: Electrophysiology represents that the odorants elicit electrophysiological response in *H. armigera* chemosensory organs. Behaviour represents that the odorants elicit behavioural response in *H. armigera*. OR-tuning represents that the odorants are identified as ligands of ORs in *H. armigera* or *B. mori*. * Reference a. Cribb et al., 2007; b. Rostelien et al., 2005; c. Bruce et al, 2002; d. Bruce and Cork, 2001; e. Bruce and Cork, 2001; f. Gregg et al., 2010; g. Deng et al., 2004; h. Hartlieb and Rembold, 1996; i. Rajapakse et al., 2006; j. Rembold et al., 1991; k. Rembold et al., 1991; l. Yan et al., 2004; m. Sun et al., 2012; n. Tanaka et al., 2009; o. Jiang et al., 2014.

as stock solutions. Before the experiments, stock solutions were diluted in Barth's solution to the indicated concentrations. Barth's solution containing 0.1% DMSO was used as a negative control.

2.7. Expression of odorant receptors in Xenopus laevis oocytes and electrophysiological recordings

Linearized modified pCS2+ vectors were used to synthesize cRNAs with mMESSAGE mMACHINE SP6 (Ambion, Austin, TX, USA) following the manufacturer's instructions. Stage V-VII oocytes were treated with 2 mg/ml of collagenase type I (Sigma–Aldrich, St. Louis, USA) in Ca^{2+}-free saline solution (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, and 5 mM HEPES, pH 7.6) for 0.5–1 h at room temperature. Oocytes were later microinjected with 25 ng *Or* cRNA and 25 ng *Orco* cRNA. The injected oocytes were incubated for 3–7 days at 18 °C in Barth's solution (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 1.8 mM $CaCl_2$ and 5 mM HEPES, pH 7.5) supplemented with 5% dialyzed horse serum, 50 mg/ml tetracycline, 100 mg/ml streptomycin and 550 mg/ml sodium pyruvate. Two-electrode voltage-clamp technique was employed to record whole-cell currents. Signals were amplified with an OC-725C amplifier (Warner Instruments, Hamden, CT, USA) at holding potential of −80 mV, low-pass filtered at 50 Hz and digitized at 1 kHz. Data acquisition and analysis were carried out with Digidata 1322A and pCLAMP software (Axon Instruments Inc., Foster City, CA, USA).

2.8. Behaviour assays

For measurement of olfactory responses, no-choice and two-choice behaviour assays were employed. For no-choice behaviour assay, 10 larvae were placed on around of a Petri dish. Odorants (or solvent control) dispensed on the 1-cm-radius filter discs were placed in the centre of the Petri dish. A 1.5-cm-radius response zone was determined around the filter disc (Fig. 3a). The number of larvae moving into response zone (no contact with the filter disc) in 10 min was counted. For two-choice behaviour assay, 10 larvae were placed in the centre of a Petri dish filled with 1% agarose. Two 0.5-cm-radius filter discs containing odorant (or solvent control) were placed in the two opposite odour zones (Fig. 4a). After 10 min, the number of larvae in respective zones was counted and a response index was calculated ((O-C)/T, where O is the number of larvae in the odorant zone, C is the number of larvae in the control zone, and T is the total number of tested larvae).

2.9. Preparation of labelled probes for in situ hybridization

The following sense (s) and antisense (as) primers were used to synthesize the gene-specific probes from the open reading frames (ORFs): Or41, s5' - GTTGGCTACGGTAAGCGTGA-3′ and as5' - ATGCTCTCGTATGGACCTCG -3'; Or52, s5′-TTGGTCAGGA-GATCCGCAAG -3′ and as5′- TTGGTCAGGAGATCCGCAAG -3'; Or42, s5′- TCGGGTTTCACTACCGCATC – 3′ and as 5' – CGCGT CAATTTCCTCCTTCG- 3'; Or31, s5′- TGGCAGAAACACTGGCATGA -3′ and as 5′- AAGGTCGGCAAAGTGAGGTT -3'. Biotin (Bio)-labelled probes were synthesized with DIG RNA labelling Kit version 12 (SP6/T7) (Roche, Mannheim, Germany), with Bio-NTP (Roche, Mannheim, Germany) labelling mixture. Antisense and sense probes were generated from linearized recombinant pGEM-T vector using the T7/SP6 RNA transcription system (Roche, Basel, Switzerland) following recommended protocols. RNA probes were subsequently fragmented to an average length of about 300 bp by incubation in carbonate buffer (80 mM $NaHCO_3$, 120 mM Na_2CO_3, pH 10.2).

2.10. In situ hybridization

Double WM-FISH were performed as described previously with a few modifications (Zielonka et al., 2016). Antennae were dissected from 5th instar larvae and transferred directly to fixation solution (4% paraformaldehyde in 0.1 M NaHCO3, pH 9.5, 0.03% Triton X-100). Incubations and washes were made in a volume of 0.25 ml (PCR tubes) with moderate shaking. After fixation for 24 h at 6 °C, antennae were shortly washed for 1 min at room temperature in PBS (phosphate-buffered saline = 0.85% NaCl, 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.1), 0.03% Triton X-100. Subsequently, the antennae were incubated for 10 min in 0.2 M HCl, 0.03% Triton X-100. Following a wash for 1 min in PBS, 1% Triton X-100, *in situ* hybridization solution (50% formamide, 5x SSC, 1xDenhardt's reagent, 50 mg/ml yeast RNA, 1% Tween 20, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps), 5 mM ethylenediaminetetraacetic acid, pH 8.0) was added and incubated at 55 °C for 30 min. Then antennae were incubated in hybridization solution containing labelled antisense RNA probes at 55 °C for at least 3–4 days. After washing four times for 15 min at 60 °C in 1x SSC solution with 0.03% Triton X-100, antennae were incubated in blocking solution [1% blocking reagent (Roche, Indianapolis, IN, USA) in Tris buffered saline (TBS: 100 mM Tris, pH 7.5, 150 mM NaCl), 0.03% Triton X-100] overnight at 4 °C. The antennae were then incubated with an anti-DIG alkaline phosphatase-conjugated antibody (Roche) diluted 1:500 in blocking solution for the detection of DIG-labelled RNA probes; for detection of biotin-labelled probes a streptavidin horseradish peroxidase-conjugate (1:100, tyramide signal amplification (TSA) kit, PerkinElmer, Boston, MA, USA) was used. After incubation for 3 days at 4 °C, antennae were washed five times for 10 min each time in TBST (TBS with 0.05% Tween 20) at room temperature. For visualization of DIG-labelled probes the antennae were subsequently incubated in 2- hydroxy-3-naphtoic acid-2'-phenylanilide phosphate (HNPP) solution [Roche; 1:100 in DIG alkaline phosphatase (DAP)-buffer (100 mM Tris, pH 8.0, 100 mM NaCl, 50 mM $MgCl_2$)] for at least 17 h at 4 °C in the dark. Biotin-labelled probes were visualized using components of the TSA Fluorescein System and incubation with a 1:50 dilution for 24 h at 4 °C in the dark. For double WM-FISH, antennae were first treated with HNPP solution and then incubated with the TSA components, with a washing step of three times for 10 min each time with TBST included between the substrate treatments. Finally, antennae were washed three times for 10 min each time in TBST. Finally, the antennae were rinsed in PBS and mounted in antifade mounting medium (Beyotime, Shanghai, China).

Antennae were analysed for epifluorescence on a Zeiss LSM710 Meta laser scanning microscope (Zeiss, Oberkochen, Germany). Figures were arranged with appropriate graphic programs.

2.11. Statistics and data analysis

Data were analysed using unpaired Student's t-test for comparing two sets of data and one-way analysis of variance (ANOVA) with Tukey LSD tests for comparing multiple sets of data. The level of significance was set as $P < 0.05$.

3. Results

3.1. Identification of Ors expressed in larval antennae and maxillae of H. armigera

Transcriptome sequencing of mixed larval antennae and maxillae yielded a total of 2,293,544 reads (average read length, 97 bp). Assembly of the reads generated 100,369 contigs larger than 200 bp, with 476,220 contigs longer than 500 bp. Seventeen

different contigs encoding candidate *Ors* were identified by querying the database with 64 and 63 known *Ors* from *H. armigera* and *B. mori*, respectively. These 17 sequences had overlapping regions with low identity, confirming that they all represented unigenes. The candidate *Or* unigenes were named according to their similarities to previously annotated *Ors*. Among them, 14 *Ors* are identical to the GenBank accessions in the amino acid sequence, but *Or9, Or38, and Or60* are not 100% identical to the GenBank accessions, with identity of 97%, 89%, and 98%, respectively. Details for all of the 17 *Ors* including unigene reference, length, and BLASTx best hit are listed in Table 2. The nucleotide sequences of all 17 *Ors* are listed in Data S1. Depending on the algorithm, the sequences contained between two and eight transmembrane domains, which is a well-characterized feature of insect *Ors*. *Or2* shared high identity with the conserved insect co-receptor, and the other 16 *Or* sequences showed low levels of similarity with each other. Full-length open reading frames (ORFs) were identified for 11 *Ors*: *Or2, Or31, Or27, Or52, Or12, Or38, Or60, Or50, Or53, Or24,* and *Or22.* Full-length ORFs of the other three *Ors*, *Or9*, *Or42*, and *Or41,* were obtained based on *Or* sequences from NCBI GenBank. Thus, full-length ORFs were acquired for 14 *Ors* expressed at the larval stage.

3.2. Expression patterns of Ors in olfactory organs of H. armigera larvae and adults

The expression patterns of the candidate 17 *Ors* in larval antennae and maxillae as well as in adult male and female antennae were evaluated by RT- PCR with specific primers (Fig. 1A). Transcripts of 16 *Ors* including the co-receptor gene *Or2* were detected in both the larval and adult stages, while that of *Or54* was detected only at the larval stage (Fig. 1A). No amplification products of *Or58* were detected in adult or larval chemosensory organs with two different pairs of specific primers.

Fifteen *Ors* were expressed in larval antennae, and 10 *Ors* were expressed in larval maxillae. Nine *Ors* (*Or2, Or34, Or31, Or9, Or12, Or42, Or60, Or53*, and *Or22*) were expressed in both larval antennae and maxillae, six *Ors* (*Or27, Or54, Or52, Or41, Or24* and *Or38*) were expressed in larval antennae but not in larval maxillae, and *Or50* was detected in larval maxillae but not in larval antennae (Fig. 1).

3.3. Odorant response spectra of Ors expressed in larval antennae and maxillae of H. armigera

The *Xenopus* oocyte expression system was used to characterize the function of 13 Ors. Each of the 13 Ors was co-expressed with the Orco Or2 in *Xenopus* oocytes, and then a panel of 48 chemical stimuli was tested using a two-electrode voltage-clamp. Table 1 lists the tested chemicals, which can be classified into four groups; green leaf volatiles (GLVs), terpenoids, aromatics, and aliphatics.

Seven Ors (Or12, Or31, Or41, Or42, Or50, Or52, and Or60) generated current responses to the 48 tested odorants (Fig. 2). Six of them (all except for Or50) were tuned to odorants from different groups. Or60 was activated by up to 25 odorants from all four groups, and the most effective ligand was *cis*-3-hexen-ol-1. HarmOr41 was activated by 11 odorants from the four groups, and the most effective ligand was *cis*-jasmone. Or52 showed a broad response to 10 odorants from the GLVs, terpenoids, and aliphatics, and the most effective ligand was 1-pentanol. Or12 was activated by nine odorants from GLVs, terpenoids, and aliphatics, where the most effective ligand was citral. Or42 responded to eight odorants from GLVs, terpenoids, aromatics, and aliphatics, and the most effective ligand was phenylacetaldehyde. Or31 was tuned to eight odorants from terpenoids and GLVs, and the most effective ligand was myrcene. HarmOr50 was tuned to (S)-*cis*-verbenol and

Table 2
Unigenes of candidate olfactory receptors in *H. armigera* larval antenna and maxilla.

Query sequence ID	Gene name	Length (bp)	ORF (aa)	FPKM	Blastx best hit (Reference/Name/Species)	E- value	Identity	full-length	TMD (No)
contig220	OR2	1419	473	242.648	gb|AIG51851.1| odorant receptor [*Helicoverpa armigera*]	0	100%	Yes	7
comp236415_c0_seq1	OR34	828	267	35.299	gb|AIG51881.1| odorant receptor, partial [*Helicoverpa armigera*]	5.00E-109	100%	No	4
comp241018_c0_seq1	OR31	1185	395	35.166	gb|AIG51879.1| odorant receptor [*Helicoverpa armigera*]	4.00E-153	100%	Yes	6
contig114	OR27	1278	426	22.816	gb|AIG51876.1| odorant receptor, partial|*Helicoverpa armigera*]	1.00E-157	100%	Yes*	6
contig55	OR54	1077	359	17.197	gb|AIG51900.1| odorant receptor, partial|*Helicoverpa armigera*]	7.00E-158	100%	No	6
contig173	OR52	1257	419	15.777	gb|AIG51898.1| odorant receptor|*Helicoverpa armigera*]	0	100%	Yes	6
comp248206_c0_seq1	OR9	768	256	15.567	gb|AIG51857.1| odorant receptor, partial|*Helicoverpa armigera*]	9.00E-172	97%	Yes	4
comp246341_c0_seq2	OR12	1224	408	10.908	gb|AIG51860.1| odorant receptor|*Helicoverpa armigera*]	0	100%	Yes	6
comp201969_c0_seq1	OR58	396	132	10.522	gb|AIG51904.1| odorant receptor, partial|*Helicoverpa armigera*]	8.00E-90	100%	No	2
contig3	OR38	1321	429	9.982	gb|ALM26234.1| odorant receptor 44|*Athetis dissimilis*]	0	89%	Yes*	5
comp247106_c0_seq2	OR42	1095	360	8.305	gb|AIG51888.1| odorant receptor|*Helicoverpa armigera*]	0	100%	Yes	5
contig58	OR41	489	163	7.361	gb|AIG51887.1| odorant receptor|*Helicoverpa armigera*]	2.00E-114	100%	Yes	3
contig4	OR60	1185	394	6.82	gb|AIG51906.1| odorant receptor|*Helicoverpa armigera*]	0	98%	Yes	5
comp77484_c0_seq1	OR50	1200	400	6.696	gb|AIG51896.1| odorant receptor, partial|*Helicoverpa armigera*]	0	100%	Yes*	6
comp242099_c0_seq1	OR53	1293	401	6.138	gb|AIG51899.1| odorant receptor|*Helicoverpa armigera*]	0	100%	Yes	6
contig25	OR24	1173	391	4.586	gb|AIG51873.1| odorant receptor|*Helicoverpa armigera*]	0	100%	Yes	4
contig39	OR22	1245	415	2.14	gb|AIG51871.1| odorant receptor|*Helicoverpa armigera*]	0	100%	Yes	4

Note: The full-length sequences labelled with * were newly obtained.

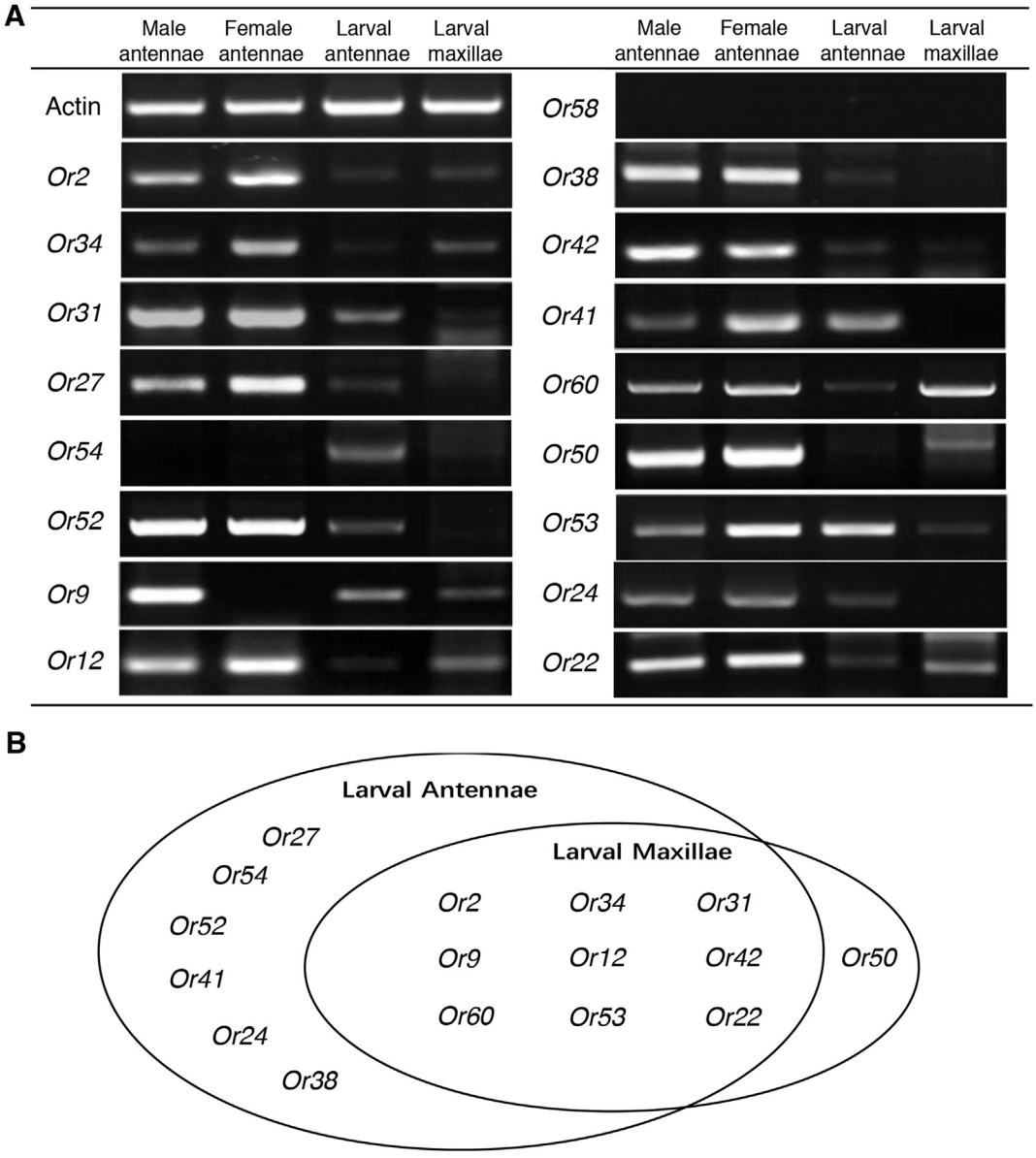


Fig. 1. Tissue-specific expression of candidate larval *Or* genes in *Helicoverpa armigera*. A. RT-PCR analysis of *Ors* transcript levels in antennae of adult males, adult females, and antennae and maxillae of fifth instar larvae; B. List of *Ors* genes transcribed in larval antennae and maxillae.

(+)-borneol with low current responses.

There were considerable overlaps among the odorant tuning spectra of the seven Ors. For example, *cis*-3-hexenyl acetate activated six Ors (Or12, Or31, Or41, Or42, Or52, and Or60), and citral activated four Ors (Or12, Or31, Or42, and Or52). However, the most effective ligands of these Ors were those that were distinctive and unique, and covered the four groups of odorants (Fig. 2, Table 3). Six Ors (Or27, Or9, Or38, Or53, Or24, and Or22) co-expressed with *Or2* did not respond to the tested compounds.

3.4. Attractiveness of individual plant odorants to larvae

A no-choice behaviour assay was established to screen odorants for their attractive or repellent activity towards *H. armigera* larvae based on the results of the odorant response spectra of Ors (Fig. 3A). We used first instar larvae, because their innate behavioural responses to plant volatiles are not affected by feeding experience. In our pilot experiments, we found that fifth instar larvae showed similar chemotaxis behaviour with decreased selectivity (data not shown). In total, 21 odorants were selected for further behavioural tests: 18 odorants that strongly evoked Or responses in the above experiments (Fig. 2), one major component emitted from green pepper, ocimene (Fig. S1), and two *H. armigera* sex pheromone components, *cis*-9-hexadecenal and *cis*-11-hexadecenal. Green pepper juice was used as positive, and paraffin oil as negative control.

Among the tested odorants and two controls, most individual chemicals attracted larvae with different strengths, but green pepper juice was the most attractive stimulus. *cis*-3-Hexen-ol-1, 1-pentanol, *cis*-jasmone, myrcene, and nonanal showed strong

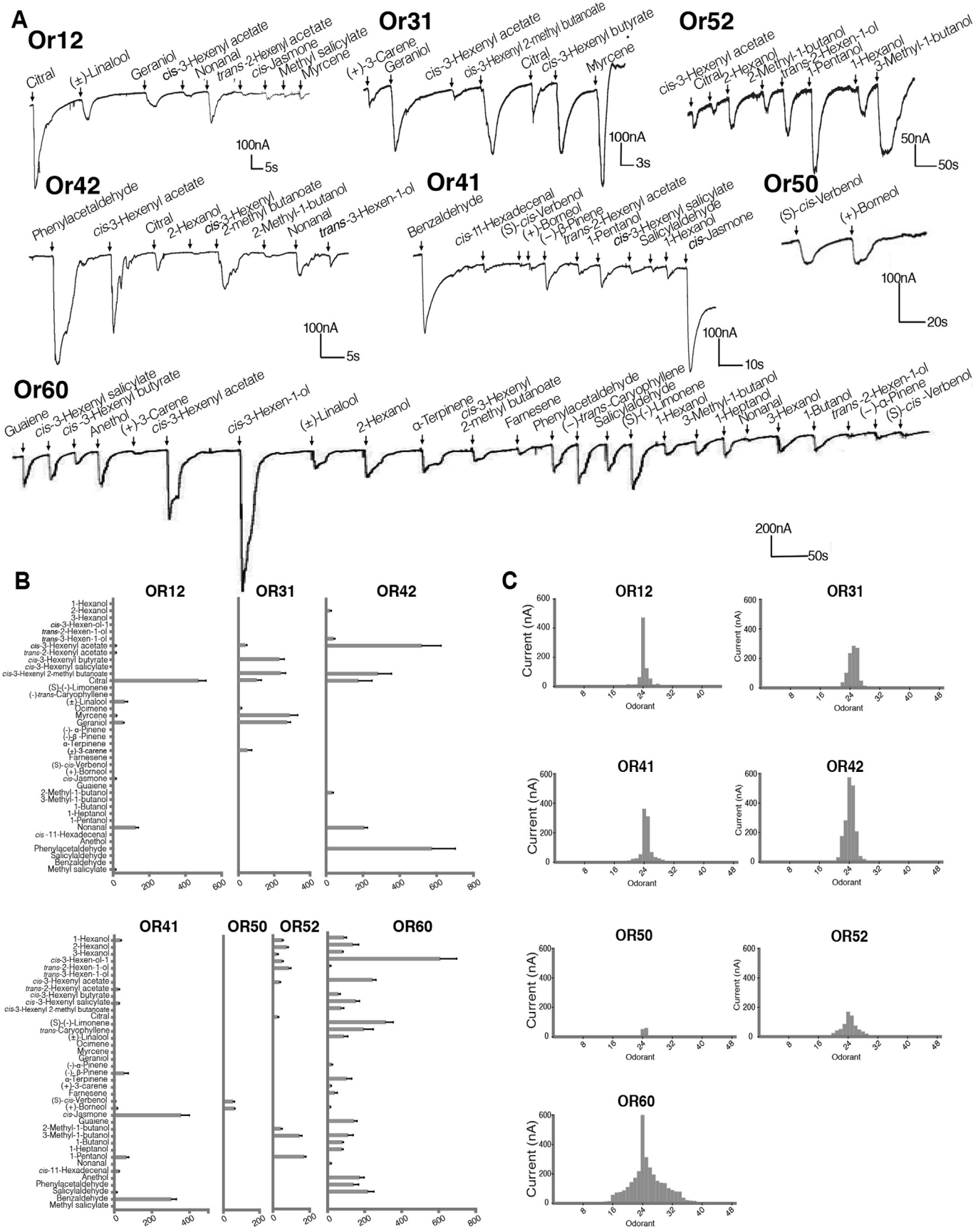


Fig. 2. Functional analysis of larval HarmOrs in *Xenopus* oocytes. A. Inward current responses of *Xenopus* oocytes in response to odorants (10^{-4} M solution). Odorants were applied for 8 s at times indicated by arrowheads; B. Odorant-response spectra of larval HarmOrs. Responses were measured as induced inward currents, expressed in nA. Error bars show standard error of the mean ($n = 4–6$).

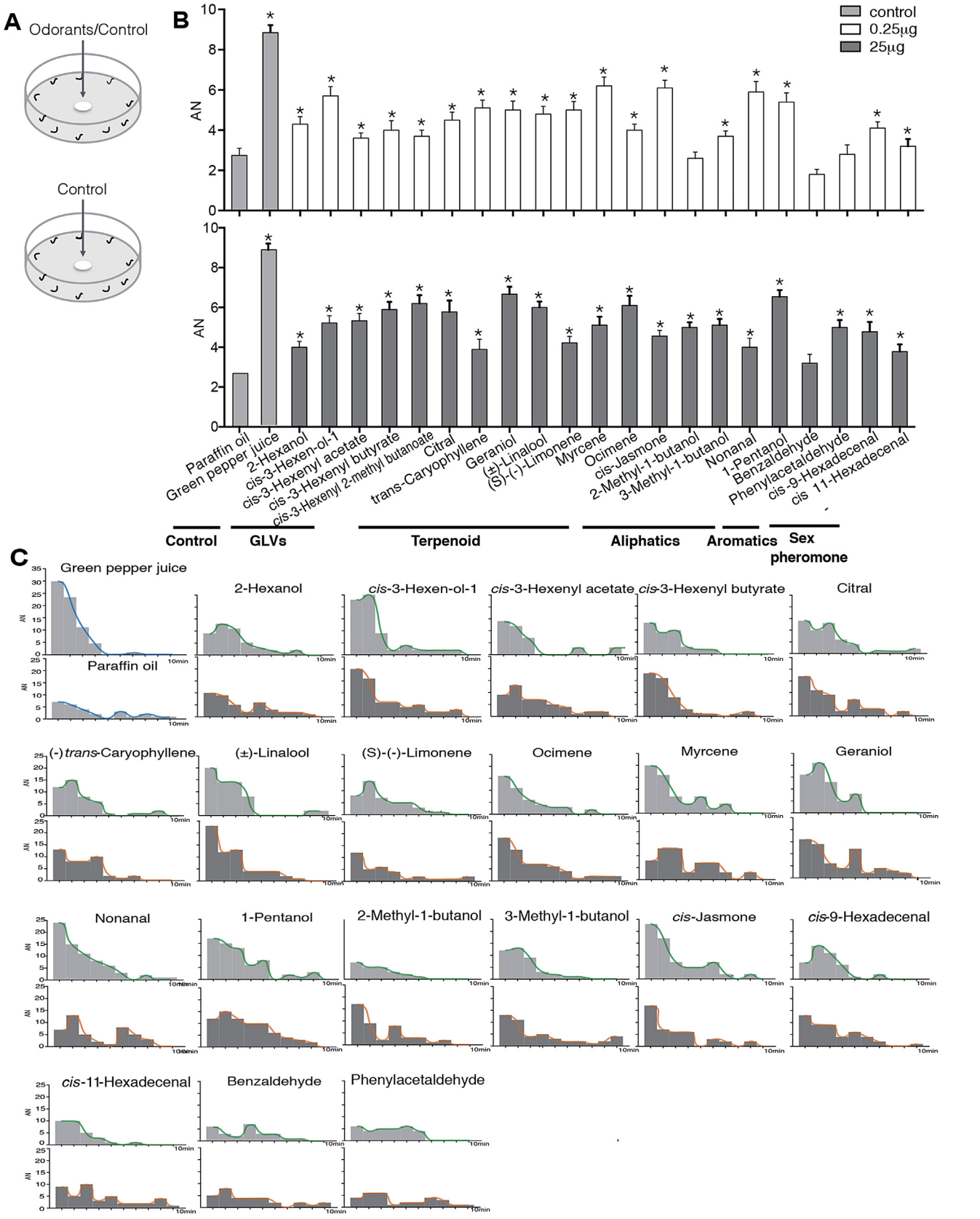


Fig. 3. No-choice chemotaxis behavioural assay for *Helicoverpa armigera* larvae. A. Schematic diagram of experimental arena of larval behavioural assay; B. Response profiles of *H. armigera* larvae to 21 odorants at doses of 0.25 μg and 25 μg. Green pepper juice and paraffin oil were used as positive and negative control, respectively. AN represents the attracted number, the number of larvae moving into the response zone. Error bars indicate standard error of the mean for $n = 10$ trials. Data were compared using two-tailed unpaired student's t tests: *, $P \leq 0.05$. C. Total number of attracted larvae per minute over a 10-min experiment.

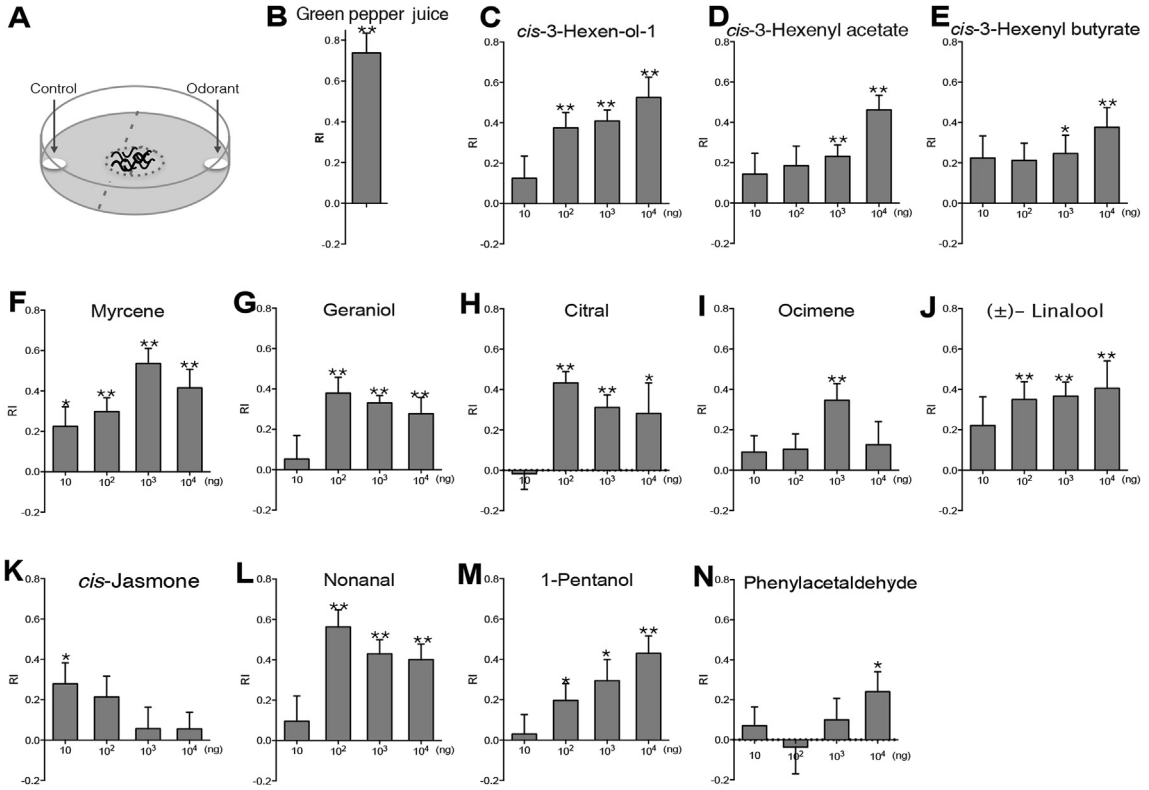


Fig. 4. Two-choice chemotaxis behavioural assay for *Helicoverpa armigera* larvae. A. Schematic diagram of experimental arena of larval behavioural assay; B. Response profiles *H. armigera* larvae to green pepper juice; C-N. Response profiles of *H. armigera* larvae to 12 odorants: *cis*-3-hexen-ol-1, *cis*-3-hexenyl acetate, *cis*-3-hexenyl butyrate, myrcene, geraniol, citral, ocimene, (±)-linalool, *cis*-jasmone, nonanal, 1-pentanol, and phenylacetaldehyde. Error bars indicate standard error of the mean for $n = 10$ trials. Significance of deviation of response indices from zero was evaluated by Student's *t*-test: *, $P \leq 0.05$; **, $P \leq 0.01$.

attractiveness at a low dose (0.25 μg); while *cis*-3-hexenyl 2-methyl butanoate, geraniol, (±)-linalool, ocimene, and 1-pentanol showed strong attractiveness at a high dose (25 μg) (Fig. 3B). The dynamics of attraction varied among the attractive odorants. *cis*-3-Hexen-ol-1, (±)-linalool, *cis*-jasmone, nonanal, and *cis*-3-hexenyl butyrate were strongly attractive in the first 3 min of the assay, while 1-pentanol and citral stably attracted larvae during the full duration of the 10-min experiment. Myrcene and geraniol strongly attracted larvae in the first 3 min at a low dose (0.25 μg), and stably attracted larvae throughout the whole experiment at a high dose (25 μg) (Fig. 3C).

We selected 12 odorants to study their attractiveness to first-instar larvae in a two-choice assay at four doses (10 ng, 100 ng, 1000 ng, and 10 000 ng) (Fig. 4). The 12 odorants included the six most effective ligands of Or12, Or31, Or41, Or42, Or52, and Or60 and another six odorants that performed well in the no-choice test. The attractiveness of the three GLVs, *cis*-3-hexen-ol-1, *cis*-3-hexenyl acetate, and *cis*-3-hexenyl butyrate, were enhanced as their doses increased (Fig. 4C–E), and *cis*-3-hexen-ol-1 was the most attractive of the three GLVs (Fig. 4C). Among the terpenoids, the larvae responded strongly to myrcene, geraniol, and citral at 10 ng or 100 ng, but the attractiveness of these compounds decreased as the dose increased (Fig. 4F–H). Ocimene was only attractive at a dose of 1000 ng (Fig. 4I). Linalool, a well-known odorant from flowers, elicited stable responses at different doses (Fig. 4J). Of the two aliphatics, *cis*-jasmone showed strong attractiveness only at the lowest dose (10 ng), while nonanal was strongly attractive at higher doses (100 ng, 1000 ng, and 10 000 ng) (Fig. 4K and L). The performance of phenylacetaldehyde, the only aromatic compound among the tested odorants, was similar to that observed in the no-choice assay, and only attracted larvae at 10 000 ng (Fig. 4N).

3.5. Attractiveness of odorant blends to larvae

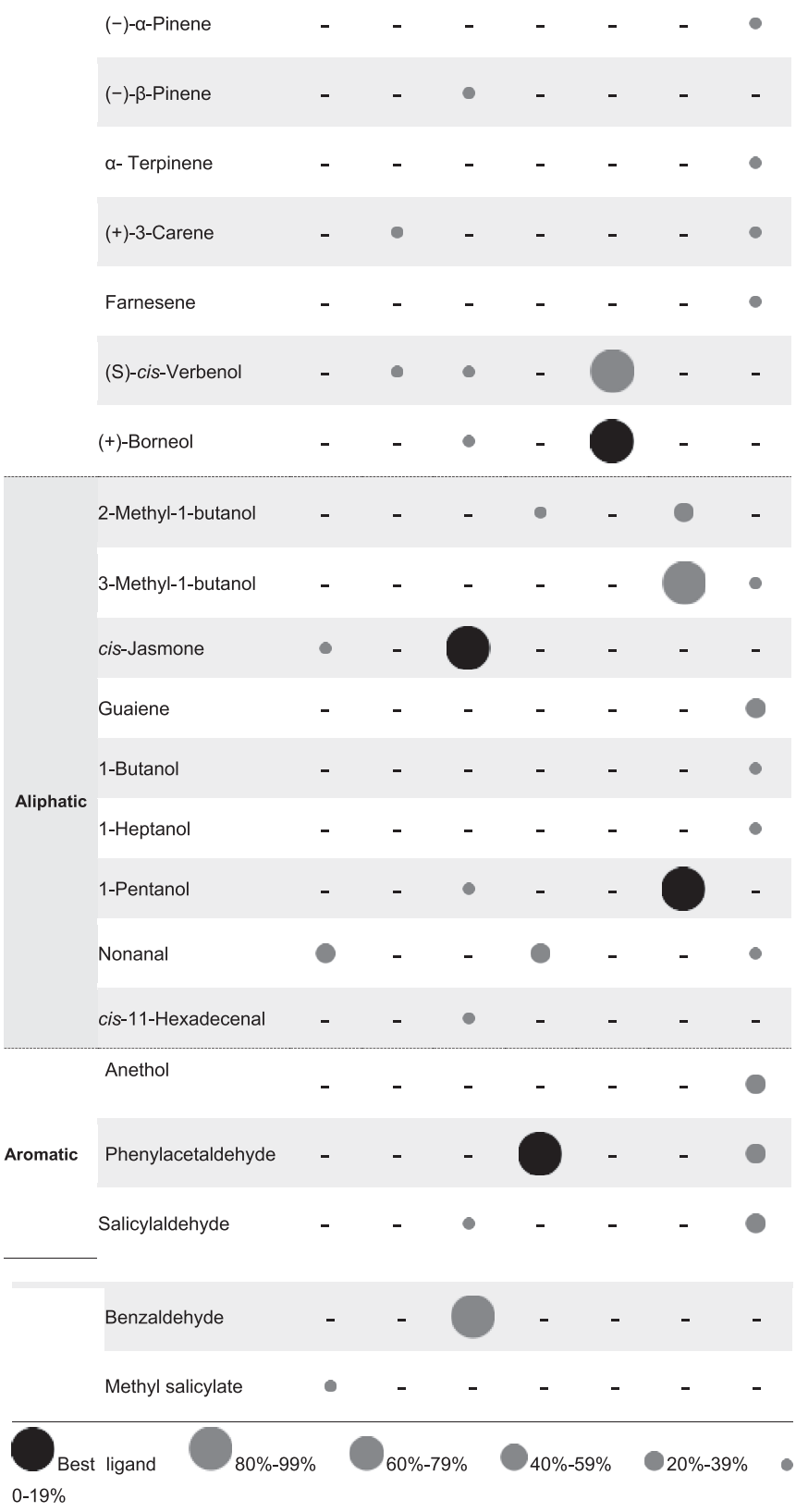
To mimic the volatiles of potential host plants, five blends of odorants mixed in equal proportions were designed and further tested in the two-choice behavioural paradigm. The first blend (MJAP) was a mixture of myrcene (M), cis-jasmone (J), phenylacetaldehyde (A) and 1-pentanol (P), the most effective ligands of HarmOr31, 41, 42, and 52, respectively; these Ors are mainly expressed in larval antennae (Fig. 1A). The second blend (CH) was a mixture of citral (C) and *cis*-3-hexen-ol-1 (H), the most effective ligands of HarmOr12 and 60, respectively; these two Ors are mainly expressed in larval maxillae (Fig. 1A). The third blend (MJAPCH) was a mixture of the above two blends. The fourth blend (HONA) was a mixture of *cis*-3-hexen-ol-1, ocimene (O), nonanal (N), and phenylacetaldehyde, one attractive odorant from each of the four chemical groups. The fifth blend (HLNCG) was a mixture of *cis*-3-hexen-ol-1, (±)-linalool (L), nonanal, citral, and geraniol (G), five odorants that stably attracted larvae at different doses (Table 4).

Among the five mixtures, MJAP was the strongest attractant (Fig. 5A). Larvae showed modest responses to HONA, CH, HLNCG, and MJAPCH. We further compared the attractiveness of MJAP against green pepper juice in the two-choice assay. Surprisingly, the larvae preferred MJAP to the green pepper juice (Fig. 5B).

To identify the attractive components in MJAP, we split this mixture into a series of mixtures with three components and two components. The mixtures MJAP, MJP, and JP were all attractive with no statistically significant difference in their attractiveness (Fig. 5C). Therefore, the common components *cis*-jasmone and 1-pentanol were identified as the crucial odorants in attraction of *H. armigera* larvae.

Table 3
Combinatorial coding of odorants in *Helicoverpa armigera* larvae.

		OR12	OR31	OR41	OR42	OR50	OR52	OR60
GLV	1-Hexanol	-	-	●	-	-	●	●
	2-Hexanol	-	-	-	●	-	-	●
	3-Hexanol	-	-	-	-	-	●	●
	cis-3-Hexen-ol-1	-	-	-	-	-	●	-
	trans-2-Hexen-1-ol	-	-	-	-	-	●	●
	trans-3-Hexen-1-ol	-	-	-	●	-	-	-
	cis-3-Hexenyl acetate	●	●	-	-	-	●	●
	trans-2-Hexenyl acetate	●	-	●	-	-	-	-
	cis-3-Hexenyl butyrate	-	●	-	-	-	-	●
	cis-3-Hexenyl Salicylate	-	-	●	-	-	-	●
	cis-3-Hexenyl 2-methyl butanoate		●	-	●	-	-	●
Terpenoid	Citral	●	●	-	●	-	●	-
	(S)-(-)-Limonene	-	-	-	-	-	-	●
	(±)-Linalool	●	-	-	-	-	-	●
	(−)-*trans*-Caryophyllene	-	-	-	-	-	-	●
	Myrcene	●	●	-	-	-	-	-
	Geraniol	●	●	-	-	-	-	-



Note: Filled circles represent the maximal response for each HarmOr. Different cycle patterns represent percentages of the maximal response of given HarmOr.

Table 4
Components of odorant blends in chemotaxis behavioural assays.

Blend	Component (dose)
MJAP	myrcene (10^3 ng) + *cis*-jasmone (10 ng) + phenylacetaldehyde (10^4 ng) + 1-pentanol (10^4 ng)
CH	citral (10^2 ng) + *cis*-3-hexen-ol-1 (10^4 ng)
MJAPCH	myrcene (10^3 ng) + *cis*-jasmone (10 ng) + phenylacetaldehyde (10^4 ng) + 1-pentanol (10^4 ng) + citral (10^2 ng) + *cis*-3-hexen-ol-1 (10^4 ng)
HONA	*cis*-3-hexen-ol-1 (10^4 ng) + ocimene (10^3 ng) + nonanal (10^2 ng) + phenylacetaldehyde (10^4 ng)
HLNCG	*cis*-3-hexen-ol-1 (10^4 ng) + (±)-linalool (10^4 ng) + nonanal (10^2 ng) + citral (10^2 ng) + geraniol (10^2 ng)
MJP	myrcene (10^3 ng) + *cis*-jasmone (10 ng) + 1-pentanol (10^4 ng)
MAP	myrcene (10^3 ng) + phenylacetaldehyde (10^4 ng) + 1-pentanol (10^4 ng)
MJA	myrcene (10^3 ng) + *cis*-jasmone (10 ng) + phenylacetaldehyde (10^4 ng)
JAP	*cis*-jasmone (10 ng) + phenylacetaldehyde (10^4 ng) + 1-pentanol (10^4 ng)
JP	*cis*-jasmone (10 ng) + 1-pentanol (10^4 ng)
JA	*cis*-jasmone (10 ng) + phenylacetaldehyde (10^4 ng)
MP	myrcene (10^3 ng) + 1-pentanol (10^4 ng)
AP	phenylacetaldehyde (10^4 ng) + 1-pentanol (10^4 ng)
MJ	myrcene (10^3 ng) + *cis*-jasmone (10 ng)
MA	myrcene (10^3 ng) + phenylacetaldehyde (10^4 ng)

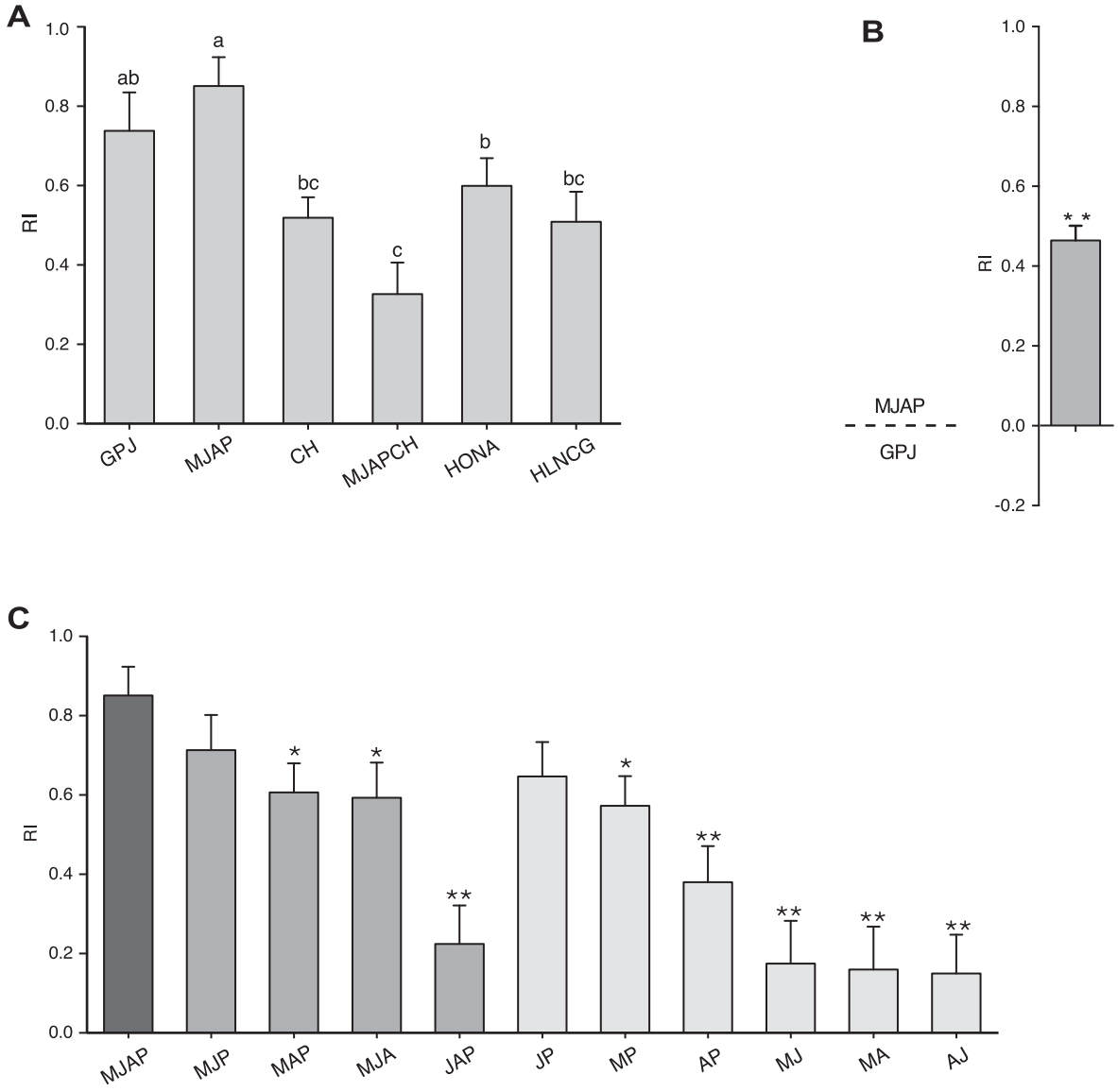


Fig. 5. Response profiles of *Helicoverpa armigera* first-instar larvae to a series of odorant mixtures. A. Response profiles of *H. armigera* larvae to five mixtures. GPJ is the abbreviation of Green Pepper Juice. Error bars show standard error of the mean (SEM) for $n \geq 10$ trials. Data were evaluated by one-way ANOVA, $P \leq 0.05$; B. Chemotaxis response indices from two-choice assay between MJAP and green pepper juice. Error bars show SEM for $n = 10$ trials. Significance of deviation of response indices from zero was evaluated by Student's *t*-test: *, $P \leq 0.05$. C. Response profiles *H. armigera* larvae to MJAP and different combinations of M, J, B, and P. Data were compared using two-tailed unpaired student's t tests: *, $P \leq 0.05$; **, $P \leq 0.01$.

3.6. Localization of four Ors in larval antenna

The most effective ligands of HarmOr31, 41, 42, and 52 are myrcene, *cis*-jasmone, phenylacetaldehyde and 1-pentanol, the four components of the most attractive mixture, MJAP (Figs. 1 and 2). Therefore, we performed whole-mount *in situ* hybridization experiments to localize the cells expressing the four *Or* genes in larval antennae. We first carried out double-labelled *in situ* hybridization with a digoxigenin (DIG)-labelled *Or41* probe and a biotin (Bio)-labelled *Or52* probe, because JP was the most attractive of the binary mixtures. When we successfully labelled cells expressing *Or52* and *Or41* in one antenna, we always found the two *Ors* are co-expressed in the same cell (Fig. 6A). We also performed double-labelled *in situ* hybridization with a DIG-labelled *Or31* probe and a Bio-labelled *Or42* probe, and found that *Or31* and *Or42* were expressed in different cells of larval antenna (Fig. 6B).

4. Discussion

The olfactory and gustatory responses of *H. armigera* larvae play a major role in food-plant selection. Ors stand on the front line in the primary discrimination of host-plant volatiles. To investigate the molecular basis of odour-coding of *H. armigera* larvae, we systematically identified and functionally characterized a major part of the Or repertoire in *H. armigera* larvae. We also formulated an odorant blend based on the most effective ligands of Ors. This blend, MJAP, strongly attracted *H. armigera* larvae. The ligand-receptor activities of Ors paralleled with the chemotaxis behaviour of the first-instar larvae, suggesting that the neural circuitry activated by these Ors govern food-search behaviour in the cotton bollworm.

We identified a set of 17 Or genes expressed in *H. armigera* larvae. Previously, 60 Or genes were identified in *H. armigera* adults (Jiang et al., 2014; Liu et al., 2012, 2014b), and so the number of Or genes expressed at the larval stage is about one third of that expressed at the adult stage. Similarly, in *D. melanogaster*, 62 Or genes are expressed in approximately 1300 OSNs while only 25 Or genes are expressed in 21 OSNs in larvae (Gerber and Stocker, 2007). Decreased numbers of Ors and OSNs in larvae have also been found in *An. gambiae* and *B. mori* (Tanaka et al., 2009; Xia et al., 2008). The simplicity of larval olfactory systems makes the larva an excellent stage to study the olfactory signal transduction and coding pathways.

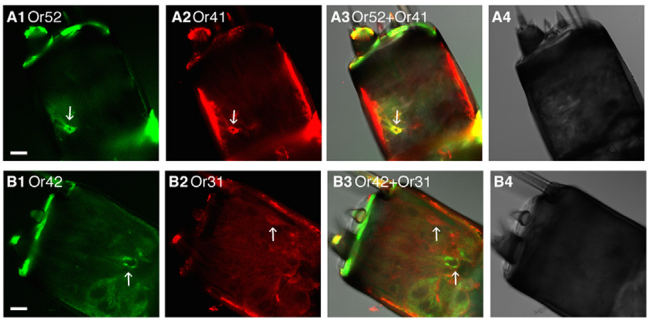


Fig. 6. Localization of Or52, Or41, Or42, and Or31 in antennae of *Helicoverpa armigera* larvae. Double fluorescence *in situ* hybridization analyses with larval antennae and combinations of differentially labelled *Or* probes and visualization of cells harbouring distinct *Or* transcripts by green (biotin) (A1, B1) and red (digoxigenin; DIG) (A2, B2) fluorescence. Co-labelling of cells by both Or probes appears as yellow/orange colour in overlay of red and green fluorescence channels (A3). Bright-field images are shown for reference (A4, B4). Arrows indicate cell location. Scale bars: 20 μm. (A1–4) Or52 and Or41 probes labelling the same cell. (B1–4) Or42 and Or31 probes labelling different cells.

Insect chemosensory systems usually use two strategies and combinations for coding neural messages, combinatorial coding and labelled-line coding (Martin N. Andersson, 2015). Most Ors of *D. melanogaster* are broadly tuned, implying that the fly may "encode" its odour environments in a combinatorial coding manner, with a modest repertoire of receptors and glomeruli to respond to numerous odour stimuli (Hallem and Carlson, 2006; Hallem et al., 2004; Kreher et al., 2005). Subsequently, researchers identified several highly specific Ors that are narrowly tuned to certain odours, especially to non-pheromone compounds, proving the existence and importance of dedicated circuits in insect olfaction (Dweck et al., 2013, 2015; Stensmyr et al., 2012). Based on the functional analyses of Ors of *H. armigera* in this study, we infer that the Ors respond to odorants in a combinatorial manner at the larval stage. There were considerable overlaps among the odorant tuning spectra of the seven Ors. Several single odorants such as citral and *cis*-3-hexenyl acetate activated multiple Ors. The combinatorial tuning map is consistent with the highly polyphagous feeding behaviour of *H. armigera* larvae. The caterpillars feed on a wide range of host plants. Thus, a simple larval olfactory system is required to discriminate key odorants from numerous plant volatiles, resulting in a wide-tuning characteristic of a number of Ors. In contrast, the larval stage of *B. mori*, a typical monophagous moth species, uses only one highly selectively tuned Or, BmOr56, to recognize *cis*-jasmone, the key volatile emitted from mulberry leaves (Tanaka et al., 2009). We speculate that chemotaxis responses toward a food source in polyphagous caterpillars such as *H. armigera* larvae are governed by a neural network fed information via a number of broadly tuned OSNs, rather than by one or only a few precisely tuned neurons like in monophagous caterpillars.

Our results also imply that antennae play a more important role than maxillae in the larval odour-coding pathway. We identified five more Ors in larval antennae than in larval maxillae (Fig. 1). Moreover, the four components in the most attractive mixture, MJAP, were the most effective ligands of Ors mainly expressed in the antennae, while the mixture CH, which was composed of the two most effective ligands of Ors mainly expressed in larval maxillae, only modestly attracted *H. armigera* larvae (Fig. 5A). It appears that cotton bollworms receive and process olfactory signals primarily from antennae, with maxillae providing supplementary information for chemotaxis behaviour. In *An. gambiae*, specific ablation of the larval antennae resulted in a dramatic loss of odorant-driven behavioural responses, while larvae subjected to maxilla ablations maintained normal responses (Xia et al., 2008).

We used a series of simple mobility assays to identify olfactory-based behavioural responses of *H. armigera* larvae to a panel of odorants that are known to activate Ors. The results of the no-choice and two-choice assays provided unambiguous initial evidence of larval olfactory-based behaviour. We designed five odorant blends based on the tissue-specific expression and odorant response spectra of Ors, combined with the olfactory-based responses of *H. armigera* larvae to individual odorants. These blends were used to further explore the behavioural responses of *H. armigera* larvae. The blend MJAP was composed of four odorants, myrcene, cis-jasmone, phenylacetaldehyde, and 1-pentanol, the most effective ligands of antennal receptors Or31, Or41, Or42, and Or52, respectively. This blend showed the strongest attractiveness to the larvae, even stronger than that of the juice of green pepper, one of the major host plants of *H. armigera* (Fig. 5B). Previous studies identified attractive blends of compounds mainly according to host plant volatiles or electrophysiological responses of *H. armigera*. A seven-component synthetic blend of electrophysiologically active compounds (benzaldehyde, (S)-(−)-limonene, (R,S)-(±)-linalool, (E)-myroxide, (Z)-β-ocimene, phenylacetaldehyde, and (R)-(−)-piperitone) identified from the host plant *Tagetes*

erecta was shown to be attractive to adult *H. armigera* in a wind-tunnel assay (Bruce and Cork, 2001). In another study where adult *H. armigera* were exposed to 31 chemical blends in a bioassay, 21 blends were significantly attractive and only one was significantly repellent (Gregg et al., 2010). The most attractive blends were those containing four to six components, including aromatic volatiles primarily found in flowers, especially 2-phenylethanol and phenylacetaldehyde, and volatiles found primarily in leaves, including green leaf volatiles and terpenoids (Gregg et al., 2010). The present study is the first report of the development of attractive blends for lepidopterous caterpillars based on the most effective ligands of Ors and expression patterns of Ors.

Several studies have shown that some components in mixtures of plant volatiles used for host recognition are more important than others, and that omitting them from a blend results in a substantial reduction in activity, whereas others play a minor role and can be omitted without any significant loss of attractiveness (Bruce and Pickett, 2011). Recent studies have suggested that there is some redundancy in the composition of host odour blends. According to the results of these previous studies, we presented *H. armigera* larvae with a series of reduced-component blends of MJAP to observe if any was present redundancy. The attractiveness of a two-compound blend of JP (*cis*-jasmone and 1-pentanol) was not significantly different from that of the full four-component blend of MJAP. This result illustrated that J and P are essential to the MJAP blend. Surprisingly, we found that *Or41* and *Or52* were co-expressed in the same OSN (Fig. 6). It is unusual to see receptors expressed in the same cell besides Orco.

In this study, we performed a comprehensive functional characterization of Ors expressed in the chemosensory organs of larval *H. armigera*. Our results provide the first evidence that many Ors in caterpillars are broadly tuned and respond to odorants in a combinatorial manner. We also designed attractants based on the most effective ligands of the Ors in larvae, and identified a four-component blend that strongly attracted *H. armigera* larvae. Our findings provide details of the molecular mechanisms of larval olfaction in *Helicoverpa/Heliothis* species, and shed light on the development of new baits targeting olfactory pathways for pest management.

Author contributions

D.C. designed and conducted experiments, analysed the data and wrote the manuscript; C.N. conducted experiments and analysed the data; L.-Q.H. contributed materials and technical support; C.-Z.W. conceived the project, designed the experiments, supervised experimental procedures, analysed the data and wrote the manuscript.

Competing financial interests

We have no conflict of interest in submitting this paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2017.03.007.

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