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Article

Highly Selective Tuning of a Silkworm Olfactory Receptor to a Key Mulberry Leaf Volatile

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Summary

Background: The olfactory system plays an important role in the recognition of leaf volatiles during the search of folivore insects for a suitable plant host. For example, volatiles emitted by mulberry leaves trigger chemotaxis behavior in the silkworms *Bombyx mori*, and as a consequence, they preferentially reside on and consume mulberry leaves. Here, we aimed to identify natural chemoattractants and their corresponding olfactory receptors (Ors) involved in silkworm behavior to mulberry leaves.
Results: Chemotaxis behavioral assays for headspace volatiles detected by gas chromatography-mass spectroscopy analysis revealed that among the volatiles that were emitted by mulberry leaves, *cis*-jasmone was the most potent attractant for silkworms, working at a threshold of 0.3 pg from a 20 cm distance. Among a total of 66 *Ors* identified in the *B. mori* genome, we found that 23 were expressed in the olfactory organs during larval stages. Functional analysis of all the larvae-expressed Ors in *Xenopus* oocytes revealed that one Or, termed BmOr-56, showed a high sensitivity to *cis*-jasmone. In addition, the ligand-receptor activity of BmOr-56 reflected the chemotaxis behavioral response of silkworms.
Conclusions: We identified *cis*-jasmone as a potent attractant in mulberry leaves for silkworms and provide evidence that a highly tuned receptor, BmOr-56, may mediate this behavioral attraction. The current study sheds light on the mechanism of the correlation between olfactory perception in folivore insects and chemotaxis behavior to a natural volatile emitted by green leaves.

Introduction

Animals utilize chemosensory systems to obtain information from their external environment. Herbivore insects, for example, are able to locate hosts or edible plants by discriminating between emitted volatiles by using olfaction. It is possible that plants may emit more than 1000 different volatile compounds [1], and thus insects must be able to discriminate between these numerous volatiles in order to select a suitable host. It has been suggested that the blend ratio of volatiles is important for host selection and that the olfactory system permits animals to detect, discriminate, and produce an appropriate behavioral response to the volatile blends [2–5]. The precise molecular mechanism underlying plant selection by folivore insects remains unclear.

In insects, the first step in odor perception occurs in the periphery olfactory neurons, in which odor molecules interact with olfactory receptor (Or) proteins. Insect *Or* genes were first discovered in *Drosophila melanogaster* (62 Ors) [6–8] and were then subsequently reported in the mosquito species *Anopheles gambiae* (79 Ors) [9] and in the honey bee *Apis mellifera* (170 Ors) [10]. A total of 48 *Or* genes have been reported in the silkworm *Bombyx mori* to date [11–13]; however, the vast majority have only been partially sequenced as a result of an incomplete silkworm genome database. With the exception of Or83b, insect *Or* genes appear to have evolved rapidly [14, 15]. In addition, there have been few orthologous relationships between insects identified. Many insect Ors form a heteromeric complex with the conserved Or83b family in single olfactory neurons [12, 14–19], and it has recently been revealed that this complex comprises a novel class of ligand-activated, nonselective cation channels [20, 21]. A combinatorial odor model, in which odor identity is encoded by the activation of distinct groups of Ors, has been suggested in insect odor perception [22–29]. Most recently, studies on larval Ors in *D. melanogaster* and *A. gambiae* have revealed the mechanistic basis of olfactory-driven behavior in the larval stage [30, 31].

B. mori is a folivore insect species that resides on and consumes mulberry leaves. In the first screening step of edible leaves, *B. mori* larvae demonstrate chemotaxis behavior by using olfaction. Once they reach their target leaves, a biting action is initiated, before a final swallowing factor such as cellulose, phosphate, and silica in leaves aids the larvae in the continuous consumption of food [32]. Unlike *D. melanogaster* and *A. gambiae* larvae, *B. mori* larvae reside in an open terrestrial environment with a low concentration of food odor signals and thus probably possess both sensitive and selective olfactory mechanisms at the receptor level that lead to chemotaxis. Because of the commercial use of silkworms, numerous studies undertaken during the mid-20th century focused on the identification of silkworm odor attractants. Several volatile attractants were identified in mulberry leaves, including C6 acetates, C6 alcohols, and terpine molecules [33, 34]. However, because of a limited understanding of olfaction at the molecular and genomic level, it has been difficult to precisely define the mechanisms underlying chemotaxis behavior.

In the current study, we raised numerous specific questions in order to increase our understanding of the molecular basis of chemotaxis behavior in silkworms in response to volatiles emitted from mulberry leaves. We specifically investigated whether *B. mori* larvae recognize mulberry leaves through a combination of emitted odors or by a single specific volatile and whether the attractants recognized by Ors is in a combinatorial fashion or via a specific receptor similar to pheromone-pheromone receptors. In combination with the chemotaxis

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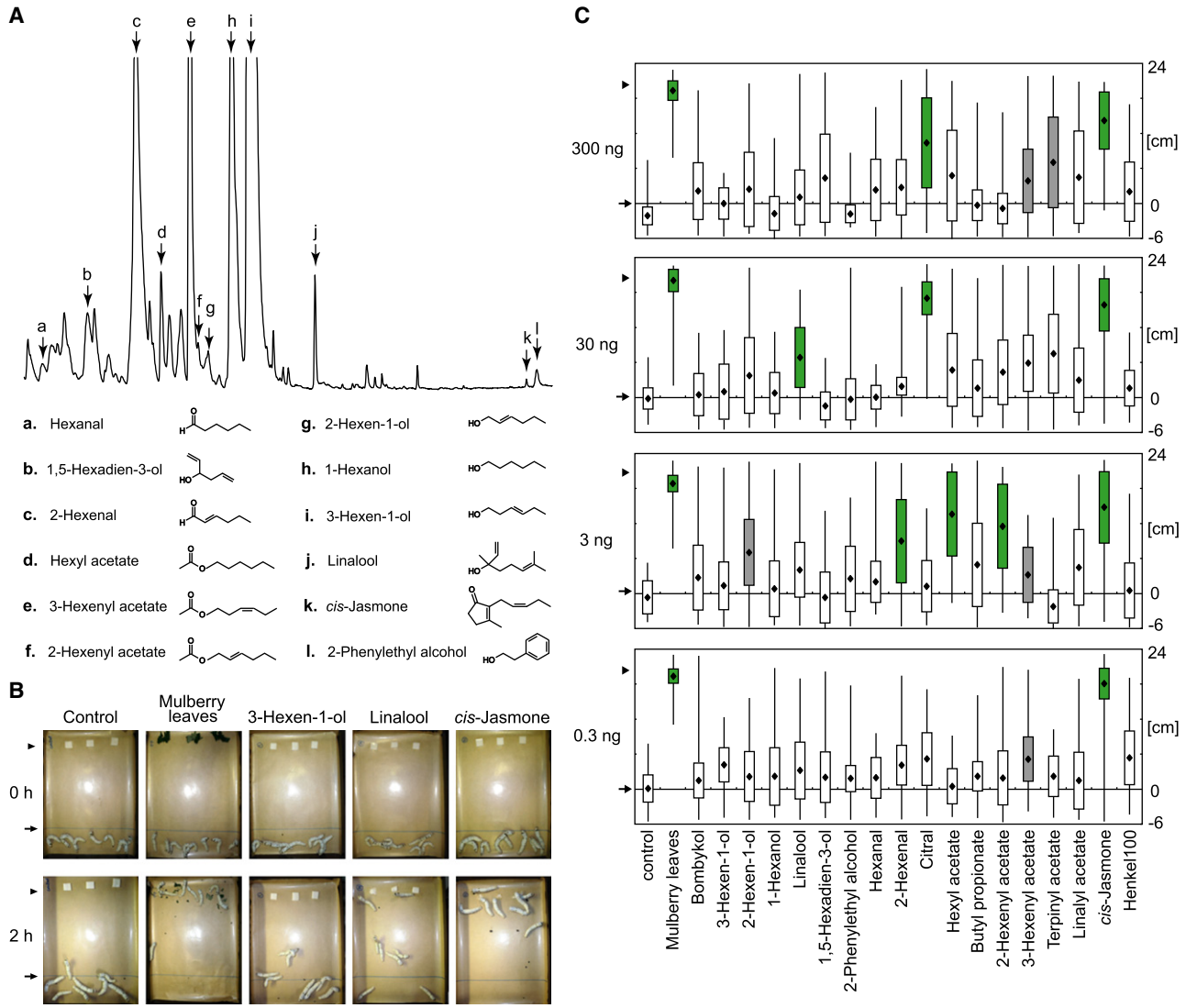

Figure 1. Analysis of Mulberry Leaf Volatiles and Silkworm Chemotaxis Behavior Assays

(A) GC-MS analysis of headspace volatiles emitted from mulberry leaves. SPME (solid-phase micro extraction) was used to collect the headspace volatiles. On GC spectra, 12 odor compounds (arrows: "a"–"l") were found to be both dominant and common to the examined mulberry leaves from three different locations. The results presented are representative of three separate experiments.

(B) Representative photographs outlining the chemoattractive behavior of fifth instar larvae. Mulberry leaves or odorant solution diluted in paraffin oil (indicated by arrowheads) were deposited in a closed box 20 cm away from the start position (arrows) where eight larvae were placed. Larvae had been food deprived for 24 hr prior to the onset of the experiment. Upper and lower panels demonstrate the position of larvae at 0 hr and 2 hr.

(C) Box-and-whisker plots of the position of larvae after the 2 hr chemotaxis assay. The boundaries of the box plots represent the first and third quartiles, and the diamonds represent the median value. The amounts of odorants are indicated. Arrowheads indicate the location where odorant or leaves were deposited; arrows indicate silkworms' starting position. Samples were individually compared to the control with a Mann-Whitney U test with Bonferroni correction for 19 comparisons after Kruskal Wallis H test. The gray ($p < 0.05$) and green boxes ($p < 0.01$) represent significant differences from the control values ($n = 24$).

behavioral assay and the functional analysis of Ors in *Xenopus* oocytes, we report the successful identification of specific receptors that recognize the most potent naturally occurring chemoattractive volatile. This study provides a receptor-based understanding of chemotaxis behavior in folivore worms.

Results

Identification of Headspace Volatiles Emitted from Mulberry Leaves

B. mori silkworms are attracted to mulberry leaves, and thus headspace volatiles emitted by the mulberry leaves should contain potent attractants. After the collection of headspace volatiles, we performed gas chromatography-mass spectroscopic (GC-MS) analysis on the odors emitted by mulberry leaves sampled from three different locations and identified 12 volatile compounds that were present in relatively significant amounts and that were also common to all of the examined samples (Figure 1A).

Behavioral Response of *B. mori* Fifth Instar Larvae to Mulberry Leaf Odorants

We next set up a behavioral assay to examine the chemotaxis activity of the identified odorants. We utilized the first or second day of fifth instar larvae that had been starved for

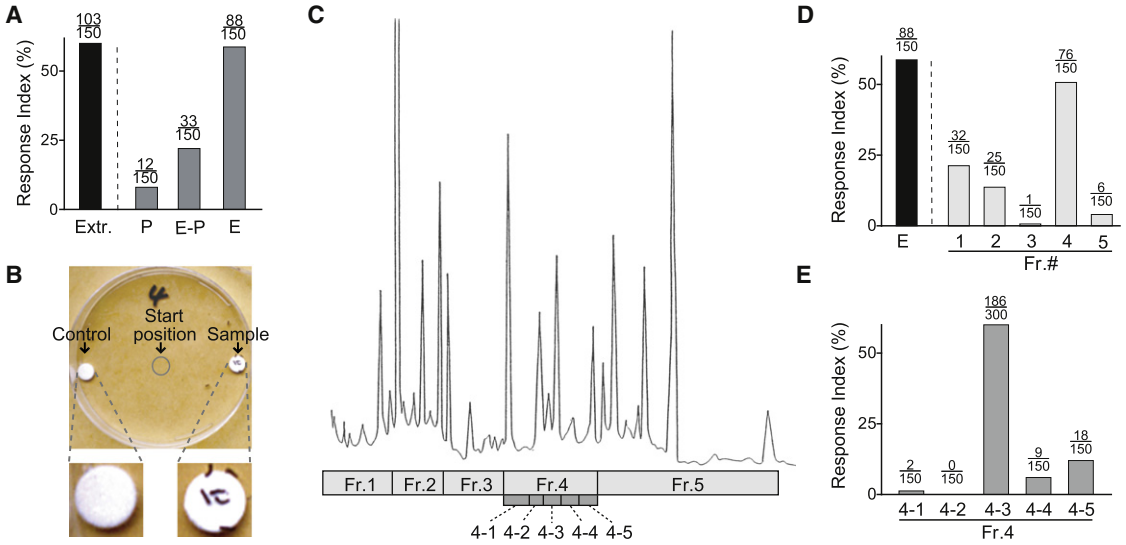

Figure 2. Behavioral Response Assay on Preparative GC Fractions from Mulberry Leaf Extract

(A) The response index (%) of the test that used mulberry leaf extract (Extr.) and with eluents from silica gel chromatography that used 100% pentane (P), 15% ether-85% pentane (E-P), and 100% ethyl ether (E).

(B) The photograph showing a behavioral response assay for first instar larvae. Water is used as the control; fraction 4 in (C) is used as the sample.

(C) Preparative GC chart of the volatiles derived from mulberry leaves. The sample eluted by 100% ethyl ether was applied onto a preparative GC chromatocolumn. Compounds were combined into five fractions (1–5) and fraction 4 further divided into five fractions (4-1–4-5) as indicated.

(D) The response index (%) of the test with fractions 1–5.

(E) The response index (%) of the test with fractions 4-1–4-5.

24 hr prior to the onset of the experiment. The odorant solution was diluted in paraffin oil and deposited in a closed box at a distance of 20 cm away from the start position where eight larvae were placed (Figure 1B). We calculated the distance that larvae had traveled at the end of a 2 hr test period by measuring the position of the head of the larva from the start position. In addition to the 12 identified volatiles, we tested odor compounds that have previously been reported to be emitted from mulberry leaves [34] and also included an odorant that has been proposed to be the most powerful attractant [35]. As a result, we tested a total of 16 odorants in the chemotaxis assays.

Mulberry leaves were found to potently attract silkworms (Figure 1B and Movies S1 and S2 available online). *cis*-jasmone demonstrated strong attractive activity when tested at amounts ranging from 0.3–300 ng ($p < 0.01$, Figures 1B and 1C) from a 20 cm distance. Citral also attracted silkworms when administered at amounts ranging from 30–300 ng ($p < 0.01$), but not at lower amounts. Linalool, 2-hexenal, hexyl acetate, and 2-hexenyl acetate showed attractive activity at either 30 ng or 3 ng ($p < 0.01$). 2-Hexen-1-ol, 3-hexenyl acetate, and terpinyl acetate demonstrated only a weak activity at some of the amounts utilized ($p < 0.05$). Repellant activity was not observed for any of the 16 odorants but clearly observed for camphene, a bicyclic monoterpene with a pungent smell, (Figure S1) that has previously been reported as a potent repellant [35]. These results suggest that the weak attractive activity observed for some odorants is not a result of avoidance to the odorant because of the enclosed environment. As a result, consistent dose-dependent chemotaxis activity was obtained only for citral and *cis*-jasmone. Similarly, first instar larvae also showed behavioral responses to *cis*-jasmone and citral but not to any of the other odorants (data not shown).

Behavioral Assay with Preparative Gas Chromatography Fractions Extracted from Mulberry Leaves

To determine whether *cis*-jasmone is the most potent attractant emitted from mulberry leaves, we investigated the chemoattractive activity of all the GC fractions derived from mulberry leaves. We utilized first instar larvae in the chemotaxis assays because of the increased speed of the assay. Mulberry leaf extract was separated with silca gel column chromatography and each of the three separated fractions was subjected to behavioral response assays. The 100% ethyl ether eluent showed similar activity to that of the original mulberry leaf extract (Figure 2A). The active ethyl ether fraction was then applied onto preparative GC and the elution combined into five fractions, as shown in Figure 2C. Each combined fraction was then assayed for a behavioral response. Strong chemoattractive activity, similar to that observed in the original extract, was identified in fraction 4 (Figures 2B–2D). Weaker responses were found in fractions 1 and 2 (Figures 2C and 2D). Next, fraction 4 was divided into five fractions, termed fractions 4-1, 4-2, 4-3, 4-4, and 4-5. Of these fractions, fraction 4-3 appeared to demonstrate the strongest activity (Figure 2E). Mass spectroscopic analysis of fraction 4-3 revealed the odorants *cis*-jasmone and 2-phenylethyl alcohol. Because 2-phenylethyl alcohol did not demonstrate chemotaxis activity (Figure 1C), we suggest that *cis*-jasmone is the most potent naturally occurring chemoattractant present in mulberry leaves.

We examined the chemosensory behavior of silkworms to other plant leaves such as *Arabidopsis thaliana*, *Nicotiana benthamiana* (Tobacco), *Citrus limon* (Lemon), and *Myrica rubra* (Yumberry). We also included leaves of *Citrus natsudaidai* (Summer tangerine), *Eriobotrya japonica* (Loquat), *Diospyros kaki* (Persimmon), and *Castanea pubinervis* (Chestnut) that have previously been reported to possess some attractive

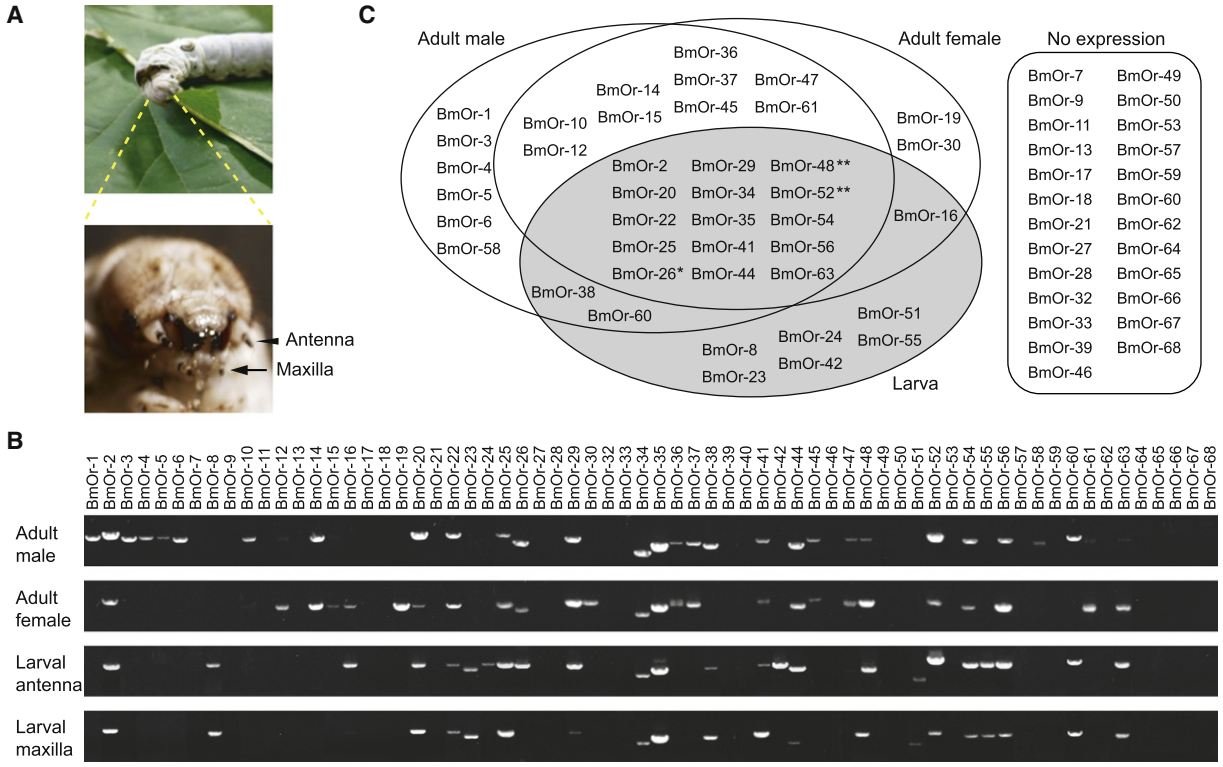

Figure 3. *Or* Gene Expression in the Chemosensory Organs of Adult and Larval *B. mori*

(A) Photographs of a fifth instar larva that is able to bite mulberry leaves (upper panel) and the head of the fifth instar larva (lower panel). The arrowhead and arrow indicate antenna and maxilla, respectively.

(B) RT-PCR analysis of *BmOr* expression in the antennae of adult males, adult females, fifth instar larvae, and the maxilla of the fifth instar larvae.

(C) A comprehensive list of *BmOrs* expressed in adult males and females, and larva. An asterisk marks those for which only a partial sequence is available; two asterisks mark pseudogenes.

activity for silkworms [36]. As a result, no leaf was as attractive as mulberry leaves (Figure S2A). We performed GC-MS analysis on the odors emitted by these leaves, after the collection of headspace volatiles. *cis*-jasmone was not detected in any of these leaves (Figure S2B).

Identification of a Complete Set of *Or* Genes and Their Phylogenetic Analysis

The next aim of our study was to identify Ors that are responsible for the detection of *cis*-jasmone in silkworms. First, we used the most recently updated *B. mori* genome database, reported by the Japan-China consortium team, to identify 187 genes that encoded putative seven-transmembrane receptors [37]. On the basis of comparison with the previously identified Ors in *D. melanogaster*, *A. gambiae*, and *A. mellifera* [6–10], 66 of the 187 genes appeared to encode *Or* genes. Two of the 66 *Or* genes appeared to be pseudogenes, whereas three *Or* genes remained truncated by the ends of contigs. Forty-eight Ors matched previously annotated *B. mori* Or (BmOr) sequences [11–13], and therefore we assigned the same number for these genes as was previously registered, even if they were reported as partial sequences. The complete sequences have been deposited (Figure S3). Newly identified BmOr sequences were named consecutively, beginning with BmOr-49. Phylogenetic analysis of Ors from four insect species revealed some species-specific clusters, indicating that a small expansion of *Or* genes occurred in each of the insect species lineages (Figure S4).

Expression of *Or* Genes in Chemosensory Organs of Adult and Larval *B. mori*

We next examined the expression of *Or* genes in adult male and female antennae and in larval antenna and maxilla (Figure 3A) by RT-PCR with primers specific for each of the 66 *Or* genes (Figure S3). *BmOr-2*, the *B. mori* ortholog to *Or83b*, was found to be ubiquitously expressed in chemosensory organs (Figure 3B). In addition, we identified amplification products of 32, 26, and 23 *Or* genes in adult males, adult females, and fifth instar larvae, respectively (Figure 3).

Of the 32 BmOrs expressed in adult males, six *BmOrs* (*BmOr-1*, *3*, *4*, *5*, *6*, and *58*) were male specific (Figures 3B and 3C). Among the 26 *BmOr* genes expressed in adult females, *BmOr-19* and *BmOr-30* were female specific. Nine *BmOrs* (*BmOr-10*, *12*, *14*, *15*, *36*, *37*, *45*, *47*, and *61*) were expressed in both male and female adult moth antenna, but not in the larval stage.

Among the 23 larval *BmOrs* analyzed, 20 *BmOrs* were expressed in both antenna and maxilla, suggesting that larval antenna and maxilla show similar *Or* expression profiles (Figure 3B). *BmOr-24*, *26*, and *42* were expressed only in larval antenna. Six larval *BmOr* (*BmOr-8*, *23*, *24*, *42*, *51*, and *55*) were expressed in larvae but not adult stages (Figures 3B and 3C). *BmOr-38* and *60* were also detected in adult males, but not in females, whereas *BmOr-16* was detected in adult females, but not males. Fourteen larval *BmOrs* (*BmOr-20*, *22*, *25*, *26*, *29*, *34*, *35*, *41*, *44*, *48*, *52*, *54*, *56*, and *63*) were expressed in both adult male and female moths.

Functional Characterization of Larval BmOr in a *Xenopus* Oocyte Expression System

Given that the aim of this study was to identify specific Ors responsible for chemotaxis behavior in silkworms, we focused on the 20 larvae-expressed *BmOrs* (except two *Ors* that appear to be pseudogenes and one *Or* that is still partial because of incomplete genome sequences) and performed functional characterization on these BmOrs by using a *Xenopus* oocyte expression system. *Xenopus* oocytes have been successfully used to characterize numerous olfactory and pheromone receptors in insects [11, 12, 29, 38, 39]. DMSO and bombykol, a silkmoth sex pheromone, were utilized as controls in these experiments. Henkel 100, which contains 100 random odor compounds, was also administered, given that it is widely used in ligand screening for Ors in both vertebrate and invertebrate systems [31, 40].

Each of the 20 larval BmOrs was coexpressed with BmOr-2, an ortholog of Or83b and also a functional partner of a conventional Or. Seven larval BmOrs (BmOr-8, 24, 29, 42, 54, 56, and 63) demonstrated current responses to at least one of the odorants applied (Figure 4). Three out of the seven *BmOrs* were larvae specific, whereas the remaining four *Ors* were expressed at both adult and larvae stages, suggesting that the functional Ors observed in larval stages were not necessarily larvae specific (Figure 3C). We also tested 38 additional odorants that have been reported to elicit current responses in *B. mori* antennae [41]. We found that BmOr-8, 24, 29, 42, and 54 responded to some of these odorants, but a response was not observed for other larvae-expressed BmOrs (data not shown). The remaining 13 BmOrs (BmOr-16, 20, 22, 23, 25, 34, 35, 38, 41, 44, 51, 55, and 60) did not respond to any of the odorants tested in this study, suggesting that they either were not functionally expressed or that they recognized additional odorants that were not tested in our study.

Among the odorants tested, BmOr-56 responded specifically to *cis*-jasmone, whereas BmOr-29 showed a nonspecific response to essentially all of the ester compounds (Figure 4). The response amplitude was also significantly different between the BmOrs; such a result is presumably a reflection of the different levels of receptor expression or the different ligand efficacy. Such differences were evident in the high response current elicited by *cis*-jasmone in the BmOr-56-expressing oocytes (~800 nA) compared to Henkel 100, the most potent activator of BmOr-54, that only generated a ~20 nA current (Figure 4). It should be noted that 3-hexen-1-ol, a typical green leaf volatile, did not activate any of the larval BmOrs and that BmOr-24 unexpectedly demonstrated a small response to bombykol (Figure 4).

Structure-Activity Relationship of BmOr-56

cis-jasmone, the most potent silkworm attractant emitted from mulberry leaves, was recognized strongly by BmOr-56 and weakly by BmOr-54 (Figure 4C). Given that BmOr-56 showed the strongest response to *cis*-jasmone, we tested a possibility that BmOr-56 might play the specific role in olfactory-mediated chemotaxis behavior in silkworms. First, we examined the expression of *BmOr-56* in olfactory neurons in antenna by in situ hybridization. *BmOr-2* was shown to be expressed in a cluster of olfactory neurons that form the neuronal perikarya from which dendrites are extended to each sensillum [42] (Figures 5A and 5B), whereas *BmOr-56* mRNA was observed in a single neuron within the neuronal perikarya (Figure 5C), confirming the larval *BmOr-56* expression.

To further investigate the structure-activity relationship of BmOr-56, we measured its response to eight additional compounds that are closely related structurally to *cis*-jasmone in receptor function and behavioral assays (Figures 5D–5G). In the *Xenopus* oocyte expression system, BmOr-56 demonstrated relatively high responses to *trans*-jasmone and dihydrojasmone (maximal response: 55% and 67% of the response to *cis*-jasmone at 100 μM, respectively; EC_{50} values: 37 and 17 μM in contrast to 6.0 μM for *cis*-jasmone, respectively; Figures 5D–5F). 2-amylcyclopentenone and amylcyclopentanone were also able to weakly activate BmOr-56 (maximal response: 11% and 13% of the response to *cis*-jasmone at 100 μM respectively; EC_{50} values: 2.2 mM and 0.1 mM, respectively; Figures 5D–5F). 3-methyl-2-cyclopentenone, methyl jasmonate, and cyclopentanone failed to activate BmOr-56. The lowest threshold concentration for response was ~100 nM for *cis*-jasmone and dihydrojasmone, ~300 nM for *trans*-jasmone, and ~1 mM for 2-amylcyclopentenone and amylcyclopentanone.

In the behavioral assay, the five odorants that demonstrated a response to BmOr-56 were able to attract larvae in a dose-dependent manner (Figure 5G). The threshold amount to attract fifth instar larvae was 0.3 pg for *cis*-jasmone, 3 pg for *trans*-jasmone, 30 pg for dihydrojasmone, 300 pg for 2-amylcyclopentenone, and 3 ng for amylcyclopentanone from a 20 cm distance ($p < 0.01$; Figure 5G). 3-methyl-2-cyclopentenone and methyl jasmonate, which showed no response to BmOr-56, were not able to attract larvae at the test amounts (Figure 5G). Cyclopentanone showed a very weak attractive activity. Taken together, our findings show that the potency observed in the behavioral assay correlates well with that observed in the receptor response assay.

Discussion

The mechanisms underlying host plant selection by folivore insects at the molecular level remain unclear. Whether folivore insects recognize the host plant by a combination of odors or via a specific odor or whether recognition is established via multiple Ors in a combinatorial fashion or by a specific receptor is not known. To reveal the molecular and receptor basis of food-searching behavior in folivore worms, we selected *B. mori* larvae that preferentially reside on and eat mulberry leaves as our insect model. We found that mulberry leaves emitted the volatile odor *cis*-jasmone, which potently attracted silkworms, and was recognized by a highly specific receptor BmOr-56 that belonged to the insect Or family. The ligand-receptor activity of BmOr-56 fairly reflected the chemotaxis behavioral activity, implicating that the neural circuitry activated by BmOr-56 may govern food-search behavior in the silkworm.

We herein reported a nearly complete set of *Or* genes that were identified in the recently completed *B. mori* genome database. We found that 6 out of 23 larval *BmOrs* were specifically expressed in larva and that the remaining 17 *BmOrs* were expressed in the adult, suggesting that although *B. mori* larva and adult demonstrate completely different life and eating styles, many *Ors* overlap in their expression. *D. melanogaster*, *A. gambiae*, and *A. aegypti* also share a significant overlap in larva and adult *Or* expression, including 11 of 25, 8 of 12, and 8 of 23, respectively [22–24, 31, 43]. Moreover, four out of seven larval BmOrs that showed current responses to at least one of the odorants tested in this study were expressed in both larvae and adults, suggesting that the functional Ors

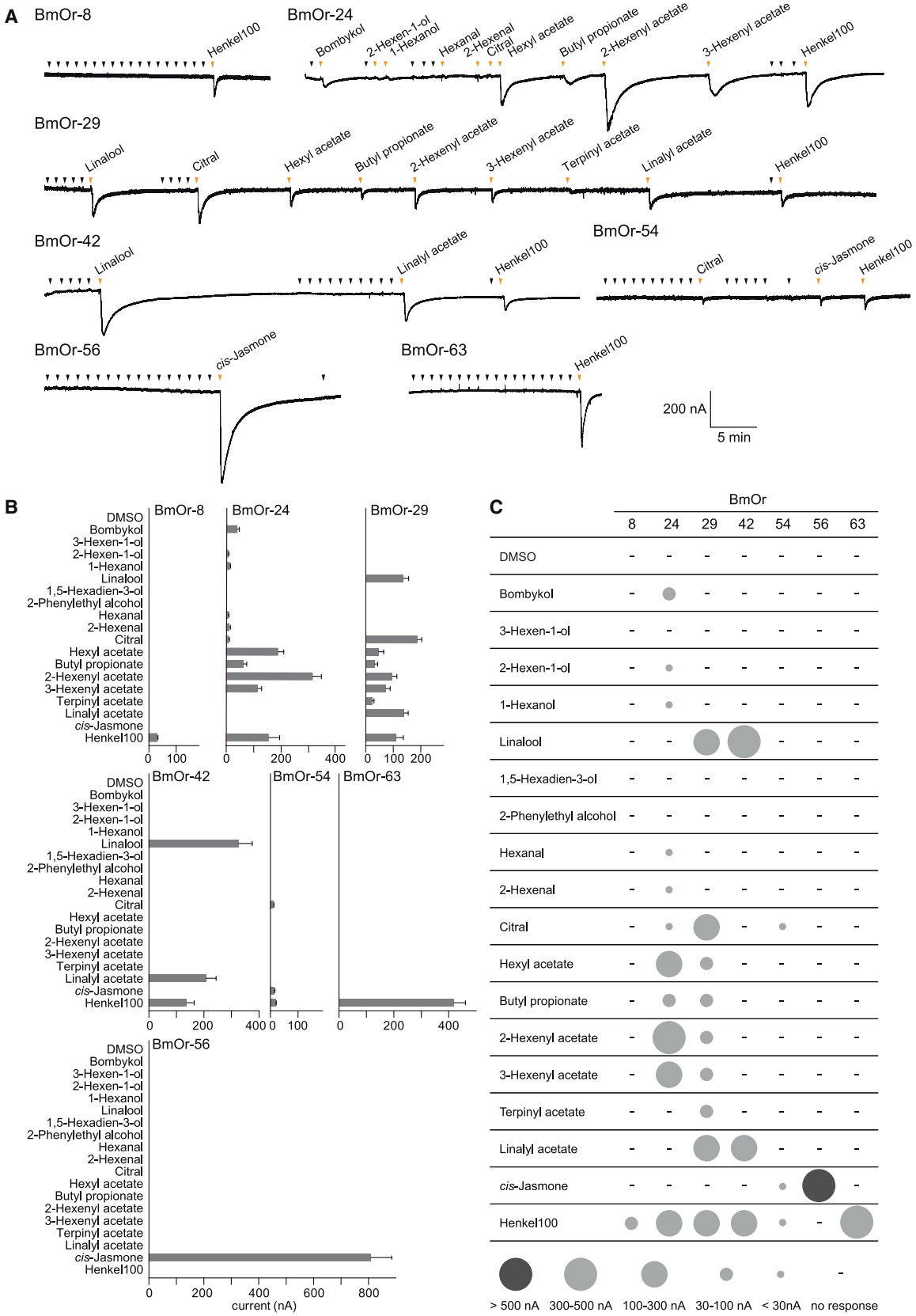

Figure 4. Functional Characterization of Larval BmOrs in *Xenopus* Oocytes

(A) Recordings of odorant-induced currents in *Xenopus* oocytes. The current trace was recorded at −80 mV with sequential application of odorants. Odorants were applied for 3 s at the time indicated by arrowheads. Bombykol was applied at 30 μM, Henkel 100 was diluted at 1:10000, and the other odorants were applied at 100 μM. The order of applied odorants is as listed in (B). Responsive odorants are indicated by orange arrowheads.

in the larval stages were not necessarily larvae specific. Among the functional Ors, BmOr-42 and 24 and BmOr-29 and 56 were found to be structurally similar to each other and were therefore categorized in the same clades in the phylogenic tree. These Ors showed strong odorant responses but with different ligand spectra, suggesting that a small expansion of *Or* genes in the lineage of each insect species resulted in the broadening of the detectable odorant repertoire to adopt eating habitats.

B. mori is one of the most commercially important insect species. Silkworm-mulberry leaf interactions have been extensively studied since the mid-20th century. Several volatiles emitted by mulberry leaves have been found to attract silkworms [34]. In the current study, however, these previously identified odorants turned out to be weak attractants at best. In addition, the optimal activity was found to be in a narrow concentration window without showing a clear dose-dependent response. Furthermore, the doses utilized were much higher than that expected to be released from mulberry leaves, and therefore, these weak attractants may not prove to be physiologically important volatiles. The results of the behavior assays for preparative GC chromatography fractions revealed that although there seemed to be a number of chemoattractant molecules, *cis*-jasmone was the most potent attractant emitted from mulberry leaves. The threshold amount of 0.3 pg (3 μl of 10^{-8} diluted solution) placed at a distance of 20 cm appeared to be significantly lower than the amount required for other insect larvae, such as *D. melanogaster* and *A. gambiae*, which typically demonstrate 10^{-2} to 10^{-5} dilution thresholds for attractants [30, 31]. This sensitivity appears to be adequate for the detection of relatively small amounts of *cis*-jasmone present in mulberry leaves.

cis-jasmone is a derivative of the plant hormone jasmonic aid and is synthesized as a metabolite from linolenic acid via the octadecanoid pathway [44]. *cis*-jasmone has been found in the volatile portion of the oil from jasmine flowers, and therefore, it is well recognized as the odor of jasmine [45]. However, emission of *cis*-jasmone from green leaves has been observed only when they are infested by insects: *cis*-jasmone is emitted from cotton leaves when they are consumed by *Spodoptera exigua* larvae [3] and is also emitted from *Nicotiana* in response to oral secretions from *Manduca sexta* larvae [46]. Thus, to the best of our knowledge, the current study appears to be the first to report the presence of *cis*-jasmone in the headspace of intact green leaves. The attractive activity of mulberry leaves was the highest among various leaves so far tested. Although more leaves should be tested in the future, *cis*-jasmone seems to be an odorant relatively unique to mulberry leaves and the key volatile for chemotaxis behavior of silkworms.

Sericulturists, who grow these animals for silk production, allow silkmoths to lay eggs on paper, and thus hatched worms must migrate and reach mulberry leaves. Therefore, we speculate that individuals with an ability to sense *cis*-jasmone emitted from mulberry leaves and to exhibit chemotaxis behavior have been evolutionary conserved over the long history of sericulture. Why silkworms have evolved to take advantage of *cis*-jasmone as an olfactory signal to approach mulberry leaves is an interesting question to be addressed in future studies. *B. mori* is the domesticated silkmoth that no longer occurs naturally in the wild, and as a consequence, they have reduced mouth parts, do not feed, cannot fly, and respond only to the sex pheromone for reproduction. Indeed, adult *B. mori* was not attracted to *cis*-jasmone (data not shown) even though *BmOr-56* was expressed in adults. In this regard, it is of great interest to investigate physiological meaning of *cis*-jasmone-BmOr-56 interaction in adult *Bombyx mandarina*, the nearest wild relative of *B. mori*.

The attractant activity of green leaf volatiles has also been established in other insect species. It appears that the method by which insects are attracted to host plants is likely to be dependent on the ratio of volatile component blends [3–5]. Thus, it is possible that in addition to the *cis*-jasmone-mediated attraction, the blend of volatile compounds emitted by mulberry leaves may also exhibit chemotaxis activity in silkworms. In order to test this hypothesis, we reconstituted a blend mixture of headspace volatiles on the basis of GC analysis and tested for chemotaxis behavior. We found that combining headspace odorants did not result in an increase in chemoattractive activity (data not shown), suggesting that in silkworms, chemotaxis behavior is not driven by a blend mixture of volatiles from mulberry leaves but rather by a single potent attractant, *cis*-jasmone. Indeed, the relative threshold for the various *cis*-jasmone-related volatiles required to attract larvae reflected the relative threshold for BmOr-56 responses. Future work will resolve whether chemotaxis behavior is ruled by a single specific volatile-Or interaction, namely *cis*-jasmone-BmOr-56, in silkworms.

In the current study, we revealed that *cis*-jasmone is the most potent attractant for silkworms among the various volatiles that are naturally emitted from mulberry leaves. A comprehensive functional characterization of all the Ors expressed in the chemosensory organs of silkworms allowed for the identification of a candidate Or responsible for the chemotaxis behavior toward *cis*-jasmone in silkworms. It is an intriguing possibility that the neural network via a single specific receptor may govern chemotaxis behavior toward a food source in silkworms. Because *cis*-jasmone seems to be a relatively specific odor to mulberry leaves, the present study may shed light on evolutionary aspects including how the folivore insect olfactory system has evolved to adopt the herbivore habitat and establish the ability to discriminate between edible leaves that exist in normal ecological environments.

Accession Numbers

The sequences reported in this paper have been deposited in the GenBank database under accession numbers listed in Figure S3.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, four figures, and two movies and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01034-3.

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(B) Odorant-response spectra of larval BmORs. Responses are measured as induced inward currents, expressed in nA. The error bars indicate the SEM (n = 4–7).

(C) Summary of the average response amplitudes of larval BmOrs to the odorants. The amplitudes of response are averaged and presented as the magnitude of closed circles.

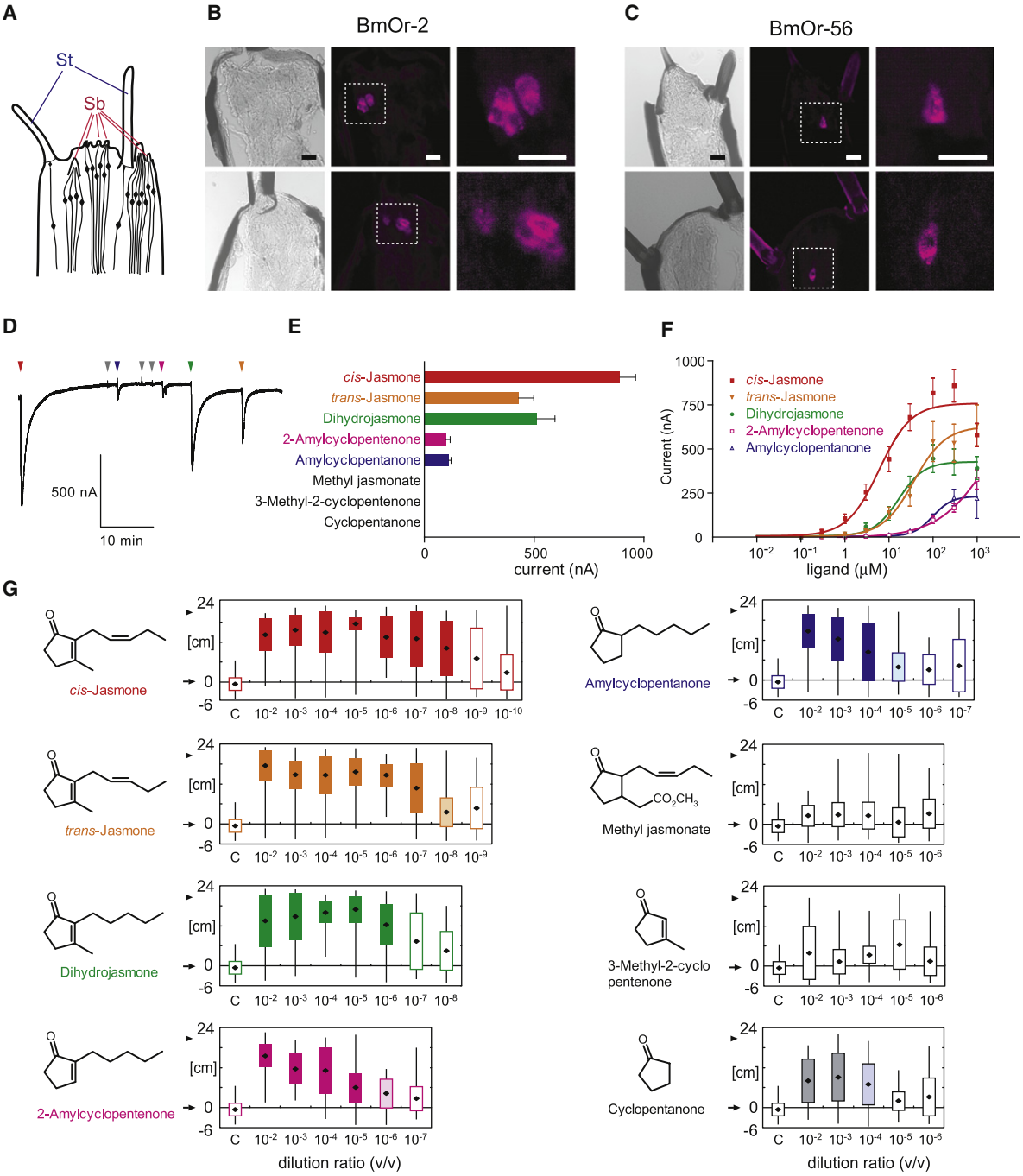

Figure 5. Expression of *BmOr-2* and *BmOr-56* in the Larval Antenna, and Structure-Activity Relationship of BmOr-56 in the Receptor Function and Behavioral Assays

(A) Schematic drawing of the larval antenna. Several olfactory neurons are clustered to form the neuronal perikarya (closed diamond) from which the dendrites were extended to each of six sensilla basiconica (Sb) and two sensilla trichodea (St) (modified from [42]).

(B) RNA in situ hybridization analysis of *BmOr-2* in antenna cryosections. The left shows transmitted-light picture, the middle shows a fluorescence confocal image, and the right shows a higher magnification of the area outlined in the dashed rectangle in the middle panel. The scale bar represents 20 μm.

(C) RNA in situ hybridization analysis of *BmOr-56* in antenna cryosections. The left shows a transmitted-light picture, the middle shows a fluorescence confocal image, and the right shows a higher magnification of the area outlined in the dashed rectangle in the middle panel. The scale bar represents 20 μm.

(D) Recordings of odorant-induced currents in *Xenopus* oocytes expressing BmOr-56 and BmOr-2. The current trace was recorded at −80 mV with sequential application of indicated odorants. Odorants (100 μM) were applied for 3 s at the time indicated by arrowheads. The colors of arrowheads correspond to those of odorants indicated in (E).

(E) Odorant-response spectra of BmOr-56. Responses are measured as induced inward currents, expressed in nA. The error bars indicate the SEM (n = 4–5).

(F) Dose-response curves of BmOr-56 to five compounds that are structurally closely related to *cis*-jasmone. The compounds were sequentially applied to the same oocytes at the indicated concentrations. Each point represents the mean current value ± SEM from four to five individual oocytes. EC_{50} values for each odorant are as follows: *cis*-jasmone (6.0 μM), *trans*-jasmone (37 μM), dihydrojasmone (17 μM), 2-amylcyclopentenone (2.3 mM), and amylcyclopentanone (100 μM).

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References

1. Dudareva, N., Pichersky, E., and Gershenzon, J. (2004). Biochemistry of plant volatiles. Plant Physiol. *135*, 1893–1902.
2. Turlings, T.C., Loughrin, J.H., McCall, P.J., Rose, U.S., Lewis, W.J., and Tumlinson, J.H. (1995). How caterpillar-damaged plants protect themselves by attracting parasitic wasps. Proc. Natl. Acad. Sci. USA *92*, 4169–4174.
3. Loughrin, J.H., Manukian, A., Heath, R.R., and Tumlinson, J.H. (1995). Volatiles emitted by different cotton varieties damaged by feeding beet armyworm larvae. J. Chem. Ecol. *21*, 1217–1227.
4. De Moraes, C.M., Lewis, W.J., Pare, P.W., Alborn, H.T., and Tumlinson, J.H. (1998). Herbivore-infested plants selectively attract parasitoids. Nature *393*, 570–573.
5. Hoballah, M.E., Tamo, C., and Turlings, T.C. (2002). Differential attractiveness of induced odors emitted by eight maize varieties for the parasitoid cotesia marginiventris: is quality or quantity important? J. Chem. Ecol. *28*, 951–968.
6. Clyne, P.J., Warr, C.G., Freeman, M.R., Lessing, D., Kim, J., and Carlson, J.R. (1999). A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in Drosophila. Neuron *22*, 327–338.
7. Gao, Q., and Chess, A. (1999). Identification of candidate Drosophila olfactory receptors from genomic DNA sequence. Genomics *60*, 31–39.
8. Vosshall, L.B., Amrein, H., Morozov, P.S., Rzhetsky, A., and Axel, R. (1999). A spatial map of olfactory receptor expression in the Drosophila antenna. Cell *96*, 725–736.
9. Hill, C.A., Fox, A.N., Pitts, R.J., Kent, L.B., Tan, P.L., Chrystal, M.A., Cravchik, A., Collins, F.H., Robertson, H.M., and Zwiebel, L.J. (2002). G protein-coupled receptors in Anopheles gambiae. Science (New York, N. Y *298*, 176–178.
10. Robertson, H.M., and Wanner, K.W. (2006). The chemoreceptor superfamily in the honey bee, Apis mellifera: expansion of the odorant, but not gustatory, receptor family. Genome Res. *16*, 1395–1403.
11. Sakurai, T., Nakagawa, T., Mitsuno, H., Mori, H., Endo, Y., Tanoue, S., Yasukochi, Y., Touhara, K., and Nishioka, T. (2004). Identification and functional characterization of a sex pheromone receptor in the silkmoth Bombyx mori. Proc. Natl. Acad. Sci. USA *101*, 16653–16658.
12. Nakagawa, T., Sakurai, T., Nishioka, T., and Touhara, K. (2005). Insect sex-pheromone signals mediated by specific combinations of olfactory receptors. Science *307*, 1638–1642.
13. Wanner, K.W., Anderson, A.R., Trowell, S.C., Theilmann, D.A., Robertson, H.M., and Newcomb, R.D. (2007). Female-biased expression of odourant receptor genes in the adult antennae of the silkworm, Bombyx mori. Insect Mol. Biol. *16*, 107–119.
14. Krieger, J., Klink, O., Mohl, C., Raming, K., and Breer, H. (2003). A candidate olfactory receptor subtype highly conserved across different insect orders. J. Comp. Physiol. *189*, 519–526.
15. Jones, W.D., Nguyen, T.A., Kloss, B., Lee, K.J., and Vosshall, L.B. (2005). Functional conservation of an insect odorant receptor gene across 250 million years of evolution. Curr. Biol. *15*, R119–R121.
16. Larsson, M.C., Domingos, A.I., Jones, W.D., Chiappe, M.E., Amrein, H., and Vosshall, L.B. (2004). Or83b encodes a broadly expressed odorant receptor essential for Drosophila olfaction. Neuron *43*, 703–714.
17. Benton, R., Sachse, S., Michnick, S.W., and Vosshall, L.B. (2006). Atypical membrane topology and heteromeric function of Drosophila odorant receptors in vivo. PLoS Biol. *4*, e20.
18. Neuhaus, E.M., Gisselmann, G., Zhang, W., Dooley, R., Stortkuhl, K., and Hatt, H. (2005). Odorant receptor heterodimerization in the olfactory system of Drosophila melanogaster. Nat. Neurosci. *8*, 15–17.
19. Pitts, R.J., Fox, A.N., and Zwiebel, L.J. (2004). A highly conserved candidate chemoreceptor expressed in both olfactory and gustatory tissues in the malaria vector Anopheles gambiae. Proc. Natl. Acad. Sci. USA *101*, 5058–5063.
20. Sato, K., Pellegrino, M., Nakagawa, T., Nakagawa, T., Vosshall, L.B., and Touhara, K. (2008). Insect olfactory receptors are heteromeric ligand-gated ion channels. Nature *452*, 1002–1006.
21. Wicher, D., Schafer, R., Bauernfeind, R., Stensmyr, M.C., Heller, R., Heinemann, S.H., and Hansson, B.S. (2008). Drosophila odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. Nature *452*, 1007–1011.
22. Fishilevich, E., Domingos, A.I., Asahina, K., Naef, F., Vosshall, L.B., and Louis, M. (2005). Chemotaxis behavior mediated by single larval olfactory neurons in Drosophila. Curr. Biol. *15*, 2086–2096.
23. Kreher, S.A., Kwon, J.Y., and Carlson, J.R. (2005). The molecular basis of odor coding in the Drosophila larva. Neuron *46*, 445–456.
24. Couto, A., Alenius, M., and Dickson, B.J. (2005). Molecular, anatomical, and functional organization of the Drosophila olfactory system. Curr. Biol. *15*, 1535–1547.
25. de Bruyne, M., Foster, K., and Carlson, J.R. (2001). Odor coding in the Drosophila antenna. Neuron *30*, 537–552.
26. Hallem, E.A., Ho, M.G., and Carlson, J.R. (2004). The molecular basis of odor coding in the Drosophila antenna. Cell *117*, 965–979.
27. Hallem, E.A., and Carlson, J.R. (2006). Coding of odors by a receptor repertoire. Cell *125*, 143–160.
28. Louis, M., Huber, T., Benton, R., Sakmar, T.P., and Vosshall, L.B. (2008). Bilateral olfactory sensory input enhances chemotaxis behavior. Nat. Neurosci. *11*, 187–199.
29. Lu, T., Qiu, Y.T., Wang, G., Kwon, J.Y., Rutzler, M., Kwon, H.W., Pitts, R.J., van Loon, J.J., Takken, W., Carlson, J.R., et al. (2007). Odor coding in the maxillary palp of the malaria vector mosquito Anopheles gambiae. Curr. Biol. *17*, 1533–1544.
30. Kreher, S.A., Mathew, D., Kim, J., and Carlson, J.R. (2008). Translation of sensory input into behavioral output via an olfactory system. Neuron *59*, 110–124.
31. Xia, Y., Wang, G., Buscariollo, D., Pitts, R.J., Wenger, H., and Zwiebel, L.J. (2008). The molecular and cellular basis of olfactory-driven behavior in Anopheles gambiae larvae. Proc. Natl. Acad. Sci. USA *105*, 6433–6438.
32. Hamamura, Y. (1959). Food selection by silkworm larvae. Nature *183*, 1746–1747.
33. Watanabe, T. (1958). Substances in mulberry leaves which attract silkworm larvae (Bombyx mori). Nature *182*, 325–326.
34. Hamamura, Y., and Naito, K. (1961). Food selection by silkworm larvae, Bombyx mori. Nature *190*, 879–880.
35. Ishikawa, S., and Hirao, T. (1965). Studies on olfactory sensation in the larvae of the silkworm, Bombyx mori (III)Attractants and repellents of hatched larvae. Bulletin of the Imperial Sericultural Experiment Station, Japan *20*, 21–36.
36. Ishikawa, S., and Hirao, T. (1964). Studies on olfactory sensation in the larvae of the silkworm, Bombyx mori (II) Chemotaxis of hatched larvae to various plant leaves (in Japanese with English summary). Journal of Sericultural Science of Japan *34*, 15–20.
37. International Silkworm Genome Consortium. (2008). The genome of a lepidopteran model insect, the silkworm Bombyx mori. Insect Biochem. Mol. Biol. *38*, 1036–1045.
38. Wetzel, C.H., Behrendt, H.J., Gisselmann, G., Stortkuhl, K.F., Hovemann, B., and Hatt, H. (2001). Functional expression and characterization of a Drosophila odorant receptor in a heterologous cell system. Proc. Natl. Acad. Sci. USA *98*, 9377–9380.
39. Wanner, K.W., Nichols, A.S., Walden, K.K., Brockmann, A., Luetje, C.W., and Robertson, H.M. (2007). A honey bee odorant receptor for the queen substance 9-oxo-2-decenoic acid. Proc. Natl. Acad. Sci. USA *104*, 14383–14388.

(G) Dose dependence of larval behavioral response in the chemotaxis test. Boundaries of box plots represent first and third quartiles, whereas the diamonds indicate the median value. Arrowheads indicate the place where 3 μl of odorant solution diluted at the indicated factor were deposited; arrows indicate silkworms' starting position; C, control. We individually compared samples to controls by using the Mann-Whitney U test with Bonferroni correction after the Kruskal Wallis H test. Each colored and shaded box represents a significant difference to the control ($p < 0.01$, 0.05, respectively) ($n = 24$). Open boxes represent no statistical difference compared to the control.

40. Wetzel, C.H., Oles, M., Wellerdieck, C., Kuczkowiak, M., Gisselmann, G., and Hatt, H. (1999). Specificity and sensitivity of a human olfactory receptor functionally expressed in human embryonic kidney 293 cells and Xenopus Laevis oocytes. J. Neurosci. *19*, 7426–7433.
41. Topazzini, A., Mazza, M., and Pelosi, P. (1990). Electroantennogram responses of five lepidoptera species to 26 general odourants. J. Insect Physiol. *36*, 619–624.
42. Waku, Y. (1991). Developmental Changes of the Antenna and Its Neurons in the Silkworm, *Bombyx mori*, With Special Regard to Larval-Pupal Transformation. J. Morphol. *207*, 253–271.
43. Bohbot, J., Pitts, R.J., Kwon, H.W., Rutzler, M., Robertson, H.M., and Zwiebel, L.J. (2007). Molecular characterization of the Aedes aegypti odorant receptor gene family. Insect Mol. Biol. *16*, 525–537.
44. Koch, T., Bandemer, K., and Boland, W. (1997). Biosynthesis of cis-Jasmone : a Pathway for the Inactivation and the Disposal of the Plant Stress Hormone Jasmonic Acid to the Gas Phase? Helv. Chim. Acta *80*, 838–850.
45. Sundt, E., Willhalm, B., and Stoll, M. (1964). Analyse des parties acides de l'essence de bergamote saponifiee. Helv. Chim. Acta *47*, 408–413.
46. Lou, Y., and Baldwin, I.T. (2003). Manduca sexta recognition and resistance among allopolyploid Nicotiana host plants. Proc. Natl. Acad. Sci. USA *100*, 14581–14586.