

Research Paper

## A male-specific odorant receptor conserved through the evolution of sex pheromones in *Ostrinia* moth species

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### Abstract

In many moths, mate-finding communication is mediated by the female sex pheromones. Since differentiation of sex pheromones is often associated with speciation, it is intriguing to know how the changes in female sex pheromone have been tracked by the pheromone recognition system of the males. A male-specific odorant receptor was found to have been conserved through the evolution of sex pheromone communication systems in the genus *Ostrinia* (Lepidoptera: Crambidae). In an effort to characterize pheromone receptors of *O. scapulalis*, which uses a mixture of (E)-11- and (Z)-11-tetradecenyl acetates as a sex pheromone, we cloned a gene (*OscOR1*) encoding a male-specific odorant receptor. In addition, we cloned a gene of the *Or83b* family (*OscOR2*). Functional assays using *Xenopus* oocytes co-expressing *OscOR1* and *OscOR2* have shown that *OscOR1* is, unexpectedly, a receptor of (E)-11-tetradecenol (E11-14:OH), a single pheromone component of a congener *O. latipennis*. Subsequent studies on *O. latipennis* showed that this species indeed has a gene orthologous to *OscOR1* (*OlatOR1*), a functional assay of which confirmed it to be a gene encoding the receptor of E11-14:OH. Furthermore, investigations of six other *Ostrinia* species have revealed that all of them have a gene orthologous to *OscOR1*, although none of these species, except *O. ovalipennis*, a species most closely related to *O. latipennis*, uses E11-14:OH as the pheromone component. The present findings suggest that the male-specific receptor of E11-14:OH was acquired before the divergence of the genus *Ostrinia*, and functionally retained through the evolution of this genus.

Key words: odorant receptor, pheromone receptor, *Ostrinia*, (E)-11-tetradecenol, functional assay

### Introduction

In many moths, mate-finding communication is mediated by the sex pheromones produced by females, which are usually a blend of a few compounds [1]. The species specificity of pheromones is conferred by the combination and blend ratio of components, and the males' response is tuned to the specific blend for ensuring correct finding of conspecific females. Since differentiation of sex pheromones is often associated with speciation, it is intriguing to know how

the changes in female sex pheromone have been tracked by the pheromone recognition system of the males.

Moths of the genus *Ostrinia* (Lepidoptera: Crambidae), which show distinct species-specific differentiation of the sex pheromone communication system [2], are good materials for studying the evolution of the pheromone recognition system. Among the 21 species of the genus *Ostrinia* recorded world-

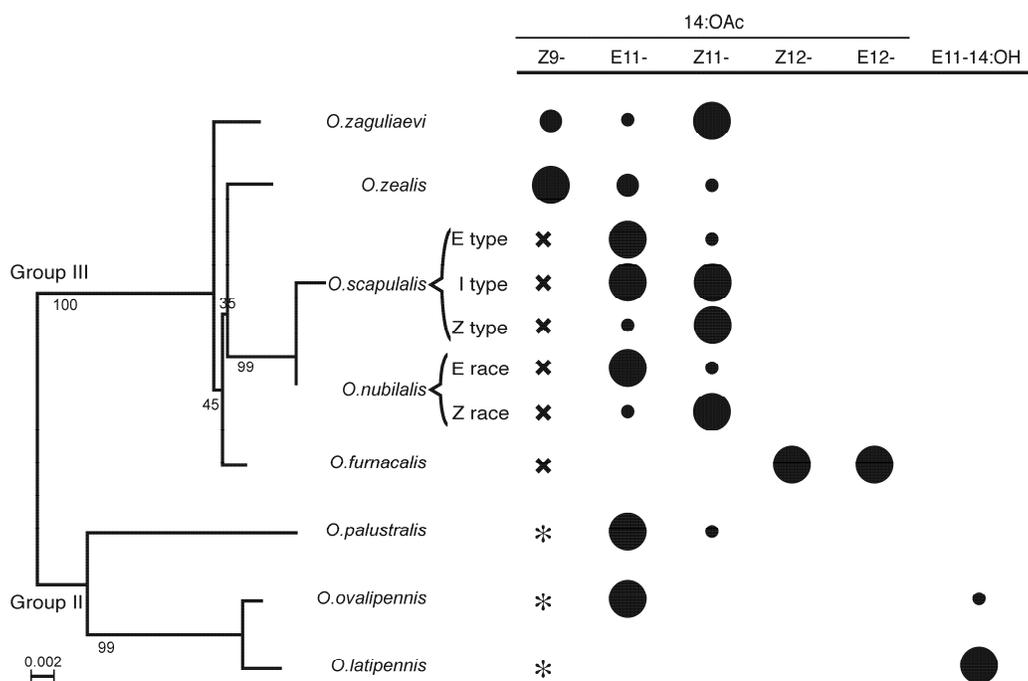
wide [3, 4], the sex pheromones of nine species (*O. nubilalis*, *O. scapularis*, *O. furnacalis*, *O. orientalis*, *O. zaguliaevi*, *O. zealis*, *O. palustralis*, *O. latipennis*, and *O. ovalipennis*) have been characterized to date. The following six compounds have been found as the components of sex pheromones produced by females; Z9-14:OAc, E11-14:OAc, Z11-14:OAc, E12-14:OAc, Z12-14:OAc and E11-14:OH (Fig. 1) [2, 5-7]. The adzuki bean borer *O. scapularis*, the species mainly focused on in the present study, uses a mixture of E11-14:OAc and Z11-14:OAc as the sex pheromone [8].

To detect and track the sex pheromones emitted by the conspecific females, males should have a system that can discriminate pheromone components and their ratios. In this system, pheromone receptors in the olfactory sensilla of male moths are likely to play an important role in the specific recognition of pheromone molecules. Recently, information on the molecular characteristics of pheromone receptors in moths, although some may be more appropriately called "male-specific odorant receptors", has amassed rapidly. Two sex pheromone receptors (BmOR1 and BmOR3) have been characterized in the silkworm *Bombyx mori* [9-12], and three pheromone receptors (HR13, HR14 and HR16) have been characterized in the tobacco budworm *Heliothis virescens* [13-15]. More

recently, pheromone receptors were characterized in the diamondback moth *Plutella xylostella* (PxOR1), the oriental armyworm *Mythimna separata* (MsOR1), and the cotton caterpillar *Diaphania indica* (DiOR1) [16].

Along with the characterization of pheromone receptors, the role of Or83b family proteins, co-expressed with odorant receptors (ORs) in most olfactory receptor neurons (ORNs), has been clarified [17-19]. Or83b is considered to act as a chaperone protein in the transport of ORs to the cell membrane of ORNs [20]. Furthermore, Or83b has been recently shown to form a heterodimer with OR, which configures a ligand-gated cation channel [21, 22].

In the present study, as a first step to understanding the evolution of the pheromone reception system in the genus *Ostrinia*, we tried to clone pheromone receptor genes and an *Or83b* homologue from the male antennae of *O. scapularis*. Since a functional assay of the cloned receptor gene (*OscOR1*) revealed that this gene, unexpectedly, encodes a receptor of a pheromone component used in a congener, *O. latipennis*, but not used in *O. scapularis*, we subsequently surveyed homologous genes in *O. latipennis* and six other congeners, in order to examine the possibility that this receptor has been retained through the evolution of the genus *Ostrinia*.



**Figure 1.** Phylogenetic relationships (left) and sex pheromone blends (right) of *Ostrinia* species examined in this study. The phylogenetic tree was constructed based on the mitochondrial COII gene sequences. The numbers near branches indicate bootstrap values. The size of circles represents rough blend ratio, and x denotes that the compound works as behavioral antagonist. \* not tested for behavioral antagonism.

## Materials and Methods

### Insects

Seven species of *Ostrinia* were reared according to previous reports [6, 23-26]. One-day-old adults and the 5th instar larvae were used in the present study.

### RNA extraction and cDNA synthesis

Total RNA was isolated from the antennae, legs, proboscis, testes, head, midgut and fat body of an individual of *Ostrinia* using an RNeasy Micro Kit (Qiagen). The first-strand cDNA was synthesized with a Takara RNA PCR kit (AMV) Ver.3.0 or a PrimeScript 1st strand cDNA Synthesis kit (Takara Bio Inc.). The quality of the cDNA was verified by testing PCR amplification of the beta-actin gene using the primers sBACT, AACTGGGATGACATGGAGAA GATCTGGC, and aBACT, GAGATCCACATCTGCT GGAAGGTGGACA [34].

### RT-PCR and cloning (*Osc*aORs)

Fragments of cDNA encoding odorant receptors of *O. scapularis* (*Osc*aORs) were amplified from the first-strand cDNA, which was prepared from the antennae of an E-type male moth of *O. scapularis*, using Ex Taq DNA polymerase (Takara-bio) and the following degenerate primers designed based on the consensus sequences of BmORs and HRs: sOR2 (TGYGARCARYTNCARCA), aOR2 (ACCATRAARTANGTNAC), sORA (TGGGSNCAYYTNAARAT), and aORA (TCCCANGGNARNNNRTANAC). The amplification was performed according to the following program: 2 min at 94°C; 1 min at 94°C, 1 min at 40°C and 1 min at 72°C (40 cycles); 15 min at 72°C. The PCR products were cloned into pGEM-T easy vector (Promega) and transformed into *Escherichia coli* JM109 cells. The sequence of the cloned cDNA was determined with an ABI Prism 310 Genetic Analyzer or ABI Prism 3700 Genetic Analyzer (Applied Biosystems).

### Tissue specificity of *Osc*aORs

To investigate the expression of *Osc*aOR1 and *Osc*aOR2 in different tissues, the following primer pairs specific for each *Osc*aOR were used to amplify cDNA from each tissue: OR1 (CAAAAATGGACCCTGAATCG and TCCTTTAGGGTCTCGCTCTG) and OR2 (CCTTCGAGGAGCTGGTGGTAGA and GTGAAGAAGCTTGGCCCCAGAGA). Negative control experiments were run using samples prepared without reverse transcriptase.

### 5'- and 3'-RACE

RACE reactions were performed using a Cap-Fishing Full-length cDNA Premix Kit (Seegene Inc.) following the manufacturer's instructions. The 5'-RACE antisense primers and 3'-RACE sense primers were as follows: aOR1-5' (TCCTTTAGGGTCTCGCTCTG), sOR1-3' (CGCAGCCTTGACAGTAACTC), aOR2-5' (CCCATGGATGCCACTAATCTC), and sOR2-3' (ACGTGTACGCGTTCAGCACAAAT).

### RT-PCR and cloning (ORs of other *Ostrinia* spp.)

The cDNAs encoding ORs were amplified from the first-strand cDNA prepared from adult male antennae of *Ostrinia* and primers specific for OR1 and OR2 designed based on the sequences of *Osc*aORs: OR1 (ATGTTTAAAATTGAAAATCA and TTAGCTGAACGTTCCGCAAGAACATGAAGTA), OR2 (ATGACCAAAGTGAAAGCT and CTACTTCAGTTGTACCAAACCATGAAATAAGT CAC). To investigate the expression of ORs in adult antennae, the following primer pairs specific for each OR were used to amplify cDNA (OR1: CAAAATGGACCCTGAATCG and TCCTTTAGGG TCTCGCTCTG; OR2: CCTTCGAGGAGCTGGTGGT AGA and GTGAAGAAGCTTGGCCCCAGAGA).

### Phylogenetic analysis

The amino acid sequences were aligned using Clustal X [28]. The phylogeny tree was constructed and evaluated by the bootstrap test with 100 re-samplings using the software PHYLIP 3.66 [29].

### *In situ* hybridization

The DNA fragment of *Osc*aORs was subcloned into pGEM-T easy vector (Promega) and used for the generation of sense or antisense RNA probes. Digoxigenin-labeled-RNA probes were prepared with the DIG RNA labeling Mix (Roche). Paraffin-embedded blocks and sections of *O. scapularis* for *in situ* hybridization were obtained by conventional means. Tissue sections were de-waxed with xylene, and re-hydrated through an ethanol series and PBS. The sections were treated for hybridization according to Hoshino et al [30]. Anti-digoxigenin antibody labeled with alkaline phosphatase and BM purple AP substrate (Roche) were used to visualize the signal. The sections were counterstained with Kernechtrot stain solution (Muto), dehydrated, and then mounted with Malinol (Muto).

### Chemicals

Z9-14:OAc and Z11-14:OAc were purchased from Sigma Chemicals Co. (St. Louis, MO). E11-14:OAc, E12-14:OAc, Z12-14:OAc and E11-14:OH

were obtained from Pherobank (Wageningen, The Netherlands). 14:OAc, Z11-16:OAc and Z11-16:Ald were purchased from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). Stock solutions of chemicals (300 mM) were prepared in DMSO and stored at  $-20^{\circ}\text{C}$ . Prior to experiments, the stock solution was diluted with bath solution (88 mM NaCl, 1 mM KCl, 0.3 mM  $\text{Ca}(\text{NO}_3)_2$ , 0.4 mM  $\text{CaCl}_2$ , 0.8 mM  $\text{MgSO}_4$ , 2.4 mM  $\text{NaHCO}_3$ , and 15 mM HEPES, pH 7.6). Bath solution containing 0.1% DMSO was used as a negative control.

### Protein expression in *Xenopus laevis* oocytes and electrophysiological recording

We used the *Xenopus* oocyte system, which can test the function of putative odorant receptors with high sensitivity [9, 16]. Stage V to VII oocytes were treated with 2 mg/ml of collagenase S-1 (Nitta gelatin) in  $\text{Ca}^{2+}$ -free saline solution (82.5 mM NaCl, 2 mM KCl, 1 mM  $\text{MgCl}_2$ , and 5 mM HEPES, pH 7.5) for 1 to 2 h at room temperature. Oocytes were then microinjected with 25 ng of cRNA for a conventional OR (OscaOR1 and OlatOR1) and 25 ng of cRNA for the Or83b family (OscaOR2 and OlatOR2). cRNA was synthesized from linearized modified pSPUTK [38]. Injected oocytes were incubated for 3 days at  $18^{\circ}\text{C}$  in bath solution supplemented with 10  $\mu\text{g}/\text{ml}$  of penicillin and streptomycin. Whole-cell currents were recorded with a two-electrode voltage-clamp filled with 3 M KCl, amplified with an OC-725C amplifier (Warner Instruments), low-pass filtered at 50 Hz and digitized at 1 kHz. The inward current was monitored at a holding potential of  $-80$  mV. Pheromones were delivered through the superfusing bath solution via a silicon tube connected to computer-driven electric valves. Data acquisition and analysis were carried out using Digidata1322A and pCLAMP software.

## Results

### cDNA cloning of a putative sex pheromone receptor of *O. scapularis*

A full-length cDNA of a putative sex pheromone receptor gene (*OscaOR1*) was cloned (Fig. 2A). The deduced amino acid sequence of *OscaOR1* showed 34–41% overall identity to male-specific BmORs of *B. mori* and HRs of *H. virescens*, the C-terminal being the most conserved region (Fig. 2B). Subsequently, a cDNA (*OscaOR2*) that encodes an

Or83b family protein was cloned (Fig. 3A). The deduced amino acid sequence of *OscaOR2* showed a high degree of identity to Or83b family proteins of various insect species belonging to different orders (Fig. 3B).

### Expression of *OscaOR1* and *OscaOR2* in different tissues

The expression of *OscaOR1* and *OscaOR2* in tissues other than adult male antennae was examined by RT-PCR (Fig.4). The mRNA of *OscaOR1* was not expressed in any tissues examined (adult female antennae, the legs, testes, proboscis of an adult moth, and the head, midgut, fat body, and testes of a larva), demonstrating that the expression of this gene is specific to the male antenna. On the other hand, the mRNA of *OscaOR2* was expressed in the larval head, adult foreleg and adult female proboscis, in addition to the antennae of adult females (Fig.4). This finding is in accord with the recent consensus that Or83b family proteins are expressed widely in chemosensory organs [19, 32–34].

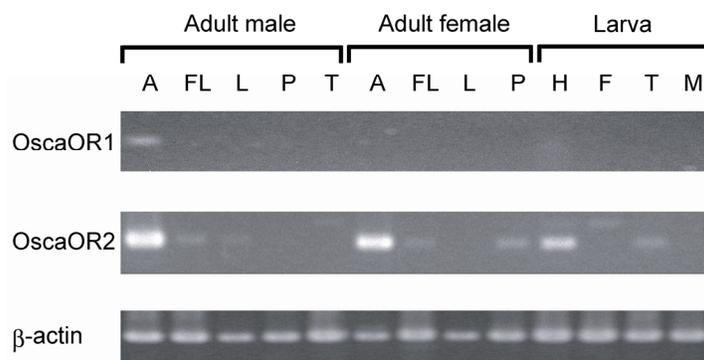
### In situ hybridization

In the longitudinal sections of antennae, many cell bodies at the base of sensilla trichodea or basiconica were stained with the antisense probes for *OscaOR1* and *OscaOR2* (Fig.5A, C, D, F), although the number of cells stained differed with the probe used. In contrast, no cell bodies at the base of sensilla coeloconica were stained with either antisense probe (Fig.5C).

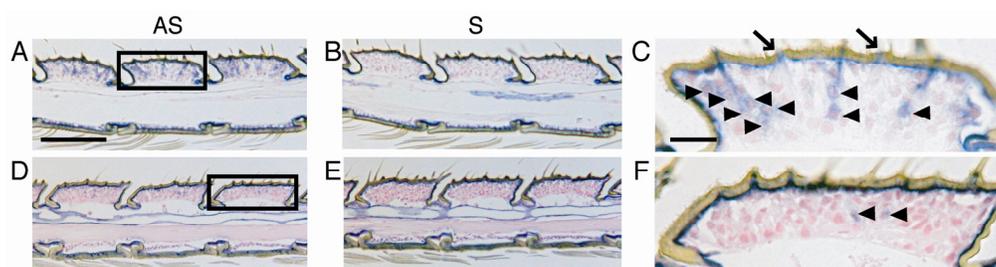
Given that the Or83b family proteins are broadly expressed in ORNs [18, 32, 33, 35, 36], the cells stained with the antisense probe for *OscaOR2* (Or83b homologue) were considered to represent most ORNs. Accordingly, the percentage of ORNs expressing *OscaOR1* was estimated to be about 20%, since the numbers of cells stained with the antisense probes for *OscaOR1* and *OscaOR2* were 1–3 (mean = 1.8; Fig.5D, F) and 9–14 (mean = 10.1; Fig.5C) per antennal segment in the longitudinal sections, respectively. Likewise, given that the number of sensilla trichodea or basiconica was 4–5 per antennal segment (Fig.5), 2–3 ORNs were estimated to be associated with each sensillum. This estimated number of ORNs per sensillum was well consistent with the results of an electrophysiological study using *O. nubilalis* [37].







**Figure 4.** Expression of *OR1* and *OR2* genes in different tissues. Expression of *OscalaOR1* and *OscalaOR2* in different tissues was tested by RT-PCR using specific primers for each gene. A, antenna; FL, foreleg; L, midleg and hindleg; P, proboscis; T, testis; H, head; F, fat body; M, midgut.



**Figure 5.** Expression of *OscalaOR1* and *OscalaOR2* genes in adult male antenna. Longitudinal sections of antennae were hybridized with antisense or sense probes for *OscalaOR2* (A, B and C). Longitudinal sections of antennae were hybridized with antisense or sense probes for *OscalaOR1* (D, E and F). Magnification of the boxed area in A (C) and D (F). Arrowheads indicate the cell bodies stained. Arrows indicate sensilla coeloconica. Scale bar in A = 10  $\mu$ m (the same scale applies to A, B, D and E). Scale bar in C = 2  $\mu$ m (the same scale applies to C and F).

### Functional assay of *OscalaOR1*

To check if the *OscalaOR1* protein is a sex pheromone receptor, we examined electrophysiological responses of *Xenopus* oocytes co-expressing *OscalaOR1* and *OscalaOR2* (*OscalaOR1/OscalaOR2* cell) to the 6 sex pheromone components used in the genus *Ostrinia* and three pheromone analogs. We confirmed beforehand that adult male antennae of *O. scapularis* respond to these 9 compounds using electroantennography (data not shown).

*OscalaOR1/OscalaOR2* cells showed a large response to E11-14:OH and a small response to Z11-16:OAc (Fig.6A), while the control cells without artificially expressed proteins showed no responses (data not shown). The responses of *OscalaOR1/OscalaOR2* cells to E11-14:OH and Z11-16:OAc were dose-dependent (Fig.6B). E11-14:OH is not a sex pheromone component of *O. scapularis*, but a single pheromone component of *O. latipennis* and a minor pheromone component of *O.*

*ovalipennis* [6, 7]. Unexpectedly, *OscalaOR1/OscalaOR2* cells did not show significant responses to E11-14:OAc and Z11-14:OAc, the sex pheromone components of *O. scapularis*.

### cDNA cloning of *OR1s* of the genus *Ostrinia*

We were able to clone homologues of *OscalaOR1* from all the *Ostrinia* species examined (Fig.7). A single *OscalaOR1* homologue was obtained from each species: *OlatOR1* (*O. latipennis*), *OfurOR1* (*O. furnacalis*), *OzagOR1* (*O. zaguliaevi*), *OzeaOR1* (*O. zealis*), *OpalOR1* (*O. palustralis*), *OovaOR1* (*O. ovalipennis*), and *OnubOR1* (*O. nubilalis*). High degrees of identity (93-99%) were observed between the deduced amino acid sequences of these *OR1s* (Fig.7). In the phylogenetic tree of odorant receptor proteins, *OR1s* of the genus *Ostrinia* formed a clade, suggesting that these genes are orthologous (*OR1* group, Fig.8). Moreover, the *OR1* group was included in a single lineage of the sex pheromone receptor subfamily.





### Functional assay of OlatOR1

In order to verify the function of *OlatOR1* as an odorant receptor of E11-14:OH, we additionally cloned an Or83b homologue from *O. latipennis* (*OlatOR2*), and examined responses of oocytes co-expressing *OlatOR1* and *OlatOR2* (*OlatOR1/OlatOR2* cell) to the 9 compounds (Fig.6C). *OlatOR1/OlatOR2* cells were found to show exactly the same response pattern as *OscAOR1/OscAOR2* cells.

### Discussion

Based on the morphology of the male genitalia, *Ostrinia* species found in Japan and the European corn borer *O. nubilalis* are classified into two groups, i.e., a simple or bifid uncus group (Group II; *O. palustralis*, *O. latipennis*, and *O. ovalipennis*) and a trilobed uncus group (Group III; *O. nubilalis*, *O. scapularis*, *O. furnacalis*, *O. orientalis*, *O. zaguliaevi*, and *O. zealis*) [3]. This grouping is also supported by molecular phylogenetic analyses of the mitochondrial cytochrome oxidase subunit II (COII) gene (Fig. 1) [38]. In the phylogenetic tree of odorant receptor proteins, the OR1 group was divided into two subgroups, the members of which perfectly matched with those of groups II and III, respectively (Fig.8). These findings suggest that speciation within the genus *Ostrinia* occurred after the acquirement of OR1.

The OR1s of *O. scapularis* and *O. latipennis*, *OscAOR1* and *OlatOR1*, strongly responded to E11-14:OH, indicating that *OscAOR1* and *OlatOR1* are indeed the receptor proteins of E11-14:OH. Since E11-14:OH is a single sex pheromone component of *O. latipennis* [6], it is concluded that *OlatOR1* functions as the sex pheromone receptor of *O. latipennis*. Although E11-14:OH is not a pheromone component of *O. scapularis*, *OscAOR1* also acts as the receptor of E11-14:OH because electroantennographic recordings showed that the male antennae of *O. scapularis* respond to E11-14:OH (data not shown). This ability of *O. scapularis* to detect E11-14:OH, a pheromone component of a congener, may suggest that change(s) in the neural circuit in the peripheral or central nervous system, rather than change(s) in the pheromone receptor, is responsible for the changes in the pheromone recognition system of *Ostrinia*. In this regard, E11-14:OH might have an unknown function in the pheromone communication system of *O. scapularis*, as discussed below.

Within the genus, *O. scapularis* and *O. latipennis* are most distantly related [2, 3]. Very high degrees of identity between the amino acid sequences of OR1s in *Ostrinia* species including *O. scapularis* and *O. latipennis*

suggest that all OR1s in this genus are receptors of E11-14:OH. It is curious that the receptor of E11-14:OH is conserved in all *Ostrinia* species examined because most of them do not use E11-14:OH as the pheromone component. Regarding the significance of OR1s in this genus, two possibilities can be considered. One is that E11-14:OH has an unknown role in the sex pheromone communication systems of the genus *Ostrinia*. For instance, this compound might serve as an antagonist in species other than *O. latipennis* and *O. ovalipennis*, although no one has systematically tested this possibility to date. The other possibility is that the common ancestor of the genus *Ostrinia* used E11-14:OH as a pheromone component, and despite that many species stopped using this component in the course of speciation, sufficient time for mutational loss of the receptor function may not have passed yet.

In *OscAOR1* and *OlatOR1*, substitutions of amino acid residues were found at 26 positions (Fig.7). Because *OscAOR1* and *OlatOR1* showed the same pattern of responses to the nine compounds tested, these 26 amino acid positions are not essential for the function of this receptor, suggesting that amino acids at these positions are not involved in the formation of the binding site of E11-14:OH or formation of the ion channel. It is difficult to tell whether the function of OR1s has been positively retained through evolution or not, since the low amino acid substitution rates found in OR1s are natural given that species in the genus *Ostrinia* diverged only within 0.4 million years [38].

*OscAOR1* was expressed in about 20% of ORNs in the antennae of male *O. scapularis*. Male-specific odorant receptors other than *OscAOR1*, which remain to be characterized, are expected to appear in the remaining 80% of ORNs. The receptors of E11-14:OAc and Z11-14:OAc, the sex pheromone components of *O. scapularis*, are likely to be included in yet unidentified receptors. All *Ostrinia* species except for *O. latipennis* and *O. furnacalis* use E11-14:OAc and/or Z11-14:OAc as sex pheromone components [2]. Hence, the use of E11-14:OAc and Z11-14:OAc as the sex pheromone components in *Ostrinia*, and accordingly, the acquisition of receptors of E11-14:OAc and Z11-14:OAc are likely to ascend to the divergence of the genus. We therefore suspect that the receptors of E11-14:OAc and Z11-14:OAc are also conserved in the genus *Ostrinia*. It is intriguing to know the repertory of pheromone receptors in the ancestors of *Ostrinia*, because it might have delimited the possible variation in the pheromone communication system in this genus.

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The abbreviations used are: 14:OAc, tetradecyl acetate; Z9-14:OAc, (Z)-9-tetradecenyl acetate; E11-14:OAc, (E)-11-tetradecenyl acetate; Z11-14:OAc, (Z)-11-tetradecenyl acetate; E12-14:OAc, (E)-12-tetradecenyl acetate; Z12-14:OAc, (Z)-12-tetradecenyl acetate; E11-14:OH, (E)-11-tetradecenol; Z11-16:OAc, (Z)-11-hexadecenyl acetate; Z11-16:Ald, (Z)-11-hexadecenal; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethyl sulfoxide; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction. The nucleotide sequences reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB467318 (*OscAOR2*), AB467319 (*OlatOR2*), AB467320 (*OscAOR1*), AB467321 (*OzagOR1*), AB467322 (*OzeaOR1*), AB467323 (*OpalOR1*), AB467324 (*OovaOR1*), AB467325 (*OnubOR1*), AB467326 (*OlatOR1*), and AB467327 (*OfurOR1*).

## Conflict of Interests

The authors have declared that no conflict of interest exists.

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