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Orco mediates olfactory behaviors and winged morph differentiation induced by alarm pheromone in the grain aphid, *Sitobion avenae*

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ABSTRACT

Olfaction is crucial for short distance host location and pheromone detection by insects. Complexes of olfactory receptors (ORs) are composed of odor-specific ORs and OR co-receptors (Orco). Orcos are widely co-expressed with odor-specific ORs and are conserved across insect taxa. A number of Orco orthologs have been studied to date, although none has been identified in cereal aphids. In this study, an Orco gene ortholog was cloned from the grain aphid, *Sitobion avenae*, and named "*SaveOrco*"; RNA interference (RNAi) reduced the expression of *SaveOrco* to 34.11% in aphids, resulting in weaker EAG (electroantennogram) responses to plant volatiles (Z-3-hexene-1-ol; methyl salicylate, MeSA) and aphid alarm pheromone (E-β-farnesene, EBF). Aphid wing differentiation induced by EBF was investigated in both RNAi treated and untreated aphids. EBF induced production of winged aphids in both pre-natal and post-natal periods in untreated aphids, but no such induction was observed in the RNAi-treated aphids. We conclude that *SaveOrco* is crucial for the aphid's response to pheromones and other volatiles, and is involved in wing differentiation triggered by EBF.

1. Introduction

Aphids are destructive pests of many economically important crops throughout the world. They damage crops by sucking phloem sap, transmitting plant viruses, and depositing sticky excreta (honeydew) on leaves which contributes to mold growth on their host plants. Aphids use plant volatiles and species-specific pheromones for interaction with their host plant and reaction to changes in their environment, especially at short distances suitable for host location (Webster, 2012) or pheromone detection (Mustaparta, 1990).

Plant volatiles (green leaf volatiles and aphid-induced defense volatiles) and insect pheromones are two types of substances perceived by the olfactory system, and could alter the behavior, physiology and finally the ecological fitness of aphids. E-β-farnesene (EBF) is one of the infochemicals which is a common component of the alarm pheromone in most aphid species, and the only volatile molecule used as an alarm pheromone in the grain aphid, *Sitobion avenae* (Francis et al., 2005). It warns neighboring aphids of attacks and overcrowding, induces defensive and avoidance responses in aphids (Pickett et al., 1992), as well as increasing the proportion of winged offspring produced by the pea aphid, *Acyrthosiphon pisum* (Kunert et al., 2005). Although the mechanism of the EBF-induced winged morph differentiation is unknown, aphids with antennae removed do not show such EBF induction, which implies that the process of aphid winged development could be mediated by the olfactory system (Kunert and Weisser, 2005).

Olfactory receptors (ORs) are one of the olfactory components, capable of receiving single or mixes of semiochemicals, filtering and transmitting olfactory cues. They respond to environmental change and trigger the activity of olfactory neurons activity inside insect olfactory organs, leading to insect responses to infochemicals. Insect ORs have novel signal transduction mechanisms. The olfactory signal transduction pathway employed in all other animals from nematodes to vertebrates is the GTP binding protein-coupled receptor (GPCR) pathway. The first insect ORs identified in 1999 (Clyne et al., 1999; Vosshall et al., 1999; Gao and Chess, 1999) were initially considered as GPCRs. However, an inverse topology to GPCR was first proposed by Wistrand et al. (2006) using a Hidden Markov Model tailored to the GPCR super family (GPCRHMM), and further identified by Benton et al., 2006 using a β-gal fusion technique to show the presence of transmembrane

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conditions in ORs. At present, it is known that insect ORs have several characteristics that differ from GPCRs. Firstly, as mentioned above, each OR has seven transmembrane domains, and a topology with the N-terminus inside the cell and C-terminus on the surface of the cell membrane (Wistrand et al., 2006; Benton et al., 2006), just the opposite of GPCRs. Secondly, OR-mediated chemoreception requires the formation of a heteromeric complex with at least an odor-specific OR, and a co-expressed OR (Orco) (Benton et al., 2006; Larsson et al., 2004). Thirdly, insect OR complex can directly form a ligand-gated ion channel (Sato et al., 2008; Wicher et al., 2008). More recently, Orco has been demonstrated to be the only functional ion channel involved in the ionotropic mechanism of mosquitos (Taylor et al., 2012; Jones et al., 2012). In the dimer OR complex, ORs function both as a filter that binds to odorants and a switch that opens the ligand-gated ion channel [eg. (Hallem et al., 2004; Carey et al., 2010)]. Heterotrimeric G-proteins are also expressed in insect olfactory neurons [eg. (Talluri et al., 1995; Kain et al., 2008)], and the related metabotropic odorant signaling has been shown to be compatible with insect ion channel signaling (Wicher et al., 2008). The connection between ionic and metabotropic signaling has also revealed that some odor-specific ORs can work as ion channels without Orco, in the presence of G proteins (Wetzel et al., 2001; Kiely et al., 2007). In addition, another class of chemosensory receptors known as ionotropic receptors (IRs) has been identified in the olfactory neurons of *Drosophila* (Benton et al., 2009). Although a number of such studies have elucidated the function of ORs in insect olfaction, the details of olfactory signaling is still unclear. Among aphids, ORs and Orco have been predicted only in *A. pisum* based on genomic DNA sequence data (Smadja et al., 2009), but no ortholog has been identified in other aphid species.

The grain aphid is an oligophagous pest of cereal plants, such as wheat, barley, oat, maize and gramineous grass, and vector of plant viruses such as barley yellow dwarf virus (BYDV). It is one of the dominant species of aphids in Chinese wheat growing areas. The grain aphid reproduces parthenogenetically with a high fecundity, and often causes serious feeding damage. Aphids have wingless and winged morphs, and the wingless aphids are the main colonizing form, and winged aphids are the flight form. When the aphid population becomes too crowded and the host plant become less nutritious, a higher ratio of winged morph occurs.

Field investigations and laboratory assays showed that *S. avenae* was attracted by plant green leaf volatile (eg. Z-3-hexene-1-ol (Visser and Fu-shun, 1995; Webster et al., 2010; Zhou, 2012)), but repelled by aphid induced defense volatiles (eg. MeSA (Liu et al., 2003)) and the alarm pheromone of *S. avenae* (eg. EBF (Wohlers and Tjallingh, 1983)). Thus, a study of the aphid's responses to pheromone and plant volatiles at the molecular level offers a promising way to explain the ecological context of aphid–aphid and aphid–plant interactions. In turn, this approach will facilitate the design and implementation of a novel, sustainable aphid management strategy, namely the push–pull strategy ("push" repel pest insect, "pull" attract natural enemies), to the benefit of the environment.

EBF is one of the potential infochemicals for a push–pull strategy, not only existing in the aphid's alarm pheromone, but also a plant essential oil. Some reports on EBF application by slow release (Bruce et al., 2005; Cui et al., 2012) or the use of genetically modified crops to release EBF automatically (Beale et al., 2006; Yu et al., 2012), indicate that EBF is a safe alternative for aphid control. An EBF induction of trans-generational wing morph differentiation has been reported in pea aphid (Kunert et al., 2005). During a preliminary test investigating aphids on wheat seedling sprayed with EBF, a higher ratio of winged aphids was found as well by our research group. Understanding the relation between EBF and the induction of winged morph differentiation in grain aphids will widen our knowledge of the mechanism of EBF repellency of aphids, and enable development of better methods to control aphids.

In an effort to obtain a deeper understanding of the physiological function of Orco in *S. avenae*, we firstly cloned *SaveOrco* from antennae of *S. avenae*. We then, carried out RNAi treatment against *SaveOrco* by feeding siRNA. Two bioassays, namely EAG test and EBF-induced winged morph differentiation were conducted. The results show that *SaveOrco* is responsible for not only olfactory reception but also wing dimorphism induced by EBF in *S. avenae*.

2. Materials and methods

2.1. Insect rearing

The grain aphid was reared as a parthenogenetic colony from aphids initially collected on wheat at the Langfang Experimental Station, Chinese Academy of Agricultural Sciences, China. Aphids were reared in low density on wheat plants (*Triticum aestivum* L.) at 22 ± 1 °C, 75% relative humidity and a 16:8 h (light:dark) photoperiod in the laboratory.

2.2. Orco gene cloning

Total RNA was isolated from 200 antennae using the PureLink RNA Mini Kit (Ambion, USA) following the manufacturers' instruction. The first strand cDNA was then synthesized from 500 ng total RNA using oligo-dT primer in the SuperScript III First-Strand Synthesis System (Invitrogen, USA). Degenerate primers (SaveOrco_core_Fwd and SaveOrco_core_Rev) to amplify the core sequences of *SaveOrco* were designed based on the conserved regions in the amino acid sequences of Orcos from several insects. Rapid amplification of cDNA ends (RACE) was performed using the GeneRacer kit (Life technologies, USA). The complete ORF (Open Reading Frame) was identified by assembling all three fragments (core, 3′ and 5′ ends) using DNAstar. Unless otherwise specified, all PCR reactions included an initial denaturation at 95 °C for 5 min, 35 cycles of 95 °C for 40 s, 55 °C for 40 s and 72 °C for 50 s followed by a final extension at 72 °C for 7 min. Denaturation temperatures for specific primer sets are shown in Table 1. All PCR products were purified using the QIAquick gel extraction kit (Qiagen, Germany), cloned into the pEASY BLUNT clone vector (Trans, China) and sequenced. A list of all primers is given in Table 1.

2.3. SiRNA of SaveOrco for feeding

The siRNA were designed online using BLOCK-iT RNAi Designer (http://rnaidesigner.invitrogen.com/rnaiexpress/) called Stealth RNAi siRNA (Invitrogen, USA). The sense sequence of siRNA and its negative control sequences were as follows, SiOrco5-AATCTCATTCGAGATTCCATGCAAA, NegOrco5-AAUUUACGAGCCUUAGUACCUCAAA, SiOrco5 and NegOrco5 were dissolved to 20 ng/μl in aphid artificial diet, respectively. Pure aphid artificial diet was employed as the blank control.

2.4. RNA interference of SaveOrco to aphid

RNAi treatment was carried out on 0–1 day old apterous *S. avenae* adults at 22 ± 1 °C and 75% relative humidity. The filtered (0.22 μm) sterilized artificial diet (100 μl) containing 20 ng/μl SiOrco5 (RNAi treatment), 20 ng/μl NegOrco5 (negative control) and nothing (blank control) were sandwiched between two layers of well stretched parafilm membrane on one end of a glass cylinder (6 cm high × 2.5 cm diameter), respectively. Ten aphids were

Table 1
List of designed primers and annealing temperatures for each pair of primers for PCR for *SaveOrco* cloning.

Primers' name	Sequence	Amplified region	Length	Annealing temperature
Saaor_core_Fwd	CAAATACTGGGTGGAACGTcayaarcaygt	Core sequence	354 bp	58 °C
Saaor_core_Rev	GATGGTCATGGCTTTTTGAcaytgytgrca			
Saaor_3race_Fwd1	AGTGAGCACCGTCATTCTAACCA	3′ end	453 bp	55 °C
Saaor_3race_Fwd2	TTACGGTTGCCACTGGTACGATG			
Saaor_5race_Rev1	GATCCATCGTACCAGTGGCAACCGT	5′ end	1083 bp	55 °C
Saaor_5race_Rev2	GATCTTGGTTGCTTGGTATGCGAGAAT			
SaveOrco_ORF_Fwd	ATGGGTTATAAGAAAGATG	ORF	1392 bp	58 °C
SaveOrco_ORF_Rev	TTATTTAAGCTGCACCAAAACCATG			
SaveOrco_qFwd	GGAACAATACCTCTCCACATC	*SaveOrco*	217 bp	56.4 °C
SaveOrco_qRev	CAAACGGGGTACTGGAACAT			
qActin_Fwd	ATCCTCACCCTGAAGTACCC	*actin*	176 bp	56.4 °C
qActin_Rev	CCACACGCAGCTCATTGTA			

placed in each tube with the end of the artificial diet sachet up and the other end covered by gauze. RNAi treatments lasted for 120 h and were performed in triplicates. After 72 h the diet was replaced by fresh ones with the same composition. Real time-quantitative PCR (RT-qPCR) was performed to detect the expression level of *SaveOrco* at the times shown below. Once the RNAi treatment was completed, aphids were transferred to wheat plants for an additional 72 h.

Expression of *SaveOrco* was determined by RT-qPCR at 0, 24, 48, 72, 96 and 120 h during the RNAi treatment period (30 aphids for each time point), as well as 72 h after the RNAi treatment.

The methods of total RNA extraction and the synthesis of the first strand of cDNA from 30 pairs of antennae were the same as described for '*Orco* cloning' above. RT-qPCR was performed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Warrington, UK). SYBR Green Real-Time PCR Master Mixes (Life technologies, USA) were used in each PCR with a reaction volume of 20 μl containing 1 μl of each primer (5 mM) and 4 μl of the first strand cDNA. A list of primers and the PCR reaction conditions used are presented in Table 1. *Actin* served as an internal reference (internal control). Generally, because all aphids from the blank control, negative control and RNAi treatments at the start time (0 h) were not affected by any treatment, we assumed that the expression levels of *SaveOrco* showed no significant differences. Relative expression was calculated using the comparative C_t method $2^{-\Delta\Delta CT}$ and the Ct values at different time points were normalized to Ct values of RNAi treatment at 0 h which was defined as the external reference (external control). All reactions were performed in triplicate.

In addition, although the result of RT-qPCR is reliable and widely acknowledged when the Ct value is less than 32 (Soong and Ladányi, 2003), semi-quantitative PCR were performed instead of RT-qPCR on cDNA from the head (with antenna) of individual aphids treated with RNAi once its EAG test finished, as the Ct value of *SaveOrco* from single aphids is more than 33. Then those EAG data from RNAi positive aphids defined by semi-quantitative PCR were selected for further statistical analysis. These PCR reactions consisted of 4 μl cDNA with 10 μl "2 × TansStart Fast *Pfu* PCR SuperMix" (Trans, China) and 1 μl of each gene specific primer.

2.5. Electroantennograhy (EAG) assays

Chlorinated silver wires in pre-elongated glass capillaries filled with Kaissling buffer were used in both reference and recording electrodes. The tips of both antennae were clipped from adult aphids and each head was excised and individually mounted on the reference electrode of a Syntech EAG platform, which was equipped with a micromanipulator and a high-impedance AC/DC preamplifier (Syntech UN-06, Nederland). The preparation was exposed to a high humidity air stream flowing at 20 ml/s to which a stimulus pulse of 2 ml/s was added for 200 ms. Dynamic changes in antennal deflection induced by the chemical stimuli or control puffs (air flow without stimuli) were recorded for 30 s. The biologically and electrophysiologically active molecules, such as EBF, Z-3-hexene-1-ol and MeSA were purchased from Sigma–aldrich, USA. Chemicals were dissolved in paraffin oil (Sigma–aldrich, USA) to a concentration of 50 ng/μl and 20 μl of each solution was applied to a filter paper strip (8 mm × 40 mm) and inserted into a 15 cm long glass Pasteur pipette. The EAG data of normal aphids (the blank control of RNAi treated aphids) served as positive control, and an average EAG value evoked by 20 μl of paraffin oil (non-volatile solvent) was used as the negative control which was considered as the background value. Original data were pooled from different batches of aphids and the EAG responses of 3 RNAi treated aphids (N = 3) and 9 of their positive controls (N = 9) were tested in each batch for statistical analysis. Every head of RNAi treated aphid was tested individually by semi-quantitative PCR after the EAG assay was completed to confirm that all EAG data collected were from RNAi positive aphids.

2.6. Winged morph differentiation induced by EBF

The bioassay was performed as described by Kunert et al. (2005) with some modifications. Two groups were tested. For group 1, EBF induction of wing morph differentiation to RNAi treated aphids was tested. Adult aphids were treated with both RNAi and EBF. The pseudo embryos inside adults in Group 1 were designated as prenatal aphids. Normal adult aphids served as controls, aphids exposed to EBF served as positive controls, and aphids not exposed to EBF served as blank controls. In addition, RNAi aphids not exposed to EBF served as negative controls. For group 2, nymphs born within the previous 24 h were treated with EBF and designated as post-natal aphids. Nymphs not treated with EBF served as blank controls.

Aphids were treated with EBF for 5 days: 1000 ng EBF (Wako, Japan) soluted in 4 μl of paraffin oil was applied to a piece of filter paper fixed by a wooden toothpick at the base of the tube/pot. Aphids were exposed to EBF starting at 9 am each day, and filter papers with EBF were replaced 5 times at 2 h intervals during the daytime (at 9:00, 11:00, 13:00, 15:00, 17:00). For group 1, the first exposure to EBF was applied 72 h after initiating the RNAi treatment, and then exposure to EBF and RNAi treatments were conducted at the same time for 48 h, till the end of the RNAi treatments (up to 120 h for RNAi). The aphids were then transferred to wheat seedlings at the two-leaf stage (10 aphids/pot) with a continuous exposure to EBF for another 72 h (total of 120 h for EBF treatment). After adults were exposed to EBF for up to 120 h (48 h on artificial diet, then 72 h on plants), newly born nymphs (F1), that had not

been directly treated, were reared on seedlings to the adult stage, when the number of winged and wingless aphids were counted, and the percentage of winged morphs was determined. The positive control and negative control were handled in the same way, but with no siRNA in the artificial diet and no EBF applied, respectively, and the blank control had neither EBF nor RNAi treatment. In group 2, newly born nymphs from plants exposed to EBF for 120 h were reared to the adult stage when the percentage of winged morphs was determined.

2.7. The behavioral response to semiochemicals of aphids treated with RNAi

EBF (a repellent from aphid alarm pheromone), MeSA (a repellent from aphid-induced wheat plants) and Z-3-hexene-1-ol(a common attractant of aphid from plant) were employed in our study to detect aphid olfactory response after RNAi treatment, as well as their mobility compared with normal aphids. A Y-tube olfactometer with a 2.7 cm diameter, 10 cm trunk length and 16.5 cm branch length was used to study. The airflow (0.1 L/min) was dried and purified using activated granular carbon and washed in distilled water before passing through a chamber where the odor source flowed into each arm (branch) of the Y-tube. A Y-shaped iron wire was placed in the center of the Y-tube to supply a trestle on which aphids could walk (Read et al., 1970). For every treatment, one arm was randomly selected to introduce 10 μl of freshly prepared odor solution (10ng/ul) into the corresponding stimuli source chamber while the other arm was used as the control to introduce 10 μl of paraffin oil (the solvent of the odor solution). Aphids were then placed at the end of the iron wire. The number of aphids in each arm was recorded after 10min. At least 3 replications (30 aphids for each group) of both RNAi treated aphids and their control were performed. The proportion of aphids in each arm to all aphids that leave the trunk area was calculated, as well as the number (out of 30) that leave the trunk area was recorded.

2.8. Statistical analysis

Results were expressed as means ± SD. The qPCR data of blank control, negative control and RNAi treatment were analyzed by one-way ANOVA followed by Tukey's test. EAG data for each compound from the RNAi treatment, positive control and negative control were compared and tested using the general linear model (PROC-GLM) using SAS 9.1 followed by the least-significant difference (LSD) method. The data of winged morph proportions in group 1 were analyzed by one-way ANOVA followed by Tukey's test and in group 2 by a two-sample *t* test.

Differences in the proportions of aphids in each arm of the Y-tube olfactometer were analyzed by two-sample *t* test. Further, the numbers of aphids that remained in each test area (both arms and trunk) were compared and tested by ANOVA followed by Duncan's new multiple range test.

3. Results and discussions

3.1. SaveOrco cloning

To understand the olfactory signal transduction component in grain aphids, we cloned the ortholog of *Orco* using degenerate primers designed from the highly conserved region of known insect *Orcos*. A cDNA sequence of 1392 bp in length containing a complete ORF that encoded 464 amino acids with a deduced MW of 53.3 kDa

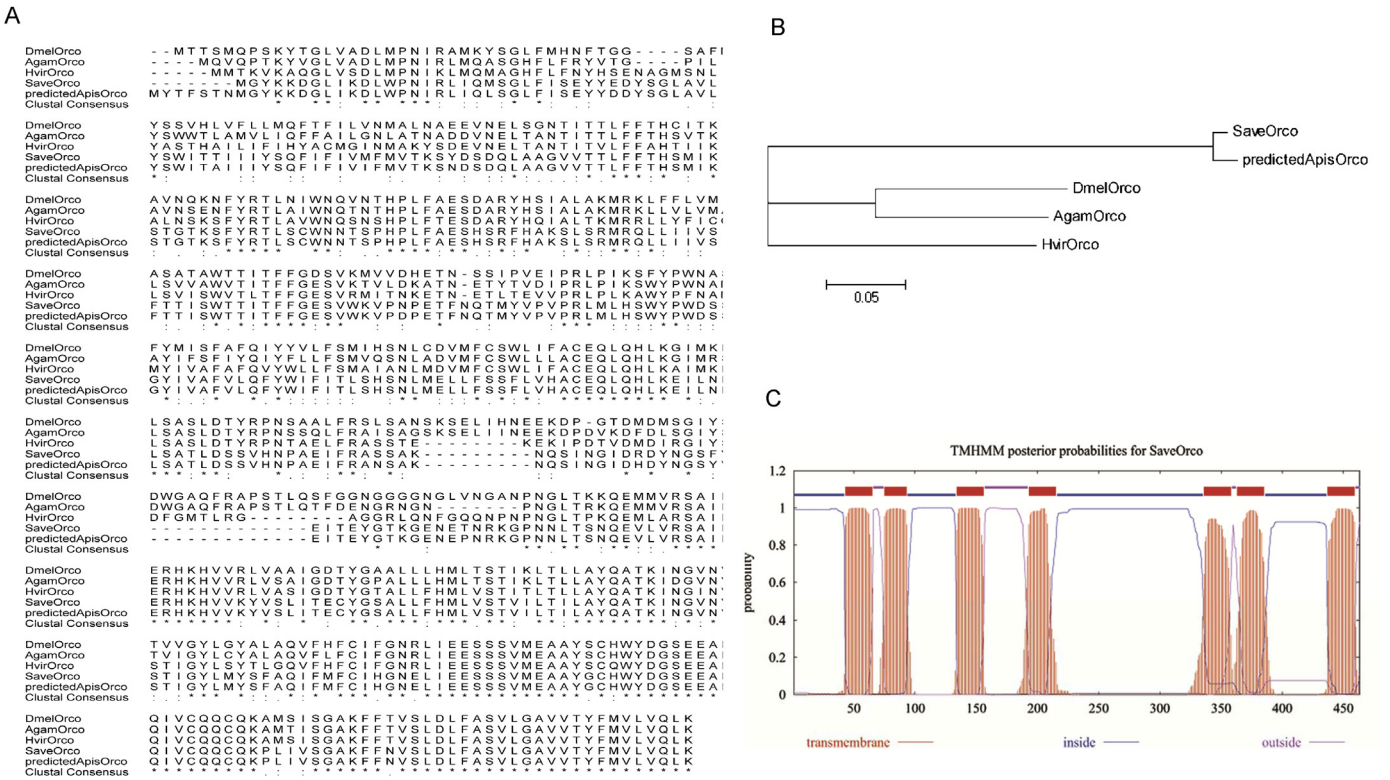

Fig. 1. Sequence analysis of *SaveOrco*. A: ClustalW comparison of the deduced amino acid sequences from 5 species of 3 orders [*SaveOrco* from *S. avenae* (Hemiptera), DmelOrco from *D. melanogaster* and AgamOrco from *A. gambiae*, (Diptera) HvirOrco from *H. virescens* (Lepidoptera) and the predicted sequence of ApisOrco from *A. pisum* (Hemiptera)]. Identical residues are marked with asterisks and similar residues with dots. B: phylogenetic tree of Orcos from five insect species mentioned above. C: Prediction of transmembrane helices in the encoded amino acid sequence.

was cloned. The predicted amino acid sequence shared a high identity (72%) with *Orcos* from other insects and greater than 90% identity at the C-terminus (final 160 aa) (Fig. 1A). The highest percentage identity of 96% was observed with the predicted ApisOrco amino acid sequence. Based on such high identity, the isolated cDNA was presumed to be an Orco ortholog of *S. avenae* and therefore named as *SaveOrco*, according to the uniform abbreviation proposed for these homologues throughout Insecta (Vosshall and Hansson, 2011) (GenBank accession no. GQ275379). A phylogenetic tree constructed based on the amino acid sequence of five Orcos from different insect orders using ClustalW, showed a grouping of the two aphid sequences together in an independent branch. Not surprisingly, the fly and mosquito sequences formed a different branch and the lepidopteran HvirOrco was in a separate branch (Fig. 1B). In this analysis, we chose ApisOrco, which is a predicted sequence as it was the only complete aphid *Orco* coding sequence registered in GenBank to date. In addition, prediction of transmembrane helices in the encoded amino acid sequence showed the presence of seven potential transmembrane helices with the N-terminus inside the cell membrane and C-terminus outside (Fig. 1C). All these characteristics of insect specific olfactory receptors further confirmed that the deduced amino acid sequence was indeed *SaveOrco*.

3.2. RNAi treatment

Feeding with siRNA has been reported to be a successful method for inducing RNAi in some sap sucking pests such as the pea aphid, *A. pisum* (Shakesby et al., 2009; Whyard et al., 2009), white fly, *Bemisia tabaci* (Upadhyay et al., 2011) and triatomine bug, *Rhodnius prolixus* (Araujo et al., 2006). Therefore, we fed siRNA to the grain aphids to carry out the RNAi treatment. For these RNAi treatments, the BLOCK-iT RNAi Designer (Life Technologies, USA) was used to design Stealth RNAi siRNA which was proposed to have higher specificity and greater stability, and therefore would be able to inhibit gene expression more effectively. Artificial diet with siRNA was renewed at the time point of 72 h to exclude possible degeneration of siRNA and also to avoid decay of the artificial diet during the RNAi treatment period. The siRNA named SiOrco5 was screened from 20 siRNAs designed for interfering with the *SaveOrco*'s transcription. Its negative control sequences were named as NegOrco5.

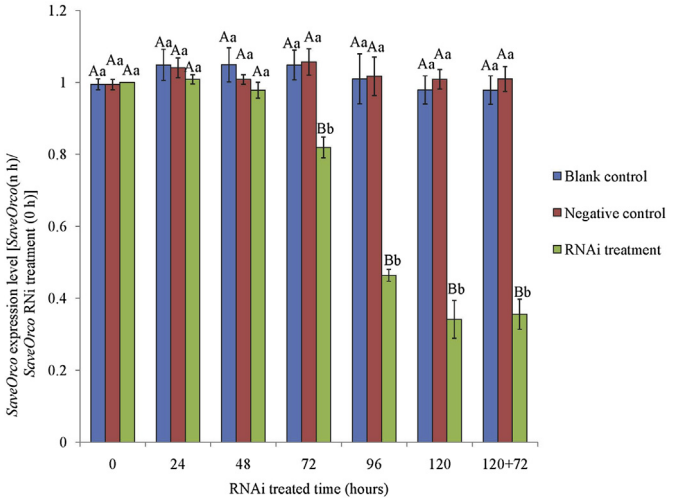

Fig. 2. Expression of *SaveOrco* in *S. avenae* (each N = 3). "0": the expression levels at start time of all treatments were not significantly different, therefore, RNAi treatment was defined as external control (external reference), "120": RNAi treated for 120 h; "120 + 72": reared on wheat seedlings for 72 h after 120 h RNAi treatment. "Blank control": aphids fed on pure artificial diet. "Negative control": aphids fed on artificial diet with 20 ng/μl NegOrco5. "RNAi treatment": aphids fed on artificial diet with 20 ng/μl SiOrco5. All data are presented as mean ± SD. Lower case ($P < 0.05$) and upper case letters ($P < 0.01$) indicate statistical significance among treatment and controls at the same time (one-way ANOVA).

3.3. RT-qPCR of SaveOrco

Expression of *SaveOrco* was determined at 0, 24, 48, 72, 96 and 120 h after continuous oral administration of siRNA, as well as 72 h after the RNAi treatment, by RT-qPCR. To obtain reliable results ($C_t < 33$) by RT-qPCR (Soong and Ladányi, 2003), cDNA from 30 aphids was used as a template for each time point instead of single aphid. In adults, the average expression level of *SaveOrco* remained unaltered until 48 h of RNAi treatment but declined to 81.9% at 72 h, 46.4% at 96 h and 34.1% at 120 h (Fig. 2) compared to the external control. The instantaneous RNAi effect of SiOrco5 lasted for an additional 72 h (35.6%) after the RNAi treatment finished (Fig. 2). The expression of the negative control did not differ significantly from the blank control at any time. Aphid feeding on siRNA induced RNA interferences in both nymphs and adults, and the RNAi effect lasted for an additional 72 h after withdrawing the application of siRNA. Such an extended effect enabled the subsequent bioassays (EBF treatment).

3.4. Electroantennography

As the cycle threshold was more than 33 (around 35–40), semi-

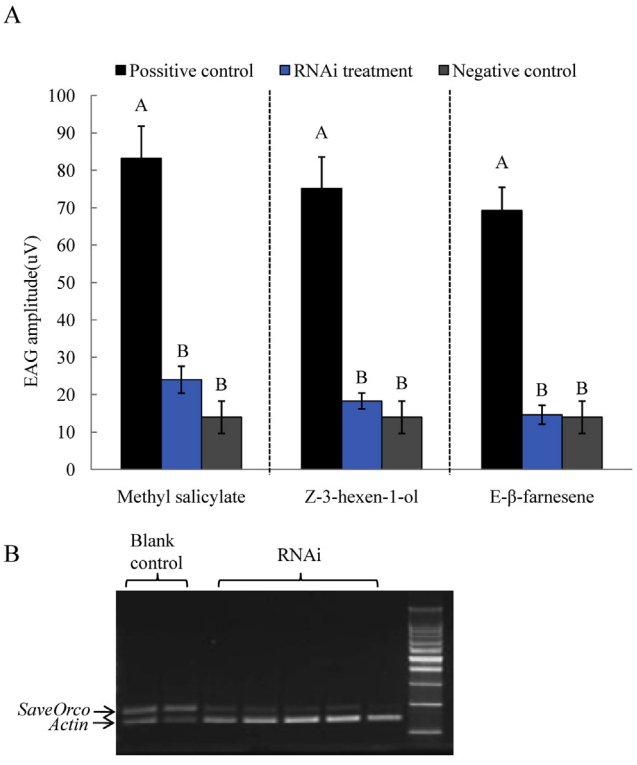

Fig. 3. Electroantennography tests of RNAi-treated aphids. A. EAG responses of the grain aphids to different odors. B. Semi-quantitative PCR (28 amplification cycle) for Screening RNAi positive aphids. ■ Positive control (natural aphids, N = 9); ■ RNAi treatment (RNAi treated aphids, N = 3); ■ Negative control (an average EAG value to solvent, N = 12). 1000 ng of each odor (10 μl, 100 ng/μl dissolved in mineral oil) was load to evoke EAG response. Data were analyzed by the general linear model (PROC-GLM) followed by the least-significant difference (LSD) method. Different capital letters represent significant difference at $P = 0.01$.

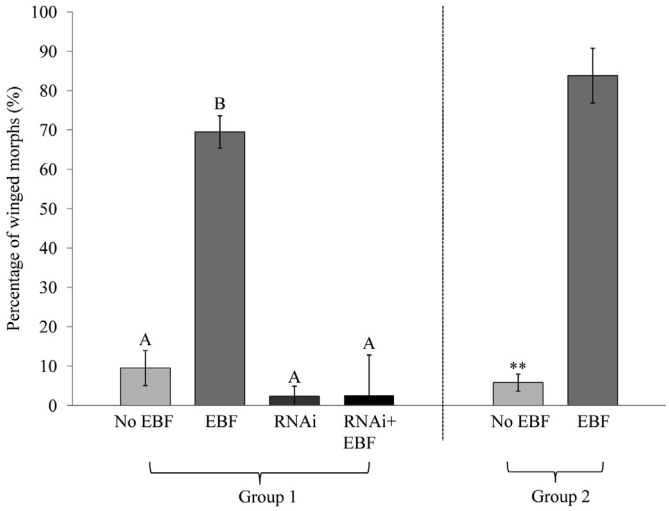

Fig. 4. The inhibition of RNAi on aphid winged development induced by EBF exposure. "Group 1": offspring born by both RNAi and EBF treated adults (pre-natal). "Group 2": EBF treated nymphs since they were newly born within 24 h of birth (post-natal). "RNAi + EBF": aphids both RNAi treated and exposed to 1000 ng EBF for 5 d "RNAi": RNAi treated aphids without EBF treatment (negative control). "No EBF": natural aphids without EBF treatment (blank control). "EBF": natural aphids exposed to 1000 ng EBF for 5 d (positive control). Bars show mean values ± SD. Different capital letters represent significant differences at $P = 0.01$ level (one-way ANOVA followed by Tukey test). "**" represents significant differences at $P = 0.01$ level (two-sample t test).

quantitative PCR was carried out instead of RT-qPCR to detect the SaveOrco's expression level in single aphids once the EAG bioassay was done, to confirm that only data from RNAi positive aphids were chosen for the statistical analysis. A reduction of *SaveOrco* expression level was observed in individual aphids treated by RNAi compared to the negative control (Fig. 3B).

RNAi treated aphids and normal aphids (the blank controls) were selected for EAG recordings. The average EAG values of RNAi aphids to all three stimuli, namely MeSA, Z-3-hexen-1-ol and EBF, were (24 ± 3.61) μV, (14.67 ± 2.52) μV and (18.33 ± 2.08) μV, respectively. The average EAG values of untreated aphids (positive control) were (75.17 ± 18.33) μV, (83.17 ± 8.62) μV and (69.28 ± 14.67) μV, respectively. And the average EAG value to paraffin oil (solvent), which is a non-volatile liquid (negative control), was (14 ± 4.34) μV. The responses of RNAi-treated aphids to MeSA, Z-3-hexene-1-ol and EBF were all significantly weaker than responses of normal aphids, and were not significantly different from the response to negative control (Fig. 3A, F = 292.37, F = 342.20 and F = 369.79, respectively, $P < 0.01$). The RNAi-treated aphids did not respond to olfactory cues, revealing the role of *SaveOrco* in olfactory perception. Such extensive olfactory dysfunction was presumed to be caused by the failure of translation of *SaveOrco* due to the knockdown of *SaveOrco* mRNA mediated by RNAi.

3.5. Winged morph differentiation induced by EBF

In group 1 where mothers (adult aphids) were exposed to EBF, the F1 generation of positive controls showed a significantly higher percentage of winged aphids than the blank control, negative control, or RNAi-treated aphids (F = 82.6, $P < 0.01$, Fig. 4). These results indicated, firstly, that exposure to alarm pheromone during the pre-natal period could induce transgenerational winged morph differentiation in *S. avenae*. This was consistent with previous reports on pea aphids (Kunert et al., 2005) that the proportion of winged offspring increased when mothers were exposed to EBF. Secondly, aphids failed to respond to EBF induction during the pre-natal period when the transcription of *SaveOrco* was interfered with by siRNA.

Similarly, in group 2 where newborn nymphs were exposed to EBF for 120 h, the positive control had a significantly higher proportion of winged morphs when they developed into adult stage than the blank control (t = 12.54, $P < 0.01$, Fig. 4). This indicated that exposure to the alarm pheromone during the post-natal period could also induce winged morph differentiation in *S. avenae*. Considering that during their post-natal period (around 4.4 days (Müller et al., 2001)), there was insufficient time to complete both RNAi and EBF treatments on nymphs [3 days (72 h) for RNAi treatment evoking significant RNAi effect before 5 days (120 h) of EBF treatment], EBF treatment was not performed on pre-RNAi-treated nymphs of the grain aphid.

Wing morph differentiation in aphids could occur when crowding or poor nutrition occurs, and generally, this phenomenon could be induced during either or both of the pre-natal and post-natal periods (Ankersmit and Dijkman, 1983). *S. avenae* responds in both periods (Kennedy and Stroyan, 1959; Kunert et al., 2007). In our study, post-natal nymphs (first 2 instars) exposed to EBF in

Table 2
Behavioral responses of *S. avenae*.

Treatments	The number of aphids in			T/(T + C)	C/(T + C)	*t* test [T/(T + S) and C/(T + C)]
	Trunk area	T	C			
RNAi aphids + EBF	24.00 ± 2.6Aa	2.67 ± 0.58	3.33 ± 2.1	0.49 ± 0.16	0.51 ± 0.16	ns, t = −0.16, $P = 0.88$
Normal aphids + EBF	17.00 ± 3.5Ab	4.90 ± 1.7	7.71 ± 2.3	0.41 ± 0.12	0.59 ± 0.12**	**, t = −3.66, $P = 0.0018$
Normal aphids + solvent control	8.71 ± 3.0 Bc	11.30 ± 3.4	10.00 ± 1.9	0.52 ± 0.11	0.48 ± 0.11	ns, t = 0.77, $P = 0.46$
Statistical analysis	F = 28.13, $P < 0.01$	NA	NA	NA	NA	NA
RNAi aphids + MeSA	24.33 ± 3.1Aa	2.67 ± 1.2	3.00 ± 2.0	0.50 ± 0.14	0.50 ± 0.14	ns, t = 0.06, $P = 0.95$
Normal aphids + MeSA	15.57 ± 3.4 Bb	6.71 ± 1.6	7.71 ± 2.2	0.46 ± 0.074	0.54 ± 0.074*	*, t = −2.68, $P = 0.0151$
Normal aphids + solvent control	11.29 ± 3.5 Bb	9.14 ± 2.8	9.57 ± 2.4	0.49 ± 0.092	0.51 ± 0.092	ns, t = −0.43, $P = 0.67$
Statistical analysis	F = 14.18, $P < 0.01$	NA	NA	NA	NA	NA
RNAi aphids + Z-3-hexene-1-ol	23.30 ± 1.5Aa	3.30 ± 1.5	3.30 ± 0.6	0.48 ± 0.12	0.52 ± 0.12	ns, t = 0.31, $P = 0.77$
Normal aphids + Z-3-hexene-1-ol	2.70 ± 1.5Bb	26.30 ± 0.6	1.0 ± 1.0	0.96 ± 0.03	0.04 ± 0.03	**, t = 32.977, $P = 0.001$
Normal aphids + solvent control	11.00 ± 2.6Cc	9.30 ± 3.0	9.70 ± 1.5	0.48 ± 0.11	0.48 ± 0.11	ns, t = 0.33, $P = 0.76$
Statistical analysis	F = 91.39, $P < 0.01$	NA	NA	NA	NA	NA

"T": Treatment arm with odors or paraffin oil. "C": Control arm with paraffin oil load. "NA": not analyzed. "ns" represents not significant. "*" represents significant difference at $P = 0.05$ level. "**" represents significant difference at $P = 0.01$ level, relative proportion of data from both arms were compared by two-sample t tests. To determine significant differences in the number of aphids staying in the trunk area among treatments (30 aphids per test, N = 3), analysis of variance (ANOVA) was performed followed by Tukey test. Lower case ($P < 0.05$) and upper case letters ($P < 0.01$) indicate statistical significance among treatment and controls at the same time.

group 2 gave rise to a higher winged morph proportion similar to the F1 nymphs in group 1 whose mother were exposed to EBF during their pre-natal period (Fig. 4). Our results suggested that EBF had a similar inducing effect as crowding and poor nutrition for *S. avenae*, and the induction of winged morph differentiation by EBF could be evoked in both post-natal and pre-natal stages.

The proportion of winged morphs in RNAi treated aphids was significantly lower than positive control but not significantly different from either blank control or negative control ($F = 82.6$, $P < 0.01$). This indicated that the failure of olfactory function blocked EBF induction of wing morph differentiation in *S. avenae*. Moreover, both winged morph proportions of RNAi-treated aphids, whether with EBF treatment or not, were lower but not significantly lower than the blank control in group 1 (Fig. 4). EBF induced behaviors such as walking and dropping from colonies increase body contacts which are similar to the effects caused by crowding. Crowding of aphids usually increases winged morph proportions (Ankersmit and Dijkman, 1983), based on which Kunert (Sloggett et al., 2004) proposed to use a classic "pseudo crowding hypothesis" (Li and Li, 2002) to decipher the connection between increase in the levels of EBF and a corresponding increase in winged offspring.

3.6. Behavioral response to semichemicals after RNAi treatment

First of all, selection behaviors of normal aphids to solvent loaded in both arms were tested. Aphids always showed no preference for either arm (Table 2, $t = 0.77$, $P = 0.46$; $t = 0.43$, $P = 0.67$; $t = 0.33$, $P = 0.76$).

Also, our results showed that repellents or attractant had significant influence on olfactory behaviors of normal aphids that were consistent with their ecological roles (Fig. 5A, B and C; Table 2). While their effects to RNAi-treated aphids were all converted into negative, the behavior data showed no significant preference (Table 2, $t = -0.16$, $P = 0.88$; $t = 0.06$, $P = 0.95$; $t = 0.31$, $P = 0.77$) to either treated or control arm, which is similar to the

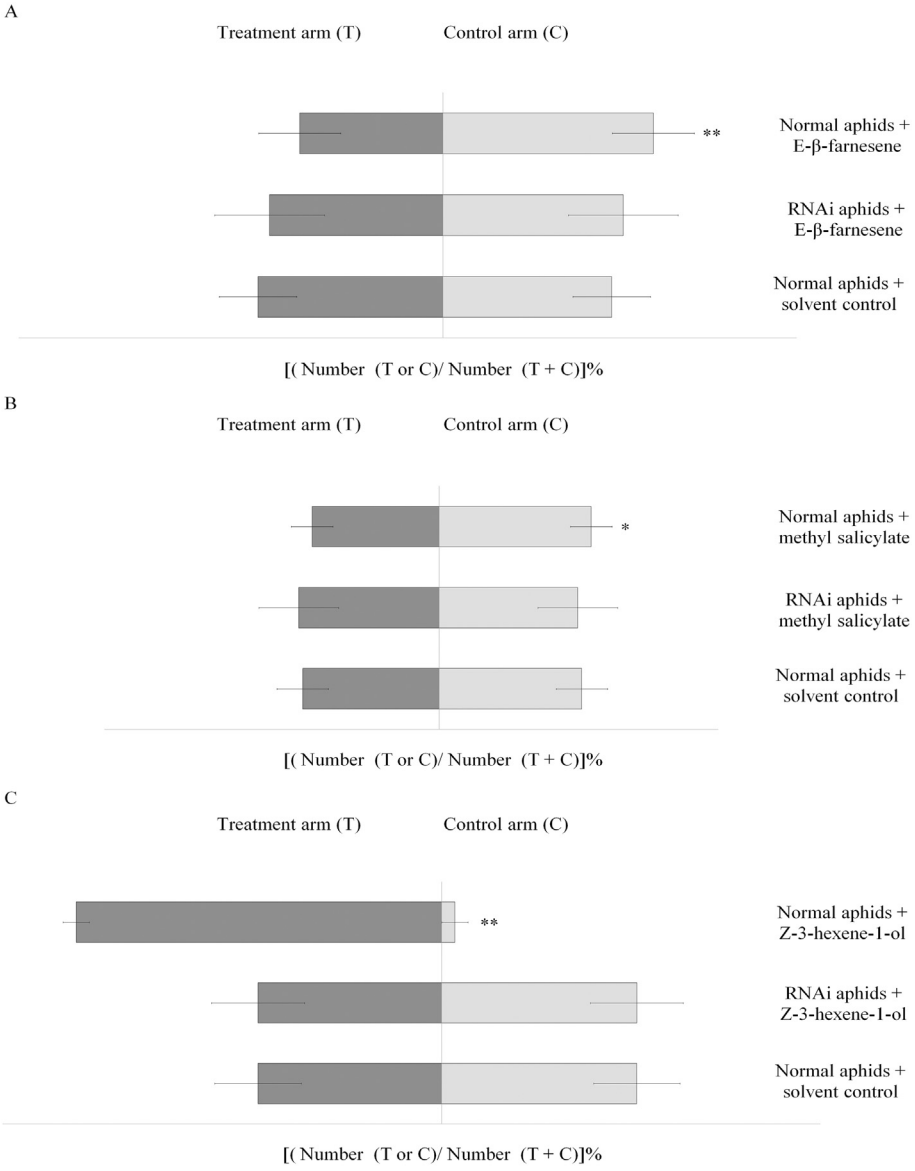

Fig. 5. Behavioral response of grain aphid to an active attractant. Aphids that stayed in the trunk area were not counted. The proportions of aphids in both arms to all aphids that left the trunk area were analyzed by two-sample *t* test at $P = 0.01$ level. "ns" represents no significance. "*" represents significant difference at $P = 0.05$ level. "**" represents significant difference at $P = 0.01$ level.

situation of normal aphids when both arms were loaded by solvent. This result indicated that *SaveOrco* was responsible for the perception of infochemicals and that RNAi treatment blocked olfactory signals thereby rendering RNAi-treated aphids insensitive to behaviorally active odors. When tested with the 3 odors, instead of response to odorant signals, significantly more RNAi-treated aphids chose to stay in the trunk area than normal aphids (Table 2). In other words, the olfaction-damaged aphids were less active even in a completely strange environment. Such a sedate character therefore enabled aphids to avoid a "pseudo crowding effect" when they were exposed to alarm pheromone(EBF) or placed into a strange environment, which could explain the abnormally stable alate ratios of RNAi treated aphid before and after a EBF treatment. This phenomenon leads to a novel idea for controlling aphids especially controlling the damage caused by dispersal of aphids. While, for further applying in pest control, more study such as behavior tests using plant volatile blends from altered aphids under a realistic ecological situation would be necessary. And we have been focusing on this work recently, and some interesting results have been achieved (data not shown here).

4. Conclusion

We report for the first time the function of an aphid *Orco* from *S. avenae* named *SaveOrco*. Down regulation of the *SaveOrco* transcript using RNAi severely damaged the aphids' olfactory signal transduction which was proved by an EAG bioassay. Our results demonstrated the crucial role of *SaveOrco* in the olfaction specifically in the perceptions of pheromone (EBF), green leaf volatile (Z-3-hexene-1-ol) and aphid-induced defense volatile (MeSA).

Although insect Orcos are highly conserved, the regions of low conservation were used in the present study to design a siRNA sequence against *SaveOrco.* This siRNA could be potentially used for the development of transgenic plants or insecticides for pest management of grain aphids. Such insect specific molecules could enable development of new pest control strategies such as the use of agonists and antagonists targeted towards Orcos. These strategies would also avoid effects on other non-target insects and non-insect organisms.

The EBF induced winged morph differentiation, indicated that induction occurs during both pre-natal and post-natal sensitive periods in *S. avenae* and that *SaveOrco* is indispensable for this physiological process. This is the first evidence at a molecular level although details of this mechanism are not yet to be uncovered. The majority of the grain aphids developed into winged morphs (migration biotype) in response to EBF induction. This indirect effect together with direct avoidance of aphids and tendency of natural enemies could decrease aphid density in EBF treated fields.

Finally, in our study, the effect of RNAi lasted for at least 3 days after the treatment which makes RNAi a powerful tool for the functional studies of target genes. Olfaction of RNAi treated aphids (feeding siRNA against *SaveOrco*) was severely damaged and these aphids were less mobile than normal aphids. Further experiments could be conducted to determine how long it takes the RNAi effect to wear off (persistence of silencing effect) and obtain some reference data for our further research such as the potential impact of olfactory gene-knockdown on host plant selection and location of the grain aphids.

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References

Ankersmit, G.W., Dijkman, H., 1983. Alatae production in the cereal aphid *Sitobion avenae*. Neth. J. Plant Pathol. 89, 105–112.
Araujo, R.N., Santos, A., Pinto, F.S., Gontijo, N.F., Lehane, M.J., et al., 2006. RNA interference of the salivary gland nitrophorin 2 in the triatomine bug *Rhodnius prolixus* (Hemiptera: Reduviidae) by dsRNA ingestion or injection. Insect Biochem. Mol. Biol. 36, 683–693.
Beale, M.H., Birkett, M.A., Bruce, T.J.A., Chamberlain, K., Field, L.M., et al., 2006. Aphid alarm pheromone produced by transgenic plants affects aphid and parasitoid behavior. Proc. Natl. Acad. Sci. U. S. A. 103, 10509–10513.
Benton, R., Sachse, S., Michnick, S.W., Vosshall, L.B., 2006. Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors *in vivo*. PLoS Biol. 4, e20.
Benton, R., Vannice, K.S., Gomez-Diaz, C., Vosshall, L.B., 2009. Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. Cell 136, 149–162.
Bruce, T.J.A., Birkett, M.A., Blande, J., Hooper, A.M., Martin, J.L., et al., 2005. Response of economically important aphids to components of *Hemizygia petiolata* essential oil. Pest Manag. Sci. 61, 1115–1121.
Carey, A.F., Wang, G., Su, C.Y., Zwiebel, L.J., Carlson, J.R., 2010. Odorant reception in the malaria mosquito *Anopheles gambiae*. Nature 464, 66–71.
Clyne, P.J., Warr, C.G., Freeman, M.R., Lessing, D., Kim, J., et al., 1999. A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. Neuron 22, 327–338.
Cui, L., Liu, Y., Yuan, H., Liu, J., Dong, J., Chen, J., Francis, F., Song, X., Liu, Y., 2012. The functional significance of E-β-Farnesene: does it influence the behavioural responses of aphid natural enemies in the fields. Biol. Control 60 (2), 108–112.
Francis, F., Vandermoten, S., Verheggen, F., Lognay, G., Haubruge, E., 2005. Is the (E)-beta-farnesene only volatile terpenoid in aphids? J. Appl. Entomol. 129, 6–11.
Gao, Q., Chess, A., 1999. Identification of candidate *Drosophila* olfactory receptors from genomic DNA sequence. Genomics 60, 31–39.
Hallem, E.A., Nicole Fox, A., Zwiebel, L.J., Carlson, J.R., 2004. Olfaction: mosquito receptor for human-sweat odorant. Nature 427, 212–213.
Jones, P.L., Pask, G.M., Romaine, I.M., Taylor, R.W., Reid, P.R., et al., 2012. Allosteric antagonism of insect odorant receptor ion channels. PLoS One 7, e30304.
Kain, P., Chakraborty, T.S., Sundaram, S., Siddiqi, O., Rodrigues, V., et al., 2008. Reduced odor responses from antennal neurons of Gqα, phospholipase Cβ, and *rdgA* mutants in *Drosophila* support a role for a phospholipid intermediate in insect olfactory transduction. J. Neurosci. Off. J. Soc. Neurosci. 28, 4745–4755.
Kennedy, J.S., Stroyan, H.L.G., 1959. Biology of aphids. Annu. Rev. Entomol. 130–160.
Kiely, A., Authier, A., Kralicek, A.V., Warr, C.G., Newcomb, R.D., 2007. Functional analysis of a *Drosophila melanogaster* olfactory receptor expressed in Sf9 cells. J. Neurosci. Methods 159, 189–194.
Kunert, G., Weisser, W.W., 2005. The importance of antennae for pea aphid wing induction in the presence of natural enemies. Bull. Entomol. Res. 95, 125–131.
Kunert, G., Otto, S., Rose, U.S.R., Gershenzon, J., Weisser, W.W., 2005. Alarm pheromone mediates production of winged dispersal morphs in aphids. Ecol. Lett. 8, 596–603.
Kunert, G., Trautsch, J., Weisser, W.W., 2007. Density dependence of the alarm pheromone effect in pea aphids, Acyrthosiphon pisum (Sternorrhyncha: Aphididae). Eur. J. Entomol. 104, 47–50.
Larsson, M.C., Domingos, A.I., Jones, W.D., Chiappe, M.E., Amrein, H., et al., 2004. Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. Neuron 43, 703–714.
Li, R., Li, S., 2002. Influence of temperature on wheat aphid's development and productivity. J. Shanxi Agric. Univ. Nat. Sci. Ed. 22, 319–321.
Liu, Y., Chen, J., Ni, H., 2003. Electroantennogram responses of *Sitobion avenae* and *Rhopalosiphum padi* to wheat plant volatiles. Acta Entomol. Sin. 46, 679–683.
Müller, C.B., Williams, I.S., Hardie, J., 2001. The role of nutrition, crowding and interspecific interactions in the development of winged aphids. Ecol. Entomol. 26, 330–340.
Mustaparta, H., 1990. Chemical information processing in the olfactory system of insect. Physiol. Rev. 70, 199–245.
Pickett, J.A., Wadhams, L.J., Woodcock, C.M., Hardie, J., 1992. The chemical ecology of aphids. Annu. Rev. Entomol. 67–90.
Read, D.P., Feeny, P.P., Root, R.B., 1970. Habitat selection by the aphid parasite *Diaeretiella rapae* (Hymenoptera: Braconidae) and hyperparsite Charips brassicae (Hymenoptera: Cynipidae). Can. Entomol. 102, 1567–1578.
Sato, K., Pellegrino, M., Nakagawa, T., Nakagawa, T., Vosshall, L.B., et al., 2008. Insect olfactory receptors are heteromeric ligand-gated ion channels. Nature 452, 1002–1006.

Shakesby, A.J., Wallace, I.S., Isaacs, H.V., Pritchard, J., Roberts, D.M., et al., 2009. A water-specific aquaporin involved in aphid osmoregulation. Insect Biochem. Mol. Biol. 39, 1–10.
Sloggett, J.J.W., Weisser, W.W., 2004. A general mechanism for predator- and parasitoid-induced dispersal in the pea aphid, Acyrthosiphon pisum (Harris). In: Simon, J.C., Dedryver, C.A., Rispe, C., Hullé, M. (Eds.), Aphids in a New Millennium Proceedings of the Sixth International Symposium on Aphids, September, 2001. Rennes, France, pp. 79–85.
Smadja, C., Shi, P., Butlin, R.K., Robertson, H.M., 2009. Large gene family expansions and adaptive evolution for odorant and gustatory receptors in the pea aphid, *Acyrthosiphon pisum*. Mol. Biol. Evol. 26, 2073–2086.
Soong, R., Ladányi, A., 2003. Improved indicators for assessing the reliability of detection and quantification by kinetic PCR. Clin. Chem. 49, 973–976.
Talluri, S., Bhatt, A., Smith, D.P., 1995. Identification of a *Drosophila* G protein alpha subunit (dGq alpha-3) expressed in chemosensory cells and central neurons. Proc. Natl. Acad. Sci. U. S. A. 92, 11475–11479.
Taylor, R.W., Romaine, I.M., Liu, C., Murthi, P., Jones, P.L., et al., 2012. Structure-activity relationship of a broad-spectrum insect odorant receptor agonist. ACS Chem. Biol. 7, 1647–1652.
Upadhyay, S.K., Chandrashekar, K., Thakur, N., Verma, P.C., Borgio, J.F., et al., 2011. RNA interference for the control of whiteflies (*Bemisia tabaci*) by oral route. J. Biosci. 36, 153–161.
Visser, J.H., Fu-shun, Y., 1995. Electroantennogram responses of the grain aphids *Sitobion avenae* (F.) and *Metopolophium dirhodum* (Walk.) (Homoptera, Aphididae) to plant odour components. J. Appl. Entomol. 119, 539–542.
Vosshall, L.B., Hansson, B.S., 2011. A unified nomenclature system for the insect olfactory coreceptor. Chem. Senses 36, 497–498.
Vosshall, L.B., Amrein, H., Morozov, P.S., Rzhetsky, A., Axel, R., 1999. A spatial map of olfactory receptor expression in the *Drosophila* antenna. Cell 96, 725–736.
Webster, B.E.N., 2012. The role of olfaction in aphid host location. Physiol. Entomol. 37, 10–18.
Webster, B., Bruce, T., Pickett, J., Hardie, J., 2010. Volatiles functioning as host cues in a blend become nonhost cues when presented alone to the black bean aphid. Anim. Behav. 79, 451–457.
Wetzel, C.H., Behrendt, H.J., Gisselmann, G., Stortkuhl, K.F., Hovemann, B., et al., 2001. Functional expression and characterization of a *Drosophila* odorant receptor in a heterologous cell system. Proc. Natl. Acad. Sci. U. S. A. 98, 9377–9380.
Whyard, S., Singh, A.D., Wong, S., 2009. Ingested double-stranded RNAs can act as species-specific insecticides. Insect Biochem. Mol. Biol. 39, 824–832.
Wicher, D., Schafer, R., Bauernfeind, R., Stensmyr, M.C., Heller, R., et al., 2008. *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. Nature 452, 1007–1011.
Wistrand, M., Käll, L., Sonnhammer, E.L.L., 2006. A general model of G protein-coupled receptor sequences and its application to detect remote homologs. Protein Sci. 15, 509–521.
Wohlers, P., Tjallingh, W., 1983. Electroantennogram responses of aphids to the alarm pheromone (E)-beta-farnesene. Entomol. Exp. Appl. 33, 79–82.
Yu, X.D., Pickett, J., Ma, Y.Z., Bruce, T., Napier, J., Jones, H.D., Xia, L.Q., 2012. Metabolic engineering of plant-derived (E)-β-farnesene synthase genes for a novel type of aphid-resistant genetically modified crop plants. J. Integr. Plant Biol. 54 (5), 282–299.
Zhou, H.B., 2012. Use of Intercropping and Infochemical Releasers to Control Aphids in Wheat (PhD dissertation). Liege University, Gembloux.