Two Odorant-Binding Proteins Mediate the Behavioural Response of Aphids to the Alarm Pheromone (\(\mathcal{E}\))-\(\beta\)-farnesene and Structural Analogues

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Abstract

Background: Aphids are agricultural pests of great economical interest. Alternatives to insecticides, using semiochemicals, are of difficult applications. In fact, sex pheromones are of little use as aphids reproduce partenogenetically most of the time. Besides, the alarm pheromone, (\(\mathcal{E}\))-\(\beta\)-farnesene for a great number of species, is difficult to synthesize and unstable in the environment. The search for novel semiochemicals to be used in population control can be efficiently approached through the study of the olfactory system at the biochemical level. Recently odorant-binding proteins (OBPs) have been shown to play a central role in olfactory recognition, thus becoming the target of choice for designing new semiochemicals.

Methodology/Principal Findings: To address the question of how the alarm message is recognised at the level of OBPs, we have tested 29 compounds, including (\(\mathcal{E}\))-\(\beta\)-farnesene, in binding assays with 6 recombinant proteins and in behaviour experiments. We have found that good repellents bind OBP3 and/or OBP7, while non repellents present different spectra of binding. These results have been verified with two species of aphids, Acyrthosiphon pisum and Myzus persicae, both using (\(\mathcal{E}\))-\(\beta\)-farnesene as the alarm pheromone.

Conclusions: Our results represent further support to the idea (so far convincingly demonstrated only in Drosophila) that OBPs are involved in decoding the chemical information of odorants and pheromones, and for the first time provide such evidence in other insect species and using wild-type insects. Moreover, the data offer guidelines and protocols for the discovery of potential alarm pheromones, using ligand-binding assays as a preliminary screening before subjecting selected compounds to behaviour tests.

Introduction

Aphids represent one of the major pests in agriculture, and their populations cannot be controlled without the extensive use of insecticides. However, their chemical communication systems are exceptionally simple, both with respect to the semiochemicals they use and to the proteins involved in their detection. Although aphids comprise a great variety of species, differing in size, shape, colour and host plants, most of them utilize the same alarm pheromone, (\(\mathcal{E}\))-\(\beta\)-farnesene [1–4], that is released in the presence of danger and induces other individuals of the same as well as of other species to immediately abandon the place. The sex pheromones of aphids are mixtures, with relative proportions typical of the species, of nepetalactone and its related alcohols nepetalactols [5–6]. Both types of pheromones are not suitable for a wide use in agriculture. The synthesis of (\(\mathcal{E}\))-\(\beta\)-farnesene is complex and expensive and the compound is relatively volatile. Moreover, its persistence in the environment is also reduced by its ease of oxidation, due to the presence of several double bonds in the molecule. It has been shown that in normal conditions of temperature and sunlight about 77% is degraded after 24 hours and only traces are left after 48 hours [7]. It is interesting that a number of plants succeed in keeping away aphids by synthesising (\(\mathcal{E}\))-\(\beta\)-farnesene, thus indicating that in principle this compound can be efficiently utilised as aphid repellent [8]. This fact has suggested the idea of producing transgenic Arabidopsis thaliana that is able to synthesise (\(\mathcal{E}\))-\(\beta\)-farnesene and defend itself from aphids [9], thus paving the way for extending such approach to crop
because aphids reproduce most of the time parthenogenetically. Only in population control, but also for monitoring purposes, plants. Sex pheromones, on the other hand, are of little use, not only in population control, but also for monitoring purposes, because aphids reproduce most of the time parthenogenetically.

Therefore, we have decided to study the aphid chemoreception system at the biochemical level in order to find new strategies for population control. In particular we have focused our research on odorant-binding proteins (OBPs), soluble proteins secreted in the lymph of chemosensilla [10–13]. In recent years, these polypeptides, first regarded as passive carriers for pheromones and odorants, have been recognised as very important elements in the process of chemodetection and recognition. Early experiments performed with recombinant PBPs of *Austroeca polyphemus* and *Bombyx mori* suggested that different pheromone components induced specific conformational changes in different PBPs [14] and that electrophysiological response of sensilla to pheromone components depended on the presence of the correct PBP [15–16]. More recently, evidence has been provided that the presence of OBPs confers higher sensitivity and specificity to the olfactory system of *B. mori* [17–18], while LUSH, one of the OBPs of *Drosophila melanogaster* has been shown to be strictly required for perception of the male pheromone vaccenyl acetate [19]. In the same protein, binding of the pheromone induces a conformational change that triggers specific interaction with the corresponding odorant receptor [20]. This has been very elegantly demonstrated by showing that a mutant of LUSH, mimicking the conformation of the protein when bound to vaccenyl acetate, efficiently activates the olfactory receptor, even in the absence of the pheromone. At the behavioural level, exchange of two genes encoding OBPs in two species of *Drosophila* produces, to some extent, switching of behaviour towards some fatty acids [21]. Finally, a very recent work analysed the response to several odorant of 17 strains of *Drosophila*, each deficient in one specific OBP [22]. The results clearly show that in each strain the olfactory response was modified in different specific way, in some cases finely tuned to one or two odorants. These fundamental papers not only provide strong evidence that OBPs are involved in odour discrimination, but also indicate that a combinatorial code is utilised by insects to recognise olfactory stimuli. Moreover, they shift the focus from olfactory receptors to OBPs as the proteins responsible for decoding the chemical information carried by odorants and pheromones, and the best target for interfering with the insect’s olfactory system.

Further support to the key role of OBPs in odour discrimination has been provided in two species of mosquitoes, *Aedes aegypti* and *Culex quinquefasciatus*, where silencing genes for OBPs selectively abolishes response to indole [23–24]. We can therefore confidently attempt to relate the binding specificities of OBPs in a given species with behavioural responses.

The genome sequencing of the pea aphid *Acyrthosiphon pisum* has revealed the presence of 15 genes encoding OBPs [25]. In a first investigation limited to three OBPs, we have found that (E)-ß-farnesene and related compounds bind specifically to OBP3, suggesting not only that this protein might be involved in the recognition of the alarm pheromone, but also that activation of other OBPs could produce a response pattern that is no longer recognised as an alarm signal [26].

We have also synthesised new insecticides combining classical aphidicidal moieties with a terpene region mimicking (E)-ß-farnesene. Such compounds retain insecticidal activity, as well as specific binding to OBP3, suggesting that they might be also endowed with repellent properties [27].

Here we describe the bacterial expression and characterization of three additional OBPs of aphids. We have also measured the binding properties of 29 pure chemicals with six recombinant OBPs and their repellent activity in two species of aphids. On the basis of the results obtained, we suggest that OBP3 and OBP7 are the proteins responsible for mediating the perception of the alarm pheromone (E)-ß-farnesene and of other repellents, and provide guidelines for the discovery and design of new aphid repellents.

**Materials and Methods**

**Insects** - *Acyrthosiphon pisum* (Harris) was reared on potted broad bean plants (*Vicia faba* L.) in a climatic chamber at 20±1°C, 75±5% RH, under an LD 18:6 h photoperiod. Aphids culture was started in 1985, with a few hundred specimens originally collected from lucerne (*Medicago sativa* L) fields, in Southern Italy (Eboli, SA). Wingless adults were used in behaviour experiments. *Myzus persicae* (Sulzer) culture was reared under constant climatic conditions (20±1°C, 60–70% RH, natural lighting) on wild cabbage (*Brassica oleracea* L) in the greenhouse of the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing. The fourth-instar and wingless adults *M. persicae* were is used in behavioural test.

**Reagents and ligands** - All enzymes were from New England Biolabs. Oligonucleotides were custom synthesized at Eurofins MWG GmbH, Ebersberg, Germany. All other chemicals, unless otherwise specified, were purchased from Sigma-Aldrich and were of reagent grade. All CAU ligands were synthesised using our previously described methods [27]. The synthetic details will be reported in another publication. The other ligands, with the exception of geranyl acetate and farnesol (*Sigma-Aldrich*), as well as (E)-ß-farnesene (Bedoukian Research, Danbury, CT, USA), were prepared using well established conventional routes.

**RNA extraction and cDNA synthesis** - Total RNA was extracted from TRI® Reagent (Sigma-Aldrich), following the manufacturer’s protocol. cDNA was prepared from total RNA by reverse transcription, using 200 units of SuperScript® III Reverse Transcriptase (Invitrogen) and 0.5 μg of an oligo-dT primer in a 50 μl total volume. The mixture also contained 0.5 mM of each dNTP (GE-Healthcare), 75 mM KCl, 3 mM MgCl2, 10 mM DTT and 0.1 mg/mL BSA in 50 mM Tris-HCl, pH 8.3. The reaction mixture was incubated at 50°C for 60 min and the product was directly used for PCR amplification or stored at −20°C.

**Polymerase chain reaction** - Aliquots of 1 μl of crude cDNA were amplified in a Bio-Rad Gene CyclerTM thermocycler, using 2.5 units of *Thermus aquaticus* DNA polymerase (GE-Healthcare), 1 mM of each dNTP (GE-Healthcare), 1 μM of each PCR primer, 50 mM KCl, 2.5 mM MgCl2 and 0.1 mg/mL BSA in 10 mM Tris-HCl, pH 8.3, containing 0.1% v/v Triton X-100. At the 5’ end, we used specific primers corresponding to the sequence encoding the first six amino acids of the mature protein. Signal peptides were predicted using the on-line programme Signal-P and not included in the segment to be amplified. The primers also contained an *Nde* I restriction site, for ligation into the expression vector and providing at the same time the ATG codon for an additional methionine in position 1. At the 3’ end specific primers were used, encoding the last six amino acids, followed by a stop codon and an *EcoR* I restriction site for ligation into the expression vector. Therefore, we used the following primers for the each protein (enzyme restriction sites are underlined):

| Peptide | Forward (Fw) | Reverse (Rev) |
|---------|-------------|--------------|
| OBP6    | 5’-AACATATGATGCCAAATATATTACC-3’ | 5’-GTCCATGGTAGTTAATTTAGGT-GTGA-3’ |
| OBP7    | 5’-AACATATGATCTTGGAGTGAGCGCGC-3’ | 5’-AACATATGATCTTGGAGTGAGCGCGC-3’ |
After a first denaturation step at 95°C for 5 min., we performed 35 amplification cycles (1 min. at 95°C, 30 sec. at 50°C, 1 min. at 72°C) followed by a final step of 7 min at 72°C. In all experiments we obtained amplification products of 350–500 bp, in agreement with the expected sizes.

Cloning and sequencing - The crude PCR products were ligated into a pGEM (Promega) vector without further purification, using a 1:5 (plasmid:insert) molar ratio and incubating the mixture overnight, at room temperature. After transformation of E. coli XL-1 Blue competent cells with the ligation products, positive colonies were selected by PCR using the plasmid's primers SP6 and T7 and grown in LB/ampicillin medium. DNA was extracted using the kit GFX Micro Plasmid Prep (GE-Healthcare) and custom sequenced at Eurofins MWG (Martinsried, Germany).

Cloning in expression vectors - pGEM plasmids containing the appropriate sequences were digested with NdeI and EcoRI restriction enzymes for two hours at 37°C and the digestion products were separated on agarose gel. The obtained fragments were purified from gel and ligated into the expression vector pET30b (Novagen, Darmstadt, Germany), previously linearized with the same enzymes. The resulting plasmids were sequenced and shown to encode the mature proteins.

Preparation of the proteins - For expression of recombinant proteins, each pET-30b vector containing the appropriate OBP sequence was used to transform BL21(DE3)pLysS E. coli cells. Protein expression was induced by addition of IPTG to a final concentration of 0.4 mM when the culture had reached a value of O.D.600 = 0.8. Cells were grown for further 2 hours at 37°C, then harvested by centrifugation and sonicated. After centrifugation, OBPs were present as inclusion bodies. To solubilise them, the pellet from 1 L of culture was dissolved in 10 mL of 8 M urea, 1 mM DTT in 50 mM Tris buffer, pH 7.4, then diluted to 100 mL with Tris buffer and dialysed three times against Tris buffer.

Purification of the proteins was accomplished by combinations of chromatographic steps on anion-exchange resins, such as DE-52 (Whatman) and QFF (GE-Healthcare), followed by gel filtration on Sephacryl-100 or Superose-12 (GE-Healthcare) along with standard protocols previously adopted for other odorant-binding proteins [28–29].

Fluorescence measurements - Emission fluorescence spectra were recorded on a Jasco FP-750 instrument at 25°C in a right angle configuration, with a 1 cm light path quartz cuvette and 5 nm slits for both excitation and emission. The protein was dissolved in 50 mM Tris-HCl buffer, pH 7.4, while ligands were added as 1 mM methanol solutions.

Fluorescence binding assays - To measure the affinity of the fluorescent ligand N-phenyl-1-naphthylamine (1-NPN) to each protein, a 2 μM solution of the protein in 50 mM Tris-HCl, pH 7.4, was titrated with aliquots of 1 mM ligand in methanol to final concentrations of 2–16 μM. The probe was excited at 337 nm and emission spectra were recorded between 380 and 450 nm. The affinity of other ligands was measured in competitive binding assays, using 1-NPN as the fluorescent reporter at 2 μM concentration and 2–16 μM concentrations of each competitor.

Figure 1. Sequences and three-dimensional models of A. pisum OBP3 and OBP7. The two OBPs, despite a low sequence identity of 15%, show a remarkable similarity in their overall folding with special reference to their binding pockets. In both cases, the hydrophobic cavity is lined with several branched chain amino acids (Val, Ile, Leu) and an aromatic residue, Tyr84 in OBP3 and Phe52 in OBP7 (highlighted in the sequences). Three-dimensional models of OBPs were generated using the on-line programme SWISS MODEL [33–35]. For OBP3 we used the structure of the PBP of Leucophaea maderae (PDB: 1ORG_A [36] as a template. Amino acid identity between the two proteins is 23%. For OBP7 the template was the structure of Drosophila LUSH (acc. No. 3b6xB [37], identity between the two proteins: 17%). Models were displayed using the SwissPdb Viewer programme “Deep-View” [34] (http://www.expasy.org/spdbv/). Residues shown in the binding pockets are highlighted in the sequences.

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For determining binding constants, the intensity values corresponding to the maximum of fluorescence emission were plotted against free ligand concentrations. The curves were drawn using the Prism software, that allowed calculation of the dissociation constants and the stoichiometry of binding, assuming that the protein was 100% active. Dissociation constants of the competitors were calculated from the corresponding IC\textsubscript{50} values, using the equation: $K_D = [IC_{50}] / [1-NPN]/K_{1-NPN}$, where $I$ is the free concentration of 1-NPN and $K_{1-NPN}$ being the dissociation constant of the complex Protein/1-NPN.

Results and Discussion

Selection of the OBPs to be expressed

Our research was aimed at understanding the molecular mechanisms responsible for the detection of (E)-ß-farnesene in aphids with specific attention to the roles of OBPs. Following our previous characterization of three OBPs in the pea aphid, we decided to express three additional proteins of the same family and measure their binding properties. The recent sequencing of the genome of the pea aphid allowed the identification and annotation of all the genes encoding OBPs. In total 13 genes encoding such proteins are reported, but for two of

Molecular modelling – Three-dimensional models of OBPs were generated using the on-line programme SWISS MODEL [33–35]. For OBPs we used the structure of the FBP of Leuconostoc mesenteroides (PDB: 1ORG_A [36] as a template. Amino acid identity between the two proteins is 23%. For OB7 the template was the structure of Drosophila Lush acc. No. 3b6x [37] (identity between the two proteins: 17%). Models were displayed using the SwissPdb Viewer programme “Deep-View” [34] (http://www.expasy.org/spdbv/).
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them (OBP14 and OBP15) the partial sequences published are too short to allow their assignment to the OBP family [25]. Of the remaining 13 sequences, three are closely related (OBP3, OBP11 and OBP12), while the others are extremely divergent with percent of identical amino acids around 10% in most cases. Moreover, OBP2, OBP4, OBP5 and OBP6 are longer than other OBPs and contain extra cysteines besides the six characteristic of all insect OBPs. Figure S1 reports the amino acid sequences alignment for the 13 OBPs of the pea aphid *A. pisum*.

Based on such analysis, we decided to express OBPs 6, 7 and 10, that, together with OBPs 1, 3 and 8, previously described [26] could be representative of the OBP repertoire in aphids. Particularly interesting is OBP7 for being similar to OBP3 in its binding pocket, although the two proteins only share 15% of their amino acids when comparing full mature sequences.

In our previous paper [26] we reported that (E)-ß-farnesene and some structural analogues (farnesol and 3,7-dimethylolyoctyl acetate) exhibit specific binding to OBP3 and suggested that this protein could be involved in the perception of the alarm pheromone. We also hypothesised that the branched amino acids (valine, leucine and isoleucine) lining the binding pocket of OBP3 could establish close interactions with the terpene branched chain of (E)-ß-farnesene, while the aromatic ring of Tyr84 would match the two terminal conjugated double bonds of the same ligand. As shown in Figure 1, OBP7 (unlike the other OBPs of *A. pisum*) also presents a set of branched residues in its binding pocket and an aromatic residue, Phe52. Although the two aromatic amino acids are situated on opposite sites in the binding pockets of the two proteins, their positions relative to the other residues lining the binding cavity suggest that OBP3 and OBP7 could exhibit some similarity in their ligand-binding specificities.

Therefore, to test our hypothesis that (E)-ß-farnesene and structurally related compounds could preferentially bind OBP3 and OBP7 and that these two proteins might be responsible for detecting the alarm pheromone, we have expressed OBP7, OBP6 and OBP10, and analysed the binding specificities of the six proteins so far available.

Expression and purification of the OBPs

Figure 2 summarizes the bacterial expression and purification of OBPs 6, 7 and 10. As in the case of other insect OBPs, these proteins were produced in high yield (at least 20 mg/L of culture) and easily purified by conventional chromatographic methods. As the proteins were produced as inclusion bodies, their solubilisation was accomplished by denaturation and refolding, according to protocols that have been verified to afford the proteins in their active forms [28,37].

Ligand-binding studies

To investigate the binding specificity of each OBP, we used two sets of potential ligands. The first series comprises a structurally homogeneous group of synthetic compounds, each containing an aromatic moiety, endowed with insecticidal activity, linked to a geranyl group, that mimicks the structure of (E)-ß-farnesene. The synthetic routes for compounds of this type have been previously described [26]. The second series includes eight pure chemical compounds, the alarm pheromone (E)-ß-farnesene and some structurally-related substances, such as farnesol, 3,7-dimethylolyoctyl acetate, 3,7-dimethylolyoctyl benzoate and geranyl acetate, as well as other compounds of different structure, namely n-butyl benzoate, n-hexyl benzoate and isopentyl 4-phenylbutyrate.

The structures of all the ligands used are reported in Table S3 and Figure 3. The results of binding experiments are reported in Figure 4 and 5, while Table S1 lists the values of 

$dissociation constants.

All three newly expressed proteins (OBP6, OBP7 and OBP10) bind the fluorescent probe with good affinity (Figure 4, upper left panel), as most of insect OBPs, thus allowing other ligands to be tested in competitive binding assays. The upper right panel of Figure 4 reports, as examples, the displacement curves of 1-NPN from the complex with OBP7 by increasing amounts of selected ligands from the first series (CAU). With some ligands, as CAU 27, we observed an increase of 1-NPN fluorescence at higher concentrations of the ligand. A likely explanation of this phenomenon would suggest that above a certain concentration some ligands might form micelles entrapping molecules of 1-NPN, that, being in a hydrophobic environment, would produce a fluorescence peak in the same region of the spectrum as that relative to 1-NPN bound to the protein. This hypothesis is supported by the fact that such peak is observed when analysing a mixture of 1-NPN and ligand in the absence of the protein. In the lower panel the reverse values of the dissociation constants of all the chemicals belonging to the first series, measured with the six proteins are plotted and compared. Figure 5 reports parallel data obtained with the same proteins and eight general odorants (second series).

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Each OBP, as expected, displayed a different spectrum of binding with a broad specificity towards a number of ligands. Looking at the selectivity of each ligand towards the six OBPs, we can observe that some compounds, including (E)-ß-farnesene and structurally similar molecules, bind only OBP3 and/or OBP7, while others also exhibit measurable affinity to the other OBPs or else do not bind any of the proteins tested.

**Behaviour experiments**

To investigate whether OBP3 or OBP7 or both could be involved in detecting the signal of alarm pheromones, we performed behaviour experiments with all the compounds used in binding studies, using two species of aphids, *A. pisum* and *M. persicae*. Both species use (E)-ß-farnesene as the alarm pheromone [2,38]. Moreover, OBPs are exceptionally well conserved in many aphid species [25]. In particular the amino acid sequence of OBP3 of *M. persicae* presents only three substitutions with respect to that of *A. pisum*, all occurring outside the binding pockets. The sequences of OBP7 are more divergent in the two species (90% identity), but only two substitutions (L57M and V88A) are located inside the binding site and are not likely to modify appreciably the hydrophobic microenvironment.

For our experiments we used a Y tube olfactometer, as described in the Materials and Methods section. Aphids of the fourth instar as well as adults were used and the chemicals were tested at the concentration of 0.5%.

In all experiments we observed that a certain number of aphids did not make any choice between the two arms of the olfactometer.
(chemical vs solvent), but remained at the base of the tube, even in the control experiments, where the solvent was used at both arms of the tube. This phenomenon, that has been previously reported [39,40], could be due to several reasons, from inability of those individuals to smell the compound to a particularly high sensitivity that enabled them to detect the chemical already at the base of the tube, or to the reduced motility caused by fungal infections [41,42].

For our calculations we decided to ignore such individuals and included only those that had reached, at the end of the experiment, one of the two arms. The repellency of each compound (R) is calculated by the formula $R = \frac{C - T}{C + T}$, where $T$ indicates the number of aphids in the arm with the compound to be tested and $C$ those in the control arm. Therefore, a value of $R = 1$ indicates that all the insects that made a choice were found in the control arm, while $R = 0$ corresponds to a situation with the aphids distributed equally between the two arms and indicates no effect of the tested substance. The results of the behaviour tests with the two species of aphids are reported in Figure 6. Compounds specifically binding OBP3 and/or OBP7 (blue colour in the graphic) were significant repellents for M. persicae (Figure 6). For A. pisum, the same compounds were also repellents except CAU-04, CAU-35 and CAU-42. However, in these cases the calculated $\chi^2$ (CAU-04

Figure 5. Binding of general odorants to the six A. pisum OBPs. Upper panel. Competition binding curves of 8 ligands to OBP7. Lower panel. Reverse values of the dissociation constants measured with the same ligands and the six A. pisum OBPs. The values of the dissociation constants are reported as supplementary information in Table S1. The structures of the 8 ligands are reported in Figure 3.

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The repellency index \( R \) was calculated by the formula \( R = (C - T)/(C + T) \), where \( T \) indicates the number of aphids in the arm with the compound to be tested and \( C \) those in the control arm; asterisks indicate that the repellence observed is statistically significant (chi-square goodness-of-fit test): * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \).

\( (E)-\beta\)-farnesene \( x^2 = 14.70 \); Farnesol \( x^2 = 13.25 \); 3,7-Dimethyloctyl acetate \( x^2 = 4.05 \); 3,7-Dimethyloctyl benzoate \( x^2 = 0.17 \); n-Hexyl benzoate \( x^2 = 6.53 \); n-Butyl benzoate \( x^2 = 2.13 \); Geranyl acetate \( x^2 = 14.72 \); Isopentyl 4-phenylbutyrate \( x^2 = 10.00 \); CAU-01 \( x^2 = 4.24 \); CAU-02 \( x^2 = 1.92 \); CAU-04 \( x^2 = 3.70 \); CAU-07 \( x^2 = 8.00 \); CAU-10 \( x^2 = 0.12 \); CAU-14 \( x^2 = 0.30 \); CAU-15 \( x^2 = 0.9 \); CAU-16 \( x^2 = 4.17 \); CAU-19 \( x^2 = 4.74 \); CAU-21 \( x^2 = 3.86 \); CAU-23 \( x^2 = 0.06 \); CAU-24 \( x^2 = 2.92 \); CAU-26 \( x^2 = 0.00 \); CAU-27 \( x^2 = 10.04 \); CAU-28 \( x^2 = 6.59 \); CAU-35 \( x^2 = 3.70 \); CAU-37 \( x^2 = 7.41 \); CAU-42 \( x^2 = 3.92 \); CAU-46 \( x^2 = 0.08 \); CAU-47 \( x^2 = 16.90 \); 3,7-Dimethyloctyl benzoate \( x^2 = 0.56 \); n-Hexyl benzoate \( x^2 = 2.78 \); n-Butyl benzoate \( x^2 = 0.12 \); Geranyl acetate \( x^2 = 13.71 \); Isopentyl 4-phenylbutyrate \( x^2 = 8.53 \); CAU-01 \( x^2 = 8.10 \); CAU-02 \( x^2 = 0.36 \); CAU-04 \( x^2 = 36.06 \); CAU-07 \( x^2 = 21.80 \); CAU-10 \( x^2 = 1.23 \); CAU-14 \( x^2 = 1.78 \); CAU-15 \( x^2 = 1.00 \); CAU-16 \( x^2 = 0.08 \); CAU-19 \( x^2 = 16.59 \); CAU-21 \( x^2 = 25.10 \); CAU-23 \( x^2 = 1.39 \); CAU-24 \( x^2 = 5.58 \); CAU-26 \( x^2 = 2.16 \); CAU-27 \( x^2 = 19.56 \); CAU-28 \( x^2 = 21.43 \); CAU-35 \( x^2 = 22.53 \); CAU-37 \( x^2 = 19.56 \); CAU-42 \( x^2 = 16.10 \); CAU-43 \( x^2 = 17.67 \); CAU-46 \( x^2 = 22.35 \); CAU-53 \( x^2 = 1.00 \).

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As shown in Figure 7 and Table S2, the repellency elicited by ligands of additional OBPs considered as a whole was much lower than the repellency elicited by \((E)-\beta\)-farnesene and ligands of OB3P and/or OB7P as a whole, for both aphid species (\( A. pisum \) \( x^2 = 13.970 \); d.f. 1, \( P < 0.001 \); \( M. persicae \) \( x^2 = 96.044 \); d.f. 1, \( P < 0.001 \) (Figure 7).

In summary, we can say that compounds selectively binding OB3P and OB7P have a much higher probability of being repellents than compounds binding other OBPs or none. This suggests that OB3P and OB7P could mediate the perception and the consequent behavioural response to \((E)-\beta\)-farnesene and other repellents, and that only a selective activation of one or both of these OBPs is...
interpreted as a specific signal of danger. Moreover, such results allow us to use this model as a working hypothesis to further investigate the mechanism of alarm pheromone recognition by aphids and devise strategies and protocols to discover new aphid repellents.

Conclusions

In our previous paper we had suggested that OBP3 could be involved in the perception of \(E\)-\(\beta\)-farnesene. The novel concept that we had proposed was that recognition of the alarm pheromone could be based not on a particularly high affinity of this compound for OBP3, but rather on the specific binding to only such OBP3 among the three tested in that report [26]. In this work we support this model with behavioural experimental evidence and include a second protein (OBP7) that could mediate perception of the alarm pheromone. OBP7 is very similar to OBP3, but only limited to their binding pockets, while identity between the two proteins is not more than 11%. Most important, however, the behaviour data reported for two species of aphids, indicate a consistency between repellency and binding characteristics. Based on these six OBPs, that are representative of the small aphids repertoire of these polypeptides, we can propose a model that allows us to predict, with reasonable confidence, whether a new chemical compound could be a repellent (for aphids that use \(E\)-\(\beta\)-farnesene as the alarm pheromone), based on binding experiments. In particular, we have shown that repellency correlates well with exclusive affinity to OBP3 or OBP7 or both. A measurable affinity to other OBPs could significantly modify the olfactory map produced in the antennal lobes to impair recognition of the stimulus as similar to \(E\)-\(\beta\)-farnesene.

The idea that each olfactory stimulus generates a complex map at the level of antennal lobes and that the map is recognised by the brain, rather than its components, is certainly not new, both in insects and in vertebrates. Each glomerulus receives the signals from each type of olfactory receptors and the map in the antennal lob is basically a processed version of the map generated at the periferal level by olfactory receptors. Now, in the light of the new specific role of OBPs in olfactory perception [14–22] and based on the results here presented, we can identify a level, between odorants and olfactory receptors, where decodification of the chemical message is performed by OBPs.

However, our model is a crude simplification of the more complex biological system. The correlation between binding data and behaviour, that we have presented in this work, although statistically significant, is not perfect. The occurrence of few exceptions to the rule we propose can be related to several factors. First, other OBPs, among those we have not examined, or also CSPs or other still unknown binding proteins, could be involved in building the olfactory image of \(E\)-\(\beta\)-farnesene and other repellents. Second, our model has considered as ligands only those compounds that could displace 50% of the bound 1-NPN at a concentration lower than 20 \(\mu\)M could be rather arbitrary and affect to some extent the classification of a compound as “ligand”. Finally, we should be aware that binding activity, as measured “in vitro” does not faithfully reproduce the “in vivo” conditions, particularly in terms of protein concentration. Given the above limitations and caveats, however, our data provide for the first time a good correlation between behavioural effects and binding properties of odorants and further support the recent view that OBPs in insects are responsible for recognising the different semiochemicals. Moreover, they can provide indications and guidelines for designing and screening new repellents for aphids. In fact, potential repellents could be first tested in simple and rapid binding experiments with the six OBPs, and then only those binding OBP3 and/or OBP7 could be further screened in more expensive and time-consuming behaviour experiments. Similar approaches could also be devised to tackle analogous problems in other insect species.

Supporting Information

Figure S1 Alignment of amino acid sequences of the OBPs of \(A.\) pisum. The 13 sequences predicted by the genome are very divergent and, in order to align the six conserved cysteines, several gaps had to be introduced. OBP2 is much longer and, together with OBP4, OBP5 and OBP6, present several additional cysteines. Signal peptides (where prediction was feasible) are underlined.

Table S1 Binding properties of the ligands utilised in ligand binding assays and behaviour experiments. For each protein, [IC]50 reports the concentration of ligand that halves the initial fluorescence intensity (both protein and 1-NPN were used at the concentration of 2 \(\mu\)M). Dissociation constants are calculated as reported in the Materials and Methods section. The structures of “CAU” ligands are reported in Table 1, those of “LIG” chemicals in Figure 3. Blank cells indicate values of [IC]50 higher than 30 \(\mu\)M. For these compounds dissociation constants have not been calculated.

Table S2 Comparison among behavioural responses elicited by all compounds tested separately or as a function of OBPs bound. Asterisks indicate that the difference is statistically significant: * \(P<0.05\), ** \(P<0.01\), ***\(P<0.001\).

Table S3 Structures of the “CAU” ligand utilised in binding assays and behaviour experiments. Abbreviations are: Me: methyl; Pr: propyl; i-Pr: isopropyl; t-Bu: tert-butylyl; Ph: phenyl; p-NO2: p-nitrophenyl.

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Author Contributions

Conceived and designed the experiments: PP PF XLY DB. Performed the experiments: YFS HLQ II FDB LR SXY YL. Analyzed the data: PP DB. Contributed reagents/materials/analysis tools: PP PF XLY DB. Wrote the paper: PP PF DB XLY.

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