Pea aphid odorant-binding protein ApisOBP6 discriminates between aphid sex pheromone components, aphid alarm pheromone and a host plant volatile

Cassie Sims[a,b], Michael A. Birkett[a], Neil J. Oldham[b], Robert A. Stockman[b] and David M. Withall[a]*

[a] Protecting Crops and the Environment, Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, UK
[b] School of Chemistry, University of Nottingham, University Park, Nottingham, NG7 2RD, UK

* Corresponding author
E-mail address: David.withall@rothamsted.ac.uk

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Abbreviations: OBP – odorant-binding protein; ApisOBP – *Acyrthosiphon pisum* odorant-binding protein; OR – odorant receptor; STD-NMR – saturation transfer difference nuclear magnetic resonance

Abstract

Olfactory perception of pheromones in insects involves odorant-binding proteins (OBPs), relatively small proteins (ca.110-240 amino acid residues) that can bind reversibly to behaviourally active olfactory ligands. In this study, we investigated the binding *in silico* and *in vitro* of the aphid sex pheromone components (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol and (4a*S*,7*S*,7a*R*)-nepetalactone and the aphid alarm pheromone (*E*)-β-farnesene by OBPs from the pea aphid, *Acyrthosiphon pisum.* Screening of protein models of ApisOBPs1-11 with the aphid sex pheromone components suggested that ApisOPB6 was a candidate**.** Fluorescence assays using ApisOBP6 suggested that ApisOBP6 was able to bind both sex pheromone components and discriminate from the aphid alarm pheromone and the generic plant compound (*R/S*)-linalool. Saturation transfer difference STD-NMR experiments with ApisOBP6 yielded results consistent to those from the fluorescence experiments, with a clear interaction between ApisOBP6 and (4a*S*,7*S*,7a*R*)-nepetalactone. These results describe a novel interaction and potential function for ApisOBP6, point to pre-receptor odorant discrimination by OBPs, and provide a platform for investigating the function of other aphid olfactory proteins involved in aphid chemical ecology.

1. Introduction

Aphids (Homoptera: Aphididae) are economically important pests of horticultural and agricultural crops worldwide, causing damage both directly and indirectly through their feeding behaviour and transmission of detrimental plant viruses, such as barley yellow dwarf virus (BYDV) (Harris and Maramorosch, 1977; Pickett et al., 2013). Pheromones and other semiochemicals are naturally-occurring behaviour-modifying chemical signals that play a critical role in the life cycle of aphids (Pickett et al., 2013). Sex pheromones for aphid pest species principally comprise (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol **1** and (4a*S*,7*S*,7a*R*)-nepetalactone **2**, whilst the main component of the aphid alarm pheromone for many pest aphids is (*E*)-β-farnesene **5**, and (*R/S)*-linalool **6** is utilised as host plant volatile cue (Figure 1) (Dawson et al., 1987; Marsh, 1972; Pickett and Griffiths, 1980).

FIGURE 1

A number of studies have shown that olfactory perception of semiochemicals in insects involves at least two distinct groups of protein, i.e. olfactory receptors (ORs), seven transmembrane receptors with an inverse topology to the G-coupled protein receptors (GPCRs) found in mammals (Benton, 2006; Buck and Axel, 1991; Butterwick et al., 2018; del Mármol et al., 2021), and odorant-binding proteins (OBPs), relatively small proteins (ca. 110-240 amino acid residues) found in high concentrations (ca. 10 mM) in the sensillum lymph of antennae (Pelosi and Maida, 1995; Vogt and Riffiford, 1981; Zhou et al., 2010). Insect OBPs can be categorised into three distinct categories including classic OBPs (possessing 6 highly conserved cysteine residues), Plus-C OBPs (possessing 8 conserved cysteine residues and one conserved proline) and Atypical OBPs (possessing 9 or 10 conserved cysteine residues) (Zhou et al., 2010). Evidence of a role for OBPs in insect olfaction has been provided by deletion of OBPs in the striped rice stem borer, *Chilo suppressalis,* the tobacco cutworm, *Spodoptera litura*, and the common fruit fly, *Drosophila melanogaster*, resulting in significant reduction in antennal electrophysiological responses, measured by observing olfactory receptor neuron (ORN) responses to their respective binding ligands (Chang et al., 2015, p. 201; Dong et al., 2017; Larter et al., 2016; Zhu et al., 2016). For the pea aphid, *Acyrthosiphon pisum*, olfactory proteins ApisOBP3, ApisOBP7 and ApisOR5 have previously been shown to be critical for perception of the aphid alarm pheromone **5** (Northey et al., 2016; Qiao et al., 2009; Zhang et al., 2017). Concurrent to these studies, in this work we tested the hypothesis that *A. pisum* OBPs play a critical role in discrimination between sex pheromone components **1** and **2**, alarm pheromone **5** and host plant volatile cue **6**, using *in silico* modelling methods, fluorescence binding assays, STD-NMR experiments and biphasic gas chromatography assays. We also investigated the potential of aphid OBPs to discriminate between **1** and **2** and their non naturally-occurring stereoisomers **3** and **4**.

2. Materials and methods

2.1 Homology models

For ApisOBP1-11, protein structures were initially predicted using the iTASSER database, which takes a hierarchical approach by identifying structural templates from the Protein Data Bank. All predicted protein structures were minimised using the Yasara minimisation server. All homology models were visualised in PyMol 2.3.4.

2.2 Molecular docking

Ligands were prepared in Chem3D 16.0 and AutoDock 4.2. Docking studies were performed using AutoDock4.2 with the Racoon Virtual screening tool using a Lamarckian genetic algorithm. Binding energies and predicted K_i values were calculated through the virtual screening tool.

2.3 *Production of OBPs*

93
94 ApisOBP6 and ApisOBP9 were expressed in *E. coli*. A hexa-histidine tag and ampicillin
95 resistance gene were included. Bl21 (DE3) competent *E. coli* were transformed with the
96 plasmids of interest. Transformation was confirmed with colony selection, PCR and induction
97 tests. Recombinant BL21 (DE3) *E. coli* was grown in LB media and expression induced with
98 ITPG (Flurochem). Cell pellets were lysed by sonication in TBS and 0.2% Triton X-100 in
99 TBS. After centrifugation, protein was initially denatured with 8 M urea and 100 mM DTT,
100 then refolded *via* rapid dilution overnight with 0.5:5 mM GSSG:GSG. The final mixture was
101 purified using a HiTrap nickel-affinity column (GE Healthcare) and elution with 500 mM
102 imidazole. The His-Tag was removed *via* overnight cleavage with enterokinase (New England
103 Biolabs) in 2 mM $CaCl_2$ in TBS and ApisOBP6/OBP9 further purified using a nickel-affinity
104 column and fast-protein liquid chromatography (Akta) with a Superdex S200 column in TBS.
105 The final protein was concentrated and buffer-exchange into 25 mM Tris using VivaSpin 20.
106
107 **2.4** ***Synthetic chemistry***
108
109 Synthetic chemistry methods and analysis can be found in Appendix B.
110
111 **2.5** ***Fluorescence measurements***
112
113 All fluorescent measurements were undertaken using a Perkin-Elmer LS50B fluorescence
114 spectrophotometer, using a 2 mL quartz cuvette, unless otherwise stated. Spectra were recorded
115 using FL WinLab software. Saturation of OBPs with fluorescent probe, 1-NPN (Sigma-
116 Aldrich) was initially measured by titrating a 2 µM protein sample (2 mL in 25 mM Tris-HCl)
117 with aliquots of 1 mM ligand in methanol to final concentrations of 1-16 µM. The fluorescence
118 intensity was recorded. Titrations were performed with aliquots of 1 mM ligand in methanol to
119 final concentrations of 1-20 µM, either after the addition of fluorescent probe to a final
120 concentration of 1 µM or in the absence of fluorescent probe. To generate K_D values, relative
121 fluorescence intensity was plotted against the concentration of ligand as a binding curve. K_D
122 values were generated in GraphPad Prism 7 using a non-linear regression.
123
124 **2.6** ***STD-NMR***
125
126 Samples were run using an AVANCE Bruker DRX-500 MHz Nuclear Magnetic Resonance
127 spectrometer equipped with a 5 mm BBO BB-1H probe and set at 500 MHz for ^{1}H spectra.
128 Analysis of Bruker data was performed using Topspin 4.0.7.
129
130 STD-NMR samples comprised of ApisOBP6 (30 µM in D_2O) and ligand (3 mM in d_6-DMSO).
131 The ApisOBP6 on-resonance frequency of 160 Hz was selected to ensure no accidental
132 excitation of ligand signals. A 3 sec saturation time and 5.12 sec relaxation delay were used.
133 For each run, 192 scans were performed. Off-resonance spectra were recorded with an
134 excitation frequency of -12,000 Hz. STD absolute values were calculated by observing the
135 change in proportions between the off-resonance spectrum and the final STD spectrum using
136 the equation $(I_0\text{-}I_{STD})/I_0$ in which the term $(I_0 - I_{STD})$ represents the ratio of peak intensity in the
137 STD spectrum and I_0 the ratio of intensity in the off resonance spectrum. A second value
138 representing the proportionate change was calculated using the equation $I_0 \text{-}(I_0\text{-} I_{STD})$.
139
140 **2.7** ***Biphasic binding assay***
141

142 High resolution gas chromatography-flame ionization detector (GC-FID) analysis was
143 performed using an Agilent 6890A GC instrument equipped with a split/splitless injector and
144 HP-1 column (320.00 µm diameter x 50 m length). The carrier gas was hydrogen (flow rate of
145 3.1 mL min^{-1}) and the GC oven temperature programmed to start at 30°C, rise to 100°C at a
146 rate of 5 °C min^{-1}, maintained at 100 °C for 10 min, then rise again to 250 °C at a rate of 10 °C
147 min^{-1}, after which it was maintained at 250 °C for 45 min. The final run time was 84.10 min.
148
149 For the biphasic assay, a solution of test ApisOBP (100 µL of 5 µM in 25 mM Tris) was added
150 to a glass vial (2 mL size). A ligand solution (80 µL of 12 µM solution in hexane) was carefully
151 added on top, to create a biphasic system. The vial was gently mixed before being centrifuged
152 (5,000 rpm, 15 minutes). Finally, samples were incubated (ambient temperature, 2h) and a
153 sample (2 µL) of the hexane layer was removed and analysed by GC-FID. Quantification of
154 the amount of ligand per sample was undertaken by generating a calibration curve for each
155 ligand across a range of concentrations (Supplemental Data Figure S2). The amount of ligand
156 present was reported in milligram and micromolar quantities.
157

158 ### 2.8 *Statistical analysis*

159
160 Statistical analysis was performed in R 3.4.4. For fluorescence data, a one-way weighted
161 analysis of variance (ANOVA) was performed between ligands for each protein, and a two-
162 way weighted ANOVA was performed to investigate the interactions between proteins and
163 ligands. For gas chromatography, a two-way ANOVA was performed. In both analyses, a
164 Tukey Test was used for post-hoc analysis.
165

166 ## 3. Results and discussion

167 ### 3.1 *In silico predictions*

168
169 Initially, *in silico* modelling was adopted to identify potential discriminatory binding
170 interactions between *A. pisum* OBPs and compounds **1**-**6**. Three-dimensional protein models
171 of ApisOBPs 1-11 were generated using iTASSER, minimised using the Yasara minimisation
172 server and visualised in PyMol (Figure 2) (Krieger et al., 2009; Pandit et al., 2006; Schrödinger,
173 2015). The generated homology models were screened using AutoDock 4.2 for their predicted
174 interaction with **1**-**6** (Figure 3) (Forli et al., 2016; Morris et al., 1998).
175
176 **FIGURE 2**
177 **FIGURE 3**
178
179 Significantly stronger binding of ApisOBP6 with sex pheromone components **1** and **2** was
180 predicted compared to the alarm pheromone **5** and the plant volatile cue **6**. Other ApisOBPs
181 were predicted to have relatively weaker binding affinities for **1, 2, 5** and **6**, with ApisOBP9
182 displaying the lowest predicted energy interactions. This was also reflected in the calculated K_I
183 values, with the lowest K_I for the sex pheromone component **1** being 2.3 µM and the K_I for **5**
184 being predicted at a higher 11.5 µM. Non-naturally occurring stereosiomers **3** and **4** were
185 predicted to bind with similar energy as sex pheromone components **1** and **2**. From these
186 predictions, ApisOBP6 was selected as a candidate for *in vitro* experiments to confirm
187 predicted discrimation ability, and ApisOBP9 was selected as a control protein, due to
188 predicted low-affinity binding activity.
189

190
191 **3.2 *Fluorescence assays***
192
193 Recombinant ApisOBP6 and ApisOBP9 were prepared *via* cloning of the required genes,
194 transformation of pET45b *E. coli* expression system and affinity purification followed by
195 subsequent cleavage of His_6 tag. Authentic samples of **1-6** were obtained with the aim of
196 studying the *in vitro* binding activity of ApisOBP6 and ApisOBP9 compared to predicted
197 binding in the *in silico* modelling. Sex pheromone component **2** was purified from *Nepeta*
198 *cataria* essential oil by flash column chromatography, whilst **1** was synthesised from **2** by
199 stereoselective sodium borohydride reduction (Appendix B) (Birkett and Pickett, 2003). Non-
200 naturally occuring steroisomer **3** was synthesised *via* a multi-step synthesis starting from
201 commercially avaliable (*R*)-citronellol **7** (Dawson et al., 1996; Schreiber et al., 1986). Allylic
202 oxidation with catalytic selenium dioxide followed by Swern oxidation yielded dialdehyde **8**.
203 Cyclisation of dialdehyde **8** proceeded via an intramolecular enamine-mediated [4+2]
204 cycloadition to yield cyclised product **9**. Hydrolysis of **9** yielded non-naturally ocurring
205 steroisomer **3** that was converted to **4** via Fétizon oxidation (Appendix B). Alarm pheromone
206 **5** was prepared by the regioselective 1,4-elimination of the allylic ether THP-(*E, E*)-farnesol
207 as previously reported (Kang et al., 1987), while (*R/S*)-linalool **6** was commercially available
208 (Sigma Aldrich).
209
210 *In vitro* fluorescence binding studies with ApisOBP6 were conducted through monitoring
211 displacement of a fluorescent probe *N*-phenyl-1-naphthylamine (1-NPN) by **1-6** (Qiao et al.,
212 2009). The sex pheromone components **1** and **2** and stereoisomers **3** and **4** yielded binding data
213 to ApisOBP6 consistent with the predicted values from the *in silico* modelling, indicating that
214 the protein models have a high degree of accuracy. A significant difference in binding was
215 observed when comparing **1-4** with the alarm pheromone **5** and the plant volatile cue **6** (Figure
216 4a). The interaction between ApisOBP6 and sex pheromone component **2** provided the lowest
217 K_D value with 1.3±0.6 μM. There was no statisitcal difference between binding constants of
218 the naturally occuring sex pheromone components **1** and **2** and thier corresponding
219 stereoisomers **3** and **4**. However, there was a potential difference between **2** and **4** ($p=0.11$),
220 althought this was not statistically significant. There was no statistical difference in measured
221 binding constants between aphid semiochemicals **1-5** and ApisOBP9 (Figure 4b). This
222 apparent stereoselectivity trend of ApisOBP6 is consistent with previously reported literature
223 of other insect OBPs. In the gypsy moth, *Lymantria dispar*, LdisOBP1 was shown to
224 preferentially bind (-)-disparlure while LdisOBP2 preferentially bound (+)-disparlure (Plettner
225 et al., 2000). Futhermore, Plettner et al demonstrated that ApolOBP3, from *Antheraea*
226 polyohemus, exhibited a lower binding affinity towards (+)-disparlure compared to (-)-
227 disparlure. Contrastingly however, OBPs from the Japanese beetle, *Popillia japnica*, and the
228 Osaka beetle, *Anomala osakana*, are incapable of discriminating between the stereoisomers of
229 japonilure, even though both beetles behaviourally discriminate the respective japonilure
230 enantiomers (Wojtasek et al., 1998). Given these previously reported observations, in
231 combination with our results detailed here, they suggest that the molecular mechanism of insect
232 semiochemical enantiodiscrimination is still not fully understood and potentially involves other
233 olfactory proteins, such as odorant receptors, to fully account for the discrimination observed.
234
235 **FIGURE 4**
236
237
238 **3.3 *STD-NMR***
239

240 STD-NMR experiments were performed to further explore *in vitro* binding between ApisOBP6
241 and **2** and **5**, selected as the strongest binder and non-binder to ApisOBP6 respectively (Figure
242 5) (Mayer and Meyer, 1999; Xia et al., 2010). For sex pheromone component **2**, strong positive
243 STD-spectra were observed for resonances 1.21, 1.50-1.59, 1.64, 1.89-1.98, 2.02-2.11, 2.31-
244 2.39 and 2.05 ppm while resonance 6.18-6.20 ppm had a negative difference. For **5**, only weak
245 positive difference spectra were observed for resonances 1.48, 1.52 and 4.95 ppm. STD-NMR
246 experiments clearly demonstrated an interaction between ApisOBP6 and **2** while only non-
247 specific interactions were observed between ApisOBP6 and **5**. Epitope mapping of the
248 attenuation of individual resonances in **2** revealed the greatest attenuation for the two methyl
249 substitutents, with all the cyclopentyl protons also demonstrating different degrees of
250 attenuation (Figure 5). Epitope mapping of the attenuation of individual protons of **2** was
251 consistent with a binding conformation predicted from the *in silico* modelling (Figure 6)
252 (Mayer and Meyer, 2001). Greatest attentuation of the two methyl substitents of **2** was
253 consistent with predicted binding conformation given these substituents point directly at the
254 protein surface, while the cyclopentyl protons also experience attenuation being located deep
255 within the binding pocket. Proton 6.18-6.20 ppm of **2** showed minimal attenuation in the STD-
256 NMR, consistent with the predicted binding orientation positioning this proton directly towards
257 the binding pocket opening and therefore having minimal interactions with the protein. This
258 low attenuation could also be explained by solvent molecules blocking the interactions with
259 the protein as previously described and was again consistent with proton-2 being located at the
260 binding pocket opening (Brecker et al., 2006; Mayer and Meyer, 2001; Puchner et al., 2015).
261
262 **FIGURE 5**
263
264 STD-NMR demonstrates which protons are involved in the binding interaction by measuring
265 distance dependence saturation-transfer. STD-NMR spectra demonstrated a clear interaction
266 between ApisOBP6 and **2**, and a lack of specific interaction between ApisOBP6 and the alarm
267 pheromone **5**. The lack of a difference spectra for **5**, indicating a lack of binding suggesting
268 that ApisOBP6 can discriminates the sex pheromone component from other important aphid
269 semiochemicals. Proton resonances for almost all protons of **2** remained in the final STD-
270 NMR spectrum, suggesting that a saturation transfer between the protein and ligand had
271 occurred. Conversely, the STD-NMR spectrum for ApisOBP6 and **5** showed only a few
272 remaining peaks, which can be explained by non-specific interactions of the protruding methyl
273 groups. An unusual result was observed with the alkene proton at the C-2 position, in which a
274 negative STD-NMR spectrum was recorded. This negative difference peak has been observed
275 in other STD-NMR experiments and was previously explained as due to a solvent molecule
276 interfering with the saturation of the ligand during spin and lock time (Mayer and Meyer, 2001;
277 Puchner et al., 2015). From our *in silico* modelling data, proton 2-H of **2** is protruding out of
278 the predicted pocket into the aqueoues external environment and is therefore accessible to
279 solvent interferance (Figure 6). In previous literature, this effect has been observed with lactose
280 ring structure, similar to the lactone structure seen here (Brecker et al., 2006).
281
282 **FIGURE 6**
283
284 **3.4** ***Biphasic Binding Assay***
285
286 Biphasic gas chromatography assays were carried out with ApisOBP6, ApisOBP9 and
287 compounds **1**, **2** and **5** presented in aqueous/hexane phases respectively as a mimic of the
288 natural biphasic system found *in vivo* (Figure 7) (Zhou et al., 2009). Significant differences in
289 the amount of compound removed from the hexane layer, and the amount removed relative to

290 the amount of protein present, were observed when hexane layers were combined with aqueous
291 layers containing ApisOBP6, ApisOBP9 or no protein. The presence of ApisOBP6 in the
292 aqueous layer resulted in a significantly greater removal of **1** and **2,** but not **5,** from the hexane
293 layer compared to the presence of ApisOBP9 or no protein at all. Furthermore, the ratio of
294 ligand (µmol per µmol protein) removed from the hexane layer was significantly higher when
295 ApisOBP6 was present in the aqueous layer compared to when ApisOBP9 was present.
296
297 **FIGURE 7**
298
299 No clear differences between the control samples and the sample containing ApisOBP9 were
300 observed. However, with ApisOBP6, the amount of **1** and **2** in the hexane layer reduced to a
301 significantly lower level than in the control or ApisOBP9. Furthermore, the ratio of molar
302 quantities of **1** and **2** taken up per mole of OBP was significantly higher in ApisOBP6 than
303 with ApisOBP9.
304
305 **4. Conclusion**
306
307 Due to the high levels of background noise experienced by the insect olfactory system in the
308 wild and the high level of specificity required, insect olfactory proteins must be sophisticated
309 in their ability to recognise and discriminate between molecules in comparison to other
310 recognition proteins (Touchet et al., 2015). Recently, OBPs and ORs from *A. pisum,* ie.
311 ApisOBP3, ApisOBP7 and ApisOR5, were shown to be critical for perception of the aphid
312 alarm pheromone, (*E*)-β-farnesene **5** (Northey et al., 2016; Wang et al., 2021; Zhang et al.,
313 2017). Our results show that not only can ApisOBP6 bind the aphid sex pheromone
314 components **1** and **2** and their respective non naturally-occuring stereoisomers **3** and **4**, but
315 ApisOBP6 can also discriminate from the aphid alarm pheromone **5** and the generic host plant
316 volatile (*R/S*)-linalool **6**. Furthermore, we observed a possible trend that ApisOBP6 has minor
317 stereoselectivity towards the naturally occuring stereoisomers over the biologically inactive
318 non-natural stereoisomer, although this was not statistically significant. To our knowledge this
319 is the first report of an interaction between an aphid OBP and aphid sex pheromone component
320 and discrimation between different aphid semiochemicals at the olfactory level. ApisOBP6 is
321 one of only two Plus-C OBPs found in aphids and is responsible for the second most abundant
322 OBP mRNA in aphid antennae (De Biasio et al., 2014). It is also a large OBP at 215 residues;
323 it has been suggested that larger OBPs may have a longer C-terminal region, which can
324 contribute to a conformational change by folding into the binding pocket when a ligand is
325 bound (Gomez-Diaz et al., 2013; Pesenti et al., 2008; Zhang et al., 2017).
326
327 In addition to exploring the ability of aphid OBPs to discriminate between multiple different
328 semiochemicals, we also explored their ability to discriminate between stereoisomers. The
329 enantiomers of the sex pheromone components **3** and **4** were tested *in silico* and *in vitro* with
330 fluorescence binding assays. There was no significant difference between the sex pheromone
331 components **2** and its enantiomers **4** interaction with ApisOBP6. This apparent slight ability
332 of ApisOBP6 to distinguish between enantiomers of the sex pheromone components suggests
333 that another olfactory protein, most likely an OR, is responsible for enantiomeric
334 discrimination. Although it is difficult to elucidate the role of ApisOBPs from these initial

335 results, the slight differences observed should be investigated further. If true enantiomeric
336 differences are seen, this would be one of the first observation of OBPs playing a discriminating
337 role at this level (Sun et al., 2012). Future work should focus on the deorphanisation of ORs in
338 *A. pisum* to find a potential corresponding sex pheromone OR that may interact with
339 ApisOBP6.
340
341 After the success of the fluorescence binding studies between ApisOBP6 and the sex
342 pheromone components **1** and **2**, STD-NMR experiments and biphasic binding assays were
343 explored to delve deeper into the specifics of the ApisOBP6 and aphid pheromone interactions.
344 The biphasic assay was uniquely designed to provide a more realistic method for investigating
345 OBP binding activities, specifically investigating polyphasic systems present in the sensory
346 organs. Solubilising ligands, typically hydrophobic in nature, from the air via the cuticular wax
347 coated antennal pore into an aqueous solution (the sensillum lymph) is one of the main roles
348 hypothesized for OBPs (Pelosi et al., 2006). Overall, these results indicate ApisOBP6 increases
349 the amount of **1** and **2** that can be solubilised into the aqueous layer than with a control or
350 ApisOBP9. This result is consistent with the other ligand binding assays with ApisOBP6, and
351 further supports the role of ApisOBP6 in binding sex pheromone components **1** and **2**.
352
353 In summary, our data shows that ApisOBP6, an OBP from the pea aphid, *A. pisum*, can
354 discriminate between aphid sex pheromone components **1** and **2**, the aphid alarm pheromone **5**
355 and the generic host plant volatile cue **6.** We also observed a slight trend, although not
356 statistically significant, in stereoselectivity between biologically active natural stereoisomers
357 and the non-naturally occuring bioligcally inactive stereoisomer that suggests the role of
358 another component of the olfactory system, potentially an OR. Our results suggest that
359 ApisOBP6 may play a role in the perception of the aphid sex pheromone and a possible role in
360 pre-receptor odorant filtering. The work also demonstrates successful prediction of
361 pheromone-OBP interactions generated from *in silico* modelling and indicates a new NMR-
362 based method for exploring olfactory protein-ligand interactions. Both these approaches may
363 be deployed in the study of the function of other insect olfactory proteins. Further work
364 including X-ray crystallography, RNAi-based silencing or CRISPR/Cas9 is required to confirm
365 ApisOBP6 function *in vivo* and the potential role of an OR in enantiomeric discrimination of
366 chiral aphid sex pheromone components.
367
368
369 **Conflicts of interest**
370
371 The authors declare that they have no conflicts of interest with the contents of this article.
372
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386
387 **Appendix A. Supplementary data**
388
389 **Appendix B. Synthetic chemistry**
390
391 **Figures**
392
393 **Figure 1:** Aphid sex pheromone components, (*1R,4aS*,7*S*,7a*R*)-nepetalactol **1** and
394 (*4aS*,7*S*,7a*R*)-nepetalactone **2,** non-natural enantiomers of the sex pheromone components
395 (*1S,4aR*,7*R*,7a*S*)-nepetalactol **3** and (*4aR*,7*R*,7a*S*)-nepetalactone **4,** the aphid alarm pheromone
396 (*E*)-β-farnesene **5** and the generic host plant volatile (*R/S*)-linalool **6**
397
398 **Figure 2:** Homology model of *Acyrthosiphon pisum* odorant-binding protein 1-11 (ApisOBP1-
399 11) generated with iTASSER and PyMol.

400 **Figure 3.** Predicted *in silico* binding interactions (shown as $1/K_i$) of key aphid semiochemicals
401 **1-6** with ApisOBP1-11.
402
403 **Figure 4:** Binding constants between (a) ApisOBP6 and (b) ApisOBP9 and aphid
404 semiochemicals (4aS,7S,7aR)-nepetalactone **2**, (4aR,7R,7aS)-nepetalactone **4**,
405 (1R,4aS,7S,7aR)-nepetalactol **1**, (1S,4aR,7R,7aS)-nepetalactol **3,** (*E*)-β-farnesene **5** and (R/S)-
406 linalool **6** calculated from fluorescence data. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; ns
407 = no significance.
408
409 **Figure 5: 2** showing the predicted epitope mapping (blue) when bound to ApisOBP6 and **5**
410 showing the predicted non-specific interactions (blue) when interacting with ApisOBP6. Raw
411 values found in Supplementary Data (Table S2; Figure S1).
412
413 **Figure 6:** (4a*S*,7*S*,7a*R*)-Nepetalactone **1** (white, with oxygens in red) in the predicted binding
414 pocket of ApisOBP6 (blue/purple).
415
416 **Figure 7: (a)** The percentage change in amount of ligand in the biphasic assay as monitored
417 by gas chromatography of ApisOBP6 and ApisOBP9 compared to a control (Tris); **(b)** The
418 amount of ligand (µmol) removed from the layer per protein (µmol). For statistical analysis, *
419 = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; ns = no significance.
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