Ionotropic receptors in the turnip moth *Agrotis segetum* respond to repellent medium-chain fatty acids

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Abstract

Background: In insects, airborne chemical signals are mainly detected by two receptor families, odorant receptors (ORs) and ionotropic receptors (IRs). Functions of ORs have been intensively investigated in Diptera and Lepidoptera, while the functions and evolution of the more ancient IR family remain largely unexplored beyond Diptera.

Results: Here, we identified a repertoire of 26 IRs from transcriptomes of female and male antennae, and ovipositors in the moth *Agrotis segetum*. We observed that a large clade formed by IR75p and IR75q expansions is closely related to the acid-sensing IRs identified in Diptera. We functionally assayed each of the five AsegIRs from this clade using *Xenopus* oocytes and found that two receptors responded to the tested ligands. AsegIR75p.1 responded to several compounds but hexanoic acid was revealed to be the primary ligand, and AsegIR75q.1 responded primarily to octanoic acid, and less so to nonanoic acid. It has been reported that the C_6-C_{10} medium-chain fatty acids repel various insects including many drosophilids and mosquitos. Our GC-EAD recordings showed that C_6-C_{10} medium-chain fatty acids elicited antennal responses of both sexes of *A. segetum*, while only octanoic acid had repellent effect to the moths in a behavioural assay. In addition, using fluorescence *in situ* hybridization, we demonstrated that the five IRs and their co-receptor AsegIR8a are not located in coeloconic sensilla as found in *Drosophila*, but in basiconic or trichoid sensilla.

Conclusions: Our results significantly expand the current knowledge of the insect IR family. Based on the functional data in combination with phylogenetic analysis, we propose that subfunctionalization after gene duplication plays an important role in the evolution of ligand specificities of the acid-sensing IRs in Lepidoptera.

Key words: *Xenopus* oocytes, fluorescence *in situ* hybridization, acid-sensing, repellent, octanoic acid, Lepidoptera, gene expression

Introduction

Insects have evolved the ability to accurately sense environmental chemical signals, which play critical roles for their survival and reproduction (Sachse and Krieger, 2011; Leal, 2013). The peripheral process of chemoreception relies on receptors that interact with external molecular cues, triggering the transduction of chemical signals into electrical signals, which may ultimately result in a behavior (Hansson and Stensmyr, 2011; Fleischer et al., 2018). In insects, airborne chemicals are mainly detected by ligand-activated receptors from two families, namely the odorant receptors (ORs) (Clyne et al., 1999) and ionotropic receptors (IRs) (Benton et al., 2009). In *Drosophila*, these two receptor families are present in the dendritic membrane of the olfactory sensory neurons (OSNs) housed in different types of sensilla with distinct chemical preferences: ORs expressed predominantly in trichoid and basiconic sensilla detect, for example, pheromones and plant volatiles, including alcohols, aldehydes, esters and aromatics; IRs, mainly expressed in coeloconic sensilla, primarily respond to acids and amines (Yao et al., 2005; Benton et al., 2009; Abuin et al., 2011; Zhang et al., 2019).

IRs originated from the ionotropic glutamate receptor (iGluR) gene family and share similar structure and mechanism of action as iGluRs. However, the expression pattern and function of the IR family differ from the three well-studied iGluR families; kainate, NMDA (N-methyl-D-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors that are expressed on the surface of synapses in animal nervous systems and play a role in signal transmission between neurons (Benton et al., 2009; Abuin et al., 2011). Insect IRs are present in different tissues and are more diverse in function. IRs were first identified in the model insect *Drosophila melanogaster* as odorant sensing receptors (Benton et al., 2009), and subsequent studies extended our knowledge on their functions to the involvement in taste (Koh et al., 2014; Stewart et al., 2015; Ahn et al., 2017; Chen and Amrein, 2017), temperature (Chen et al., 2015; Ni et al., 2016), humidity (Enjin et al., 2016; Knecht et al., 2016; 2017) and

74 even auditory sensing (Senthilan et al., 2012). Similar to the ORs that are co-expressed with
75 the conserved odorant receptor co-receptor (Orco) to form a functional ligand-gated ion
76 channel (Larsson et al., 2004; Sato et al 2008; Wicher et al., 2008), co-expression in neurons
77 is also observed among insect IRs. These receptors display more complex combinations since
78 at least three IRs (IR8a, IR25a and IR76b) can function as co-receptors of specific tuning IRs.
79 IR8a is normally co-expressed with IRs tuned to acids (Grosjean et al., 2011; Silbering et al.,
80 2011; Rytz et al., 2013; Ai et al., 2013), while IR25a and IR76b are co-expressed with IRs
81 responding to amines (Abuin et al., 2011; Hussain et al., 2018). In addition to the role in
82 olfaction, IR25a was also reported as a co-receptor for IRs detecting temperature, humidity,
83 and taste molecules (Ni et al., 2016; Enjin et al., 2016; Ganguly et al., 2017).

84 Based on their phylogeny and expression profiles, *Drosophila* IRs are classified into two
85 groups: antennal IRs (A-IRs) and divergent IRs (D-IRs). A-IRs are highly conserved across
86 insects and mainly expressed in the antennae, whereas the D-IRs have radiated to different
87 extents in different species and are present in various tissues (Benton et al., 2009; Croset et al.,
88 2010; Abuin et al., 2011; Rytz et al., 2013; Koh et al., 2014; Andersson et al., 2019; He et al.,
89 2019). A number of the so-called Lepidoptera-specific IRs (LS-IRs) are absent in *Drosophila*
90 and were originally identified in Lepidoptera (Olivier et al., 2011; Bengtsson et al., 2012;
91 Poivet 2013; van Schooten et al., 2016; Liu et al., 2018; Zhu et al., 2018a). Despite the name,
92 some of them are also present in Trichoptera and mosquitos with overall high support values,
93 although in some cases the orthology remains somewhat ambiguous (Yuvaraj et al., 2018; Yin
94 et al., 2020). The LS-IRs do not represent a single monophyletic IR subfamily, because they
95 are scattered across the phylogenetic tree, with some (IR1, IR75p, IR75q and IR87a) located
96 within the A-IR while others (IR7d, IR100b-j and IR143) nested within the D-IR clade, and
97 also include a putative pseudogene (IR2) (Liu et al., 2018; Zhu et al., 2018a). The large IR75p

98 and IR75q expansions are of particular interest due to their close relationship with the
99 characterized acid-sensing IRs (Liu et al., 2018; Zhu et al., 2018a).

100 Over the past decade, much progress has been made in elucidating the functions of IRs in
101 *Drosophila* and a few mosquito species. Although this major gene family spans across
102 protostomes (Croset et al., 2010; Eyun et al., 2017), the functional information of IRs beyond
103 dipterans is still limited, restricting our knowledge concerning the evolution and specialization
104 of this ancient gene family. To our knowledge, only two IRs from the parasitoid wasp
105 *Microplitis mediator* have been functionally investigated (Shan et al., 2019). The second
106 largest insect order, Lepidoptera, rely on their remarkable olfactory abilities to sense the
107 external environment. However, the function of IRs in Lepidoptera remains largely unexplored,
108 although recent studies in *Manduca sexta* and *Mythimna separata* suggested a role for the co-
109 receptor IR8a in acid-sensing (Zhang et al., 2019; Tang et al., 2020). In the present study, we
110 report the first functional characterization of IRs in Lepidoptera. We show that two IRs from
111 the turnip moth *Agrotis segetum* respond to medium-chain fatty acids and that these fatty acids
112 elicit antennal responses, with octanoic acid being significantly repellent to moths in an
113 olfactory bioassay. Further, we find that in this moth the acid-detecting IRs are expressed in
114 basiconic or trichoid sensilla, but not in coeloconic sensilla as was found in *Drosophila*. Based
115 on our phylogenetic analysis and receptor characterization, we suggest that
116 subfunctionalization occurred in Lepidoptera after a gene duplication event in the IR75p/q
117 clade.
118

119 ## Results

120 ***Characterization of the* A. segetum *IR repertoire from antennal and ovipositor***
121 ***transcriptomes***

122 Studies on *M. sexta* have shown that in addition to the antennae, the ovipositor plays an
123 important chemosensory role (Klinner et al., 2016). Hence, in the present study we profiled
124 replicated transcriptomes of the antennae (N=4 for both sexes) and ovipositors (N=2) from
125 *A. segetum* ('Aseg') by mRNA sequencing (RNA-Seq) for an exhaustive search of IRs
126 involved in chemosensation. This produced a total of over 50 gigabases of sequence data with
127 the yield of each sample reaching over 10 million paired-end (100 bp) reads. Clean reads from
128 all 10 samples were assembled together to produce a transcriptome comprised of 83,645 non-
129 redundant transcripts having a mean length of 976 bp and an N_{50} of 1,788 bp.

130 In total, 26 AsegIR genes were identified, including the three co-receptors, IR8a, IR25a
131 and IR76b. Of the 26 assembled IR transcripts, 23 encoded full-length genes. We followed the
132 nomenclature previously used to name lepidopteran IRs (Liu et al., 2018). The raw sequence
133 reads have been deposited in the SRA database at NCBI under the Bioproject accession number
134 PRJNA707654.

135 ***The IR75p and IR75q lineages grouped with acid-sensing IRs***

136 We performed phylogenetic analyses of AsegIRs along with the IR sequences from *Bombyx*
137 *mori* (Bombycidae), *Plutella xylostella* (Plutellidae), *H. armigera* (Noctuidae), *D.*
138 *melanogaster* and *Anopheles gambiae* (both Diptera) (Fig. 1). The two IR classes, A-IRs and
139 D-IRs, identified in previous studies (Croset et al., 2010; Liu et al., 2018; Zhu et al., 2018a)
140 can be recognized in the tree. A single AsegIR orthologue from all the 18 monophyletic groups
141 of conserved A-IRs (IR1.1, IR1.2, IR21a, IR31a, IR40a, IR41a, IR60a, IR64a, IR68a, IR75d,
142 IR75p, IR75p.1, IR75p.2, IR75q.1, IR75q.2, IR76b, IR87a, IR93a) were identified. Fewer
143 AsegIRs were identified in the D-IR class, which was expected because D-IRs are typically
144 expressed in tissues other than antennae and ovipositors, and presumably mediate non-
145 olfactory behaviors. The large expansions of IR7d, IR75p and IR75q have been evident in

146 almost all the reported antennal transcriptomes of Lepidoptera, and these IRs typically have
147 relatively high levels of gene expression. The expression of IR75p and IR75q genes is
148 predominantly restricted to the adult antennae, whereas IR7d genes seem to be generally
149 distributed in different body parts of adults and larvae (van Schooten et al., 2016; Liu et al.,
150 2018; Zhu et al., 2018a). Notably, the lineage expansions IR75p and IR75q, along with IR1.1
151 and IR1.2, grouped within a larger cluster together with IR75a, IR64a and IR31a, which have
152 been reported as acid-sensing IRs in *Drosophia* and mosquitos (Abuin et al., 2011; Ai et al.,
153 2013; Silbering et al., 2011), although IR75d in the neighbouring expansion responded to an
154 amine (pyrrolidine) in *Drosophila* (Silbering et al., 2011).

155 ***AsegIRs in 75p/q expansions were predominantly expressed in adult antennae***

156 The expression levels of the 26 AsegIRs were normalized across sequencing libraries using
157 Transcripts Per Million (TPM) scaling factor (Li and Dewey, 2011). Surprisingly, one of the
158 LS-IRs, AsegIR7d.3, was found to be the most abundantly expressed IR, with much higher
159 levels of expression than that of the three co-receptors, and with higher expression in
160 ovipositors than in both male and female antennae. Among the three co-receptors, AsegIR25a
161 showed the highest expression, with higher TPM values compared to AsegIR8a and
162 AsegIR76b. AsegIR25a and AsegIR76b had non-biased expression in the antennae of both
163 sexes, while AsegIR8a showed female-biased expression. AsegIR75q.1 and AsegIR75q.2 were
164 identified from a large polycistronic transcript with comparatively high expression in both male
165 and female antennae. The other three IRs in the IR75 clade, i.e., IR75p, IR75p.2 and especially
166 IR75p.1, were expressed at much lower levels (Fig. 2A).

167 Quantitative PCR was conducted to further investigate the expression pattern of the
168 AsegIRs from the IR75p/q expansions and their putative co-receptor IR8a in various tissues of
169 both sexes, including chemosensory organs (antennae and proboscises) of both sexes, female

170 ovipositor, as well as male abdomen, head and thorax (without antennae), legs and wings.
171 GADPH (glyceraldehyde 3-phosphate dehydrogenase) and RPS3 (ribosomal protein S3) were
172 used as reference genes. The results showed that AsegIRs from the IR75p/q expansions and
173 AsegIR8a were expressed predominantly in adult antennae of both sexes, although IR75p.1
174 and IR75p.2 showed a broader tissue distribution, being weakly expressed in almost all assayed
175 non-chemosensory tissues (Fig. 2B). Consistent with the results of the transcriptome analysis,
176 AsegIR8a had higher expression level in female antennae than in male antennae; AsegIR75q.1
177 and AsegIR75q.2 were much more abundantly expressed than the other IR genes in the
178 expansion, while IR75p.1 had a lower expression level and was female-biased (Fig. 2B).

179 ***AsegIRs are expressed in basiconic or trichoid sensilla***

180 In *Drosophila*, IRs are mainly located in the antennal coeloconic sensilla (Benton et al., 2009;
181 Rytz et al., 2013), whereas it was previously unknown which sensillum type the IRs in moths
182 are associated with. To address this question, we first studied the morphology and distribution
183 of the sensilla on *A. segetum* antennae by scanning electron microscopy (SEM). The antennae
184 of *A. segetum* are sexually dimorphic, with setaceous antennae in females and plumose
185 antennae in males (Fig. 3A-D). There is one pair of branches on most of the segments of male
186 antennae, whereas the segments towards the tip of the antennae are unbranched (Fig. 3B and
187 3D). The SEM images indicated that the trichoid sensillum was the most abundant type on both
188 female and male antennae and it was the only sensillum type present on the branches of male
189 antennae (Fig. 3E, 3F and 3H). Only two or three coeloconic sensilla were observed on each
190 segment of the male antennae, including the unbranched segments and the stem parts of the
191 branched segments, but not on the branches; they were more abundant (six observed) on each
192 segment of the female antennae (Fig. 3E and 3F). Basiconic sensilla were present throughout
193 the entire female antennae and male antennae (except for the branches) but in lower abundance
194 compared to trichoid sensilla in both sexes (Fig. 3E and 3F).

195 To further explore the distribution of AsegIR75p/q and their putative co-receptor
196 AsegIR8a on female and male antennae and to confirm their co-expression pattern, we
197 performed two-color fluorescence *in situ* hybridization (FISH) with digoxigenin (DIG)-
198 labelled antisense RNA-probes for the five AsegIRs and biotin-labelled antisense probes for
199 AsegIR8a. Corresponding sense probes were used as control. On female antennae, AsegIR8a
200 was abundantly expressed across the whole segment, while signals of the five AsegIRs were
201 much fewer (Fig. 4 and Fig. 5). On the male antennae, AsegIR8a and the five AsegIRs were
202 predominantly expressed at the branched segments but not the unbranched segments toward
203 the tip of the antennae (Fig. 4, Fig. 5 and Fig. S1). They were not expressed on the stretches of
204 the branches (Fig. S1) but distributed at the bases of the branches and at the stem parts (Fig. 4
205 and Fig. 5). Fewer signals of the five AsegIRs were detected compared to AsegIR8a signals,
206 consistent with their expression levels. The two-color FISH confirmed that the five AsegIRs
207 were indeed co-expressed with AsegIR8a in both sexes (Fig. 4 and Fig. 5). Intriguingly, all the
208 five AsegIRs as well as AsegIR8a were in both sexes not expressed in coeloconic sensilla on
209 the antennae as in *Drosophila* (Fig. 4, Fig. 5 and Fig. S1). On the female antennae, they seemed
210 to be expressed in the OSNs located in basiconic sensilla. On the male antennae, however,
211 because the bases of the branches have high density of hair-like sensilla, we were not able to
212 distinguish from the FISH images whether the IRs were expressed under basiconic sensilla or
213 trichoid sensilla.

214 ***IR75p.1 and IR75q.1 responded to medium-chain fatty acids in* Xenopus *oocytes***

215 The five AsegIRs from the IR75p/q expansions were each co-expressed with AsegIR8a in
216 *Xenopus* oocytes, and screened against a panel of 46 compounds, most of which are common
217 plant volatiles, including acids, aldehydes and alcohols. Oocytes co-expressing
218 AsegIR75p.1/AsegIR8a showed responses to acids and aldehydes with exclusively C_5-C_7
219 straight chains at a concentration of 100 μM. For the alcohol ligands, only C_6 unsaturated

220 compounds elicited responses. The strongest response was elicited by hexanoic acid, followed
221 by (*Z*)-3-hexenol, (*E*)-2-hexenal and hexanal (Fig. 6A and B). Dose-response trials indicated a
222 higher sensitivity of AsegIR75p.1 to hexanoic acid compared to the other active ligands (Fig.
223 6B). Oocytes co-expressing AsegIR75q.1/AsegIR8a showed a strong response to octanoic acid
224 and a weaker response to nonanoic acid at a concentration of 100 μM. This receptor also
225 responded slightly to heptanoic acid and octanal, with response magnitudes much lower than
226 that to the primary ligand (Fig. 6A and C). The responses to octanoic acid and nonanoic acid
227 were dose-dependent, with a threshold concentration at 1 μM (Fig. 6C). The oocytes injected
228 with the cRNAs of AsegIR75q.1, AsegIR75p.1 and AsegIR8a alone did not show any response
229 to the tested compounds. In addition, the other three receptors in the same expansions,
230 AsegIR75p, AsegIR75p.2 and AsegIR75q.2, did not respond to any of the tested compounds
231 when co-expressed with AsegIR8a in oocytes.

232 ***Medium-chain fatty acids elicit antennal responses***

233 Due to the responses of AsegIR75p.1 and AsegIR75q.1 to medium-chain fatty acids (C_6-C_{10})
234 in the oocytes, we further tested whether these acids could elicit antennal responses in the
235 moths by gas chromatography coupled to electroantennographic detection (GC-EAD). Both
236 female and male antennae showed clear responses to the tested acids (Fig. 7A). The responses
237 to decanoic acid were smaller in both sexes, while the response magnitudes to the other tested
238 acids were similar. Male moths seemed to have larger responses to hexanoic acid and octanoic
239 acid than female moths, but the responses to the other tested acids were not significantly
240 different between the two sexes (Fig. 7B).

241 ***Octanoic acid is behaviorally repellent to* A. segetum**

242 In order to reveal the biological significance of the observed IR responses to fatty acids, we
243 performed two-choice Y-tube bioassays. In an initial experiment, we tested whether the odor

244 of honey solution (10%) was attractive to the adult moths. Among 51 individuals (29 females
245 and 22 males), most of *A. segetum* from both sexes (female: χ^2=12.45, d.f.=1, p= 0.0004; male:
246 χ^2=4.545, d.f.=1, p=0.033) were attracted to the honey solution and only two males chose water.
247 In another control experiment, cotton balls with 10% honey solution were placed in each of the
248 two arms of the Y-tube and were equally attractive to both female and male *A. segetum* (Fig.
249 7C), demonstrating no side-preference. In the treatment groups, individual female and male
250 moths were given the choice between 10% honey solution and 10% honey solution loaded with
251 one of the C_6-C_{10} fatty acids at the dose of 100 μg. Our results indicated that among the five
252 tested acids, only octanoic acid showed a significant repellent effect on both sexes, with
253 significantly fewer individuals choosing the honey solution with octanoic acid compared to
254 honey solution alone (female: χ^2=11.7647, d.f.=1, p=0.0006; male: χ^2=9.3226, d.f.=1,
255 p=0.0022). The moths showed no significant preference/avoidance in treatments with other
256 acids (Fig. 7C). The subsequent dose-response experiment showed that adults of both sexes
257 were significantly less attracted to the 10% honey solution with octanoic acid above 1 μg. No
258 significant difference was observed between the control and the dose of 100 ng octanoic acid
259 in either sex (female: χ^2=0.1538, d.f.=1, p=0.6949, male: χ^2=0.36, d.f.=1, p=0.5485) (Fig. 7D).
260

261 **Discussion**

262 IRs have been well studied in *Drosophila* and in some mosquito species, whereas the function
263 of this gene family remains poorly understood in other insects including the Lepidoptera.
264 Through phylogenetic analysis of lepidopteran IRs, several large receptor lineage expansions
265 are observed (Liu et al., 2018), among which the IR75p and IR75q lineages are the most salient
266 due to their close relationship with acid-sensing IRs and enriched expression in adult antennae.
267 The tandem duplications in these expansions were considered to be associated with the

268 discrimination of structurally similar chemicals in high-resolution (Rytz et al., 2013; van
269 Schooten et al., 2016). Here we present the first comprehensive investigation of the ligand
270 specificities of IRs from the IR75p/q expansions.

271 We identified a set of 26 AsegIRs from the antennal and ovipositor transcriptomes of *A.*
272 *segetum*. Based on the number of IRs identified in the antennal transcriptomes of other moth
273 species and *Drosophila* (e.g., 17 in *Spodoptera littoralis*, 12 in *Helicoverpa armigera* and 19
274 in *D. melanogaster*) (Poivet et al., 2013; Liu et al., 2012; Menuz et al., 2014), our dataset
275 appears to include most of the IRs expected to be expressed in the antennae and ovipositor of
276 *A. segetum*. This number is, however, lower compared to those identified from the genomes of
277 the other species (e.g., 45 in *S. litura*, 39 in *H. armigera* and 66 in *D. melanogaster*; Croset et
278 al., 2010; Zhu et al., 2018a; Liu et al., 2018), which include the IRs expressed in the other body
279 parts and pseudogenes. From the phylogenetic analysis, the IR75p/q expansions together with
280 IR1.1 and 1.2 clades were grouped into a large cluster in which some receptors including IR31a,
281 IR64a and IR75a,b,c have been reported as acid-sensors in *D. melanogaster*; IR64a.1 and
282 IR64a.2 in the parasitoid wasp *M. mediator*; and IR75k in the mosquito *A. gambiae* (Abuin et
283 al., 2011; Silbering et al., 2011; Rytz et al., 2013; Ai et al., 2013; Pitts et al., 2017; Shan et al.,
284 2019). This suggested that the receptors within the IR75p/q expansions may also be involved
285 in acid detection.

286 Based on the hypothesis of an "acid-sensing" cluster of receptors, all five AsegIRs within
287 the IR75p/q expansions were functionally investigated in *Xenopus* oocytes. We characterized
288 two functional IRs, with AsegIR75q.1 being specific for octanoic acid, and AsegIR75p.1
289 responding relatively broadly to C_5-C_7 straight chain acids and aldehydes, as well as
290 unsaturated C_6 alcohols. On top of this *in vitro* test at the receptor protein level, our *in vivo*
291 GC-EAD experiments showed that both sexes of *A. segetum* were able to detect C_6 to C_{10} fatty

292 acids, with no significant difference in the response magnitudes. We further explored the
293 behavioral significance of the responses to these fatty acids and found that only octanoic acid
294 had a significant repellent effect on female and male *A. segetum* moths, indicating that
295 AsegIR75q.1 might mediate the behavioral repellency of octanoic acid.

296 Fatty acids are common in nature and are normal constituents of vertebrate skin (Mullens
297 et al., 2009; Venter et al., 2011). It has been shown in a number of studies that the medium-
298 chain fatty acids, in particular C_8-C_{10}, are highly repellent or toxic to many arthropods,
299 including flies, mosquitos, ticks, ants, midges, and bees to name a few (Legal and Plawecki,
300 1995; Legal et al., 1999; Mullens et al., 2009; Venter et al., 2011; Zhu et al., 2018b; Ramadan
301 et al., 2020). For instance, octanoic acid and hexanoic acid are highly abundant in the fruit of
302 *Morinda citrifolia* and function as secondary defence compounds which are lethal or toxic to
303 most drosophilids, with the exception of *D. sechellia* which has evolved physiological
304 adaptations to specialize on the "toxic" Morinda fruit (Matsuo et al., 2007; Andrade et al.,
305 2017). Also, the C_6-C_{10} saturated fatty acids act as negative signals for ovipositional responses
306 of female mosquitos (Skinner et al., 1970; Hwang et al., 1982). In this study, we show that
307 octanoic acid is also a repellent for a lepidopteran species, using a 'reverse chemical ecology'
308 approach. IR75q.1 is well conserved across the Lepidoptera, and further studies are needed to
309 address the question of whether the repellency of octanoic acid is conserved in lepidopteran
310 insects, or if it has a specific ecological importance to *A. segetum*. The biological significance
311 of the response of AsegIR75p.1 to hexanoic acid for *Agrotis* moths is still elusive as this
312 compound was behaviorally inactive in our assay. However, a recent study suggested that
313 hexanoic acid deterred female hawkmoths (*M. sexta*) from ovipositing on plants (Zhang et al.,
314 2019).

315 We also explored the antennal distribution patterns of AsegIR75p/q and the co-receptor
316 AsegIR8a, which showed signals from hair-like (basiconic or trichoid) sensilla, but not from
317 coeloconic sensilla, on female and male antennae of *A. segetum.* In *D. melanogaster*, ten
318 antennal IRs (IR31a, IR41a, IR75a, IR75b, IR75c, IR75d, IR76a, IR76b, IR84a and IR92a) are
319 expressed in subsets of coeloconic sensilla on the antennae. The other four antennal IRs are not
320 expressed in coeloconic sensilla but found in neurons of the arista (IR21a) and sacculus (IR40a,
321 IR93a and IR64a) together with their co-receptors (Benton et al., 2009; Rytz et al., 2013; Ai et
322 al., 2013). The two co-receptors, IR8a and IR25a, are broadly expressed in coeloconic sensilla
323 in *D. melanogaster*; IR8a is also expressed in sacculus neurons while the IR25a is additionally
324 detected, but more weakly so, in basiconic and trichoid sensilla as well as aristal and sacculus
325 neurons (Benton et al., 2009; Rytz et al., 2013). Electrophysiological recordings from *D.*
326 *melanogaster* showed that the neurons housed in coeloconic sensilla detect acids, ammonia
327 and humidity, which indirectly suggests that the IRs define the response profiles of the
328 coeloconic neurons (Yao et al., 2005; Benton et al., 2009). In the desert locust *Schistocerca*
329 *gregaria*, IR25a and IR8a were shown to be expressed in coeloconic sensilla (Guo et al., 2014).
330 Beyond these two species, no additional evidence suggest that IRs are generally expressed in
331 coeloconic sensilla. However, accumulating evidence indicates that in some insects, IRs might
332 be expressed in non-coeloconic sensilla. In the mosquitos, trichoid sensilla and grooved-peg
333 sensilla both responded to amines and acids (Qiu et al., 2006) and it was speculated that the
334 responses were underlined by IRs expressed in these sensilla (Liu et al., 2010; Rytz et al., 2013).
335 In a basal insect species, *Lepismachilis y-signata* (Archaeognatha), in which IRs are the main
336 olfactory receptors detecting different chemical classes of odors, IRs are expressed in OSNs
337 underneath basiconic sensilla and no coeloconic sensilla were observed on the antennae
338 (Missbach et al., 2014). It was suggested that the evolutionary origin of IRs might have been

339 associated with basiconic sensilla and then their expression switched to coeloconic sensilla
340 after the neopteran insects originated (Missbach et al., 2014).

341 Based on the limited information regarding the sensory physiology of coeloconic sensilla
342 in Lepidoptera, it seems that the response profiles of coeloconic, basiconic and trichoid sensilla
343 partly overlap despite their striking morphological differences. In *B. mori*, although some
344 coeloconic sensilla were specialized towards C_4-C_9 carboxylic acids, coeloconic sensilla
345 responding to aldehydes and alcohols were also abundant; both coeloconic and basiconic
346 sensilla responded to hexanoic acid in *B. mori* and *M. sexta* (Pophof, 1997; Zhang et al., 2019).
347 In addition, no thermo- or hygroreceptors were found in coeloconic sensilla of *B. mori* (Pophof,
348 1997). It is therefore likely that the expression of IRs is not restricted to coeloconic sensilla.
349 Our study provides the first evidence from Lepidoptera that AsegIR75p/q and the co-receptor
350 (AsegIR8a) are expressed in OSNs located in basiconic or trichoid sensilla, suggesting that the
351 expression of IRs in specific sensillum types might be taxon-specific. This also suggests that
352 the coeloconic structure is not a prerequisite for IR function in neopteran insects, at least not
353 for acid-sensing IRs. However, further studies are needed to investigate at which taxonomic
354 level or where in the insect phylogeny this change in expression pattern has occurred.

355 Gene duplications are considered an important mechanism through which organisms can
356 acquire new genetic material for specific adaption and evolution of new functions (Crow and
357 Wagner, 2006; Moleirinho et al., 2011). After gene duplication, the duplicated genes are less
358 constrained by purifying selection and may start to accumulate mutations resulting in novel
359 functions, or in most cases to accumulate loss-of-function mutations generating pseudogenes
360 (Moleirinho et al., 2011; Andersson et al., 2015; Benton, 2015). Gene subfunctionalization can
361 also happen which means that both pre-existing and duplicated genes distinctively but
362 complementarily retain some of their original function (Force et al., 1999; Moleirinho et al.,

363 2011). Our phylogenetic tree shows that two mosquito (*A. gambiae*) IRs, AgamIR75k and
364 AgamIR75l clustered within the IR75p/q expansions and are closer to the IR75q lineage.
365 AgamIR75k is also an acid-sensing receptor, showing primary responses to octanoic and
366 nonanoic acids, and secondary responses to hexanoic, heptanoic, and decanoic acids (Pitts et
367 al., 2017). The response spectrum of AgamIR75k seems to be a combination of the response
368 profiles of AsegIR75p.1 and AsegIR75q.1, which indicates functional conservation of the
369 IR75p/q expansions across different insect orders and also suggests that subfunctionalization
370 has occurred after gene duplication in the IR75p/q expansions in Lepidoptera. The expression
371 level of AsegIR75q.1 was much higher than that of AsegIR75p.1 and other IRs within the same
372 clade, implying that the key ligand of AsegIR75q.1, octanoic acid, plays a biologically
373 important role for *A. segetum* and needs to be detected with high-resolution. The other three
374 receptors, AsegIR75p, AsegIR75p.2 and AsegIR75q.2 showed no response to any of the 46
375 tested compounds although they have higher expression levels than AsegIR75p.1, probably
376 because the ligands of these receptors were not included in the test panel. An alternative
377 explanation could be that nonfunctionalization occurred after the gene duplication.

378 ***Concluding remarks***

379 We present the first comprehensive investigation of ligand specificities of IR75p and IR75q
380 expansions and found that these IRs responded to medium-chain fatty acids. All the C_6-C_{10}
381 saturated fatty acids elicited antennal responses in the *A. segetum* moths, whereas only the
382 primary ligand of AsegIR75q.1, octanoic acid, acted as a behavioral repellent. We also provide
383 direct evidence that the acid-sensing receptor AsegIR75q.1 and its co-receptor are expressed
384 in basiconic or trichoid sensilla, indicating that the coeloconic structure is not a prerequisite for
385 IR function in neopteran insects. Our study expands the acid-sensing IR cluster in insects and
386 we propose that gene subfunctionalization may have played an important role after gene
387 duplication events during olfactory specialization in the IR75p/q expansions in Lepidoptera.

388

389 ## Materials and Methods

390 ***Chemicals***

391 A set of 46 odorants from different chemical classes were tested in this study, including acids,
392 aldehydes and alcohols. Most of them are common plant volatiles and can elicit responses of
393 neurons housed in coeloconic sensilla in *Drosophila* and *B. mori* (Table S1) (Pophof, 1997;
394 Yao et al., 2005). For oocyte recordings, stock solutions were prepared by dissolving each
395 compound to 100 mM in dimethyl sulfoxide (DMSO), which were stored at −20 °C. Before
396 each experiment, the stock solution was diluted to indicated concentration in Ringer´s buffer
397 (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 0.8 mM $CaCl_2$, 5 mM HEPES, pH 7.6) with the final
398 stimuli containing 0.1% DMSO. Ringer´s buffer containing 0.1% DMSO was used as negative
399 control.

400 ***Insects***

401 The *A. segetum* used in the experiments were from the continuous culture at the Pheromone
402 Group, Department of Biology, Lund University. Larvae were reared on an artificial bean-
403 based diet, under a 16L:8D photoperiod, 50% relative humidity and a temperature of 25 ℃.
404 Pupae were sexed and females and males kept separately, and emerged adults were fed with
405 10% honey solution. Individuals used in all experiments were unmated.

406 ***RNA extraction, transcriptome sequencing, assembly and annotation***

407 Male and female antennae, and ovipositors were collected from 3-day old virgin *A. segetum*
408 under a stereomicroscope. In total, four antennal samples for each sex and two ovipositor
409 samples were prepared. Each antennal sample contained 5 pairs of antennae and each
410 ovipositor sample contained 40 ovipositors. The collected tissues were immediately frozen on
411 dry ice and then stored at - 80℃ until use. The samples were homogenized and the total RNA

412 was extracted and purified using RNeasy Micro Kit (Qiagen, GmbH, Hilden, Germany). The
413 quality and quantity of the DNase treated RNA samples were verified using a BioAnalyzer
414 (Agilent).

415 Library preparation using the Illumina TruSeq RNA poly-A selection kit and sequencing
416 over two lanes on an Illumina HiSeq2500 with Rapid SBS Kit v2 chemistry was performed at
417 SciLifeLab (Stockholm, Sweden). Quality of the raw read data was assessed using FastQC
418 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimming of adaptors, filtering
419 of low-quality reads and removing contaminants were performed using Trimmomatic (v0.36)
420 (Bolger et al. 2014) with a custom screening database. Clean reads were assembled using
421 Trinity (v2.8.2) with default settings. Contigs from the Trinity output were clustered to remove
422 redundancy using CD-HIT-EST (v 4.6.8) (Li and Godzik, 2006) with a sequence identity
423 threshold of 0.95. The non-redundant transcript assembly was screened for putative protein-
424 coding regions using the program TransDecoder (release 5.0.1)
425 (http://transdecoder.github.io/) with the option of retaining hits to the PfamA domain database
426 (Finn et al. 2014). Completeness was assessed using the BUSCO (v3.0.2b) (Simão et al., 2015)
427 tool searching against the insecta_odb9 database of 1658 genes. Clean read data from each
428 pooled sample was then mapped to the non-redundant transcripts using the
429 align_and_estimate_abundance.pl script from the Trinity v2.8.2 software package using
430 default parameters except for --est_method RSEM --aln_method bowtie2 --trinity_mode.
431 Reads not mapping in pairs were discarded from DE analysis. The output was analyzed using
432 the Bioconductor (Huber et al. 2015) package DESeq2 (v1.22.2) (Love et al. 2014) in R.

433 Initial functional annotation of assembled sequences was performed by blasting against
434 the pooled database of nr (NCBI non-redundant protein sequences), as well as KOG/COG
435 (Clusters of Orthologous Groups of proteins) and Swiss-Prot with threshold E-value < 1e−5.

436 Additional blast searches were performed using the identified *A. segetum* IR genes as queries
437 and the transcriptome as a custom database to ensure that all IR genes were discovered. The
438 IRs were labelled following the nomenclature convention of lepidopteran IRs (Liu et al., 2018).

439 ***Phylogenetic analysis***

440 A phylogenetic tree was built based on the amino acid sequences of IRs from *A. segetum*, *B.*
441 *mori*, *P. xylostella*, *H. armigera, D. melanogaster* and *A. gambiae*. The sequence alignment
442 was performed using ClustalW, a built-in plugin with default settings in Geneious R9
443 (Biomatters Ltd. Auckland, New Zealand). To ensure the quality of the alignment and
444 subsequent construction of the tree, short sequences (< 300 amino acids) were excluded. A
445 maximum-likelihood tree was constructed using MEGA 7 with the WAG+G+F substitution
446 model that was chosen using "Find best protein model" in MEGA 7. The tree was rooted with
447 the IR8a and IR25a receptor lineage and further viewed and edited using FigTree 1.4.4.

448 ***Quantitative PCR (qPCR)***

449 For analysis of tissue expression patterns of IR8a and the five AsegIRs from the IR75p and
450 IR75q lineages, tissues were dissected from 3-5 day old virgin moths, including chemosensory
451 organs (antennae and proboscises) of both sexes, female ovipositors, as well as male abdomens,
452 heads+thoraxes (without antennae), legs and wings. Total RNA of these tissues was extracted
453 using the RNeasy Mini kit or RNeasy Plus Micro kit (Qiagen, GmbH, Hilden, Germany)
454 depending on the weight of starting material and treated with DNase. First-strand cDNAs were
455 synthesized from 250 ng total RNA with the SuperScript™ IV First-Strand Synthesis System
456 (Invitrogen, Carlsbad, CA, USA) following the manufacturer´s instruction. Gene expression
457 patterns were examined by qPCR on a Stratagene Mx3005P Real-Time PCR System (Agilent
458 technologies), with GADPH and RPS3 as reference genes. Primers used for qPCR are shown
459 in Table S2 and the primer efficiencies were validated by standard curves with 10X serial

460 dilutions of the templates. Three biological replicates were performed in separate plates, with
461 three technical replicates on each plate. The reaction was carried out in a 20 μL system
462 containing 1 μL cDNA template, 10 μL Power SYBR Green PCR 2X Master Mix (Life
463 Technologies), and 0.3 μM of each primer. The thermal cycling parameters consisted of an
464 initial denaturation step at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C
465 for 1 min. No-template controls were run in parallel for each primer pair. A subsequent melting
466 curve analysis was performed to ensure the primer specificity. The relative expression level of
467 each gene to the average of reference genes was calculated by the comparative CT method
468 (Schmittgen and Livak, 2008).

469 ***Scanning electron microscopy (SEM)***

470 The antennae from 2-3 day old adults were dissected and airdried. The dried samples were
471 carefully mounted on SEM stubs and then sputter-coated with gold (Cesington 108 auto, 45
472 seconds, 20 mA). The preparations were examined by a scanning electron microscope (SEM;
473 Hitachu SU3500) at 5 kV in the Department of Biology, Lund University.

474 ***Whole mount fluorescence* in situ *hybridization (WM-FISH)***

475 Whole mount fluorescence *in situ* hybridization was performed to monitor the antennal
476 expression pattern of the IR8a and genes in IR75p/q expansions on both female and male *A.*
477 *segetum* antennae (Karner et al., 2015; Zhang et al., 2016). Digoxigenin (DIG)- and biotin-
478 labelled antisense probes of target IRs were transcribed from linearized pCS2+ vectors with
479 the coding region of the corresponding receptors using the T7 RNA transcription kit (Roche,
480 Mannheim, Germany). Labelled probes were subsequently fragmented to an average length of
481 about 800 bp by incubation in sodium carbonate buffer (80 mM $NaHCO_3$, 120 mM Na_2CO_3,
482 pH 10.2). DIG-labelled probes were visualized using anti-DIG AP-conjugated antibodies and
483 the Vector Red Alkaline Phosphatase (AP) Substrate Kit (Vector Laboratories, Burlingame,

484 CA, US); biotin-labelled probes were visualized by streptavidin-horseradish peroxidase (HRP)
485 conjugates with Cyanine 5 Plus Amplification Reagent in a TSA kit as the substrate
486 (PerkinElmer, Boston, MA, USA). Sense probes labelled with DIG and biotin by SP6 RNA
487 transcription kit were used as control. Antennae were dissected from the head and cut into
488 pieces of about 4-5 mm length and then transferred to fixation solution (4% paraformaldehyde
489 in 0.1 M Na_2CO_3, pH 9.5, 0.03% Triton X-100). The antennae were gently squeezed by fine
490 tweezers to improve tissue penetration. The following fixation, hybridization, blocking,
491 antibody and substrate incubation steps were performed according to previously described
492 protocols (Karner et al., 2015). Finally, the tissues were mounted in Mowiol mounting medium.
493 A confocal microscope (Leica SP8 DLS, Germany) was used to analyze the sections (Zhang et
494 al. 2016).

495 ***Functional characterization in* Xenopus *oocytes***

496 First strand cDNA of female antennae was synthesized from 500 ng total RNA using
497 SuperScript™ IV First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) following
498 the manufacturer's instructions. Primers containing Kozak sequence ('GCCACC') and
499 restriction sites (Table S3) were designed to amplify the full-length IR genes using Platinum
500 Pfu Polymerase (Thermo Fisher Scientific). The amplified genes were then sub-cloned into the
501 pCS2+ expression vectors. All DNA sequences were verified by Sanger sequencing, using a
502 capillary 3130xL Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) at the
503 sequencing facility at the Department of Biology, Lund University. The sequences of the co-
504 receptor AsegIR8a and genes in the IR75p/q clade that were cloned and functionally tested
505 (AsegIR75p, AsegIR75p.1, AsegIR75p.2, AsegIR75q.1 and AsegIR75q.2) have been
506 deposited in GenBank under the accession numbers MW659848-MW659853. Large quantities
507 of purified plasmids containing AsegIRs and AsegIR8a with verified sequences were obtained
508 using the PureLinkTM HiPure Plasmid Filter Midiprep Kit (Thermo Fisher Scientific). cRNAs

509 of AsegIRs and AsegIR8a were synthesized from NotI (Promega) linearized recombinant
510 pCS2+ plasmids using the mMESSAGE mMACHINE SP6 transcription kit (Thermo Fisher
511 Scientific).

512 Each of the five AsegIRs from the IR75p and IR75q lineages were co-expressed with the
513 putative co-receptor AsegIR8a in *Xenopus* oocytes, and two-electrode voltage clamp
514 recordings were performed following previously described protocols (Zhang et al., 2010;
515 Zhang and Löfstedt, 2013; Hou et al., 2020). In brief, oocytes were collected from ovarian
516 tissue of female *X. laevis* (frogs purchased from European *Xenopus* Resource Centre,
517 University of Portsmouth, UK) and treated with 1.5 mg/mL collagenase (Sigma-Aldrich Co.,
518 St. Louis, MO, USA) in Ca^{2+} free Oocyte Ringer 2 buffer (containing 82.5 mM NaCl, 2 mM
519 KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) at room temperature for 15-18 minutes. Mature
520 healthy oocytes (stage V-VII) were isolated and co-injected with 50 ng IR75 cRNA and 50 ng
521 IR8a cRNA and incubated for 4-7 days at 18 °C in Ringer's buffer containing sodium pyruvate
522 (550 mg/L) and gentamicin (100 mg/L). Two-electrode voltage clamp recordings were
523 performed to measure the ligand-induced whole-cell inward currents from oocytes in good
524 condition at a holding potential of −80 mV, with a TEC-03BF signal amplifier. The test
525 compounds and Ringer's buffer were applied to the oocytes through a computer-controlled
526 perfusion system with exposure to stimuli at a rate of 2 mL/min for 20 s and extensive washing
527 in Ringer's buffer at a rate of 4 mL/min between stimulations. Data were collected and analyzed
528 by Cellworks software (npi electronic GmbH, Tamm, Germany).

529 ***GC-EAD***

530 The antennal electrophysiological responses of male and female *A. segetum* to the synthetic
531 mixture of C_6-C_{10} straight chain acids were recorded on an Agilent 7890 gas chromatograph
532 equipped with a flame ionization detector (FID) (Agilent Technologies, USA) and an

533 electroantennographic detector (EAD) (Syntech, Germany). An Agilent J&W HP-5 column
534 (30 m × 0.25 mm i.d., 0.25 µm film thickness; Agilent Technologies, USA) was used in the
535 GC, where the inlet temperature was set at 250 °C, the transfer line was heated at 255 °C and
536 the detector was set at 280 °C. Hydrogen was used as the carrier gas at a constant flow of 1.8
537 mL/min, and a 1:1 division of the GC effluent was directed to the FID and EAD, respectively.
538 A PRG-2 EAG (10x gain) probe (Syntech, Germany) was used in the recording. After cutting
539 off the tips, both antennae associated with the head of a 1-2 d old adult were mounted on the
540 probe using conductive gel (Blågel, CefarCompex, Sweden). Charcoal-filtered and humidified
541 air passed over the antennal preparation. The GC oven was programmed from initial hold at
542 100 °C for 2 min, then increased to 160 °C at a rate of 10 °C/min and then held for 2 min at the
543 final temperature. For each recording, a synthetic mixture containing 500 ng of each acid was
544 injected in split mode (ratio of 40:1). Data were collected with the software GC-EAD Pro
545 Version 4.1 (Syntech, Germany). Statistical analysis between stimuli within each group was
546 performed by one-way-ANOVA followed by an LSD test at 0.05 level. Differences between
547 males and females responding to the same stimulus was analyzed by Student´s t-Test at $p <$
548 0.05 level. N=6 for female, N=4 for male.

549 ***Two-choice Y-tube experiments***

550 A Y-tube olfactometer was used to investigate the behavioral responses to acids. The
551 olfactometer consisted of a 14 cm main arm with an insect introduction chamber attached and
552 two 14 cm long arms with an interior angle of 120° for the treatment and control odors. The
553 inner diameter of the Y-tube was 1.6 cm. The tests were always conducted in a dark room under
554 red light illuminating the olfactometer from above (～26 Lux). Tests were performed during
555 the first two hours of the scotophase at the following experimental conditions: 24°C air
556 temperature, 55% relative humidity and 0.8 m/s airflow.

557 To test whether the honey solution is attractive to the adult moths, two cotton balls
558 (loaded 1mL 10% honey solution with 10 μL acetone vs 1 mL water with 10 μL acetone) was
559 placed at inside of each Y-arm. Individual adults of *A. segetum* were released from the induce
560 chamber at the end of main arm (29 females and 22 males). Another control experiment (cotton
561 balls loaded 1 mL 10% honey solution with 10 μL acetone were placed at both sides of Y-tube)
562 were performed to check whether there is a side-bias.

563 In this bioassay, five fatty acids were tested individually at the dose of 100 μg, including
564 hexanoic acid, heptanoic acid, octanoic acid, nonanoic acid and decanoic acid ($N \geq 26$ for each
565 acid and each sex). Stock solutions of these fatty acids were prepared by diluting the neat
566 compounds to 10 mg/mL in acetone. Before each experiment, 10 μL stock solution was mixed
567 in 1 mL 10% honey solution, then the mixture was loaded on a cotton ball. 10% honey solution
568 with 10 μL acetone was used as control. The cotton balls were left for 10 min for the
569 evaporation of acetone before tests and placed at inside of the ends of the Y-arms. The Y-tube
570 and cotton balls were renewed after every 10 tests. 16-20 h starved naïve individual
571 males/females, 2-5 days old, were used in the experiment. After the screening-assay, octanoic
572 acid was tested at a series of doses in 10-fold increments from 100 ng to 100 μg. Insects were
573 considered to have made a choice only when they entered either of the arms and fed on the
574 cotton within 5 min. By contrast, during the observation period (5 min), if the insect kept
575 moving back and forth or stayed in the main arm without movement, it was considered as no
576 choice had been made. The proportions of no choice for each treatment and each sex were
577 lower than 5%, so we excluded the no choice data when we did the Chi2 test for statistical
578 significance.

579 **Declarations**

580 ***Ethics approval and consent to participate***

581 The care and use of *Xenopus laevis* frogs in this study were approved by the Swedish Board of
582 Agriculture, and the methods were carried out in accordance with the approved guidelines.

583 ***Consent for publication***

584 Not applicable.

585 ***Availability of data and materials***

586 The datasets used and/or analysed during the current study are available from the
587 corresponding author on reasonable request. The sequence reads have been deposited in the
588 SRA database at NCBI (BioProject accession number PRJNA707654). The amino acid
589 sequences of the IRs identified from *Agrotis segetum* antennal and ovipositor transcriptomes
590 are available in Additional file 2; sequences of the co-receptor AsegIR8a and IRs in the 75p/q
591 clade (AsegIR75p, AsegIR75p.1, AsegIR75p.2, AsegIR75q.1 and AsegIR75q.2) have been
592 deposited in GenBank (accession numbers MW659848-MW659853).

593 ***Competing interests***

594 The authors declare no competing interests.

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603 ***Authors' contributions***

604 XQH, DDZ and CL conceived the study. XQH, DDZ, MNA, DP and CL contributed to
605 experimental design. XQH and DP performed transcriptomic data analysis. XQH performed

606 the molecular work, oocyte recordings, scanning electron microscopy imaging, phylogenetic
607 analysis and behavioural assay. DDZ performed qPCR experiment. DDZ and XQH did *in situ*
608 hybridization. HLW performed the GC-EAD. XQH and DDZ analyzed the data. XQH drafted
609 the manuscript with contributions from HLW and DP. DDZ revised the manuscript according
610 to reviewers' comments. All authors provided editorial and scientific input on the final version
611 of the manuscript.

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617

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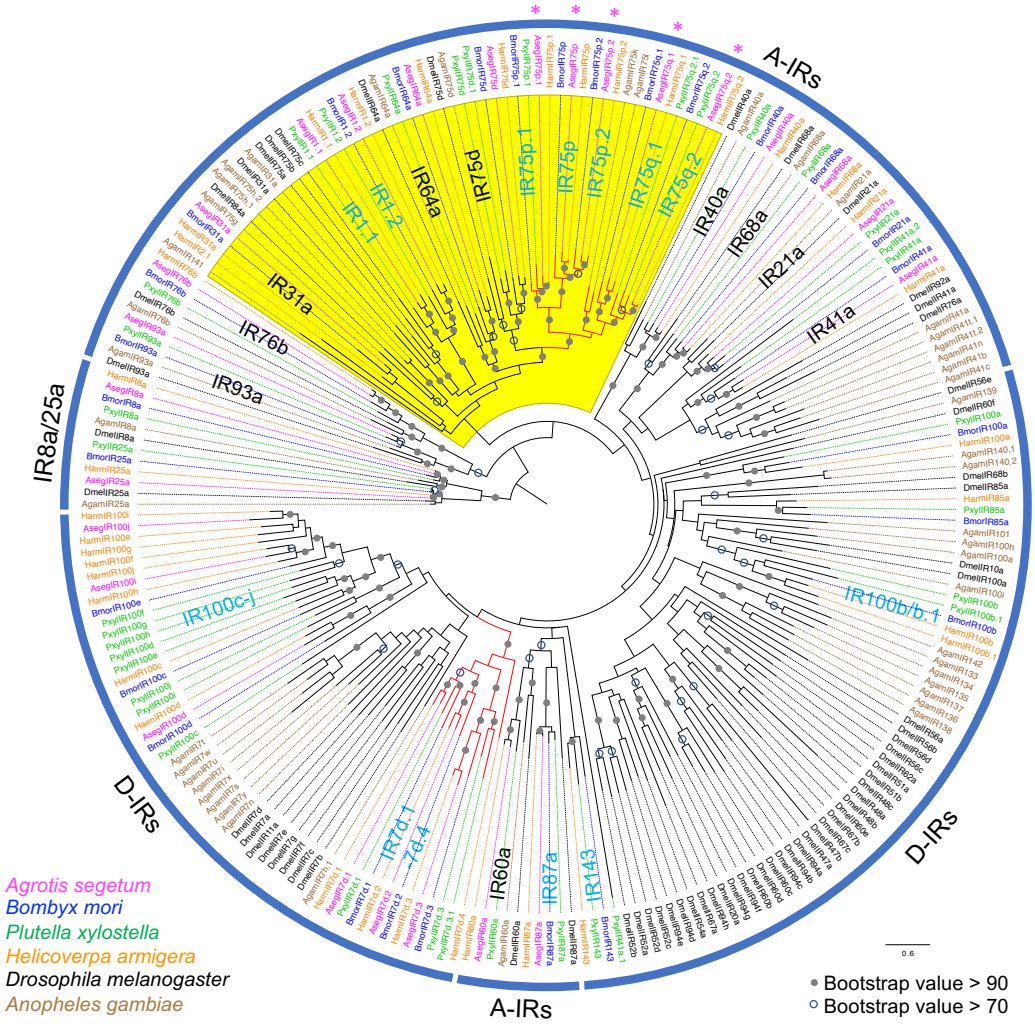
824

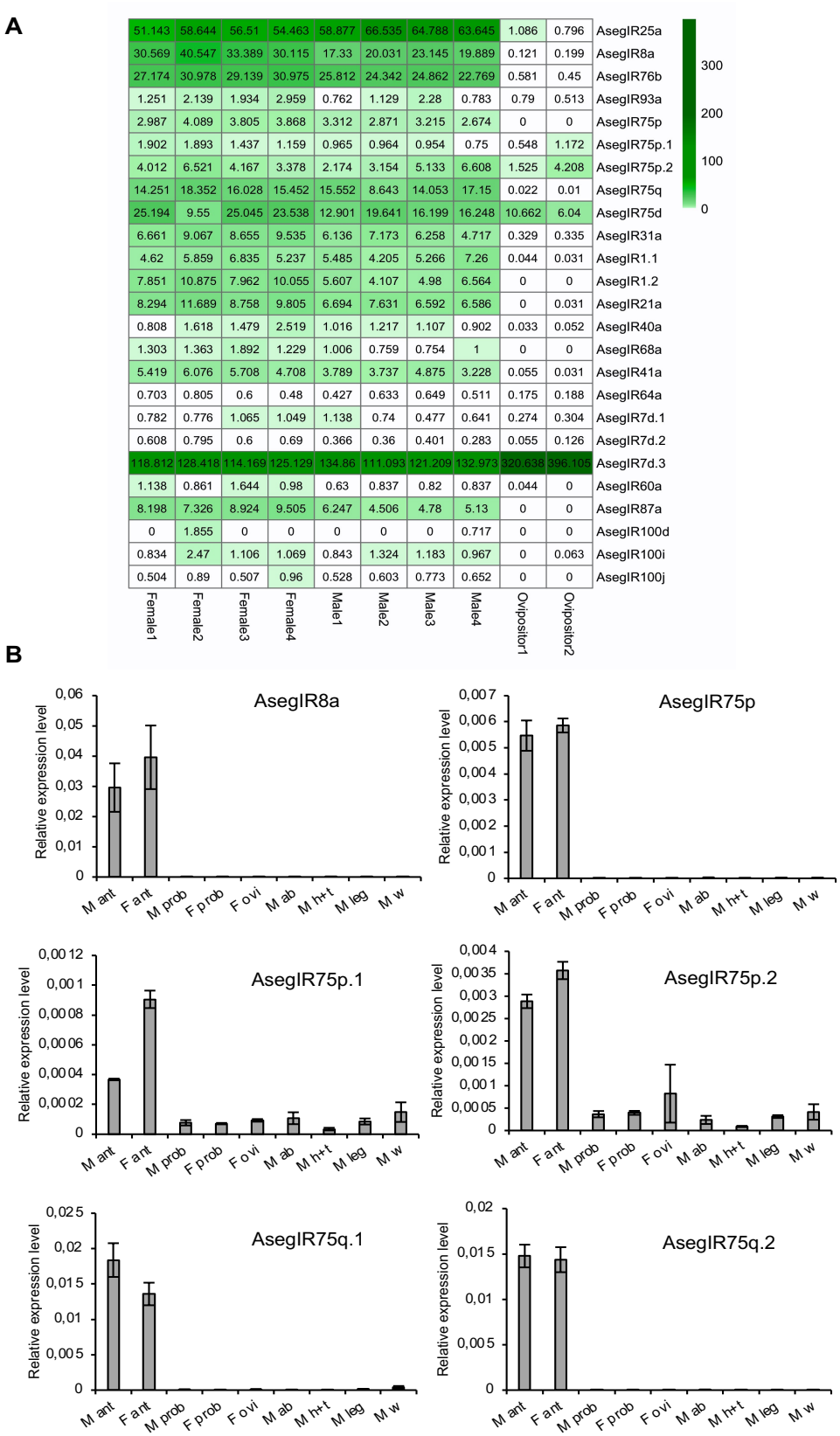
825 **Figure legends**



826

827 **Fig. 1. Phylogeny of Lepidoptera IRs.** The maximum-likelihood phylogenetic tree is based
828 on a protein sequence alignment of ionotropic receptors (IRs) from *A. segetum*, *B. mori* (Croset
829 et al., 2010), *P. xylostella* (Liu et al., 2018), *H. armigera* (Liu et al., 2018), *D. melanogaster*
830 (Benton et al., 2009; Croset et al., 2010) and *A. gambiae* (Croset et al., 2010; Liu et al., 2010).
831 The tree was rooted with the lineage of IR8a and IR25a receptors. The IRs from different
832 species are color coded as indicated in the figure. The monophyletic IR groups are noted on

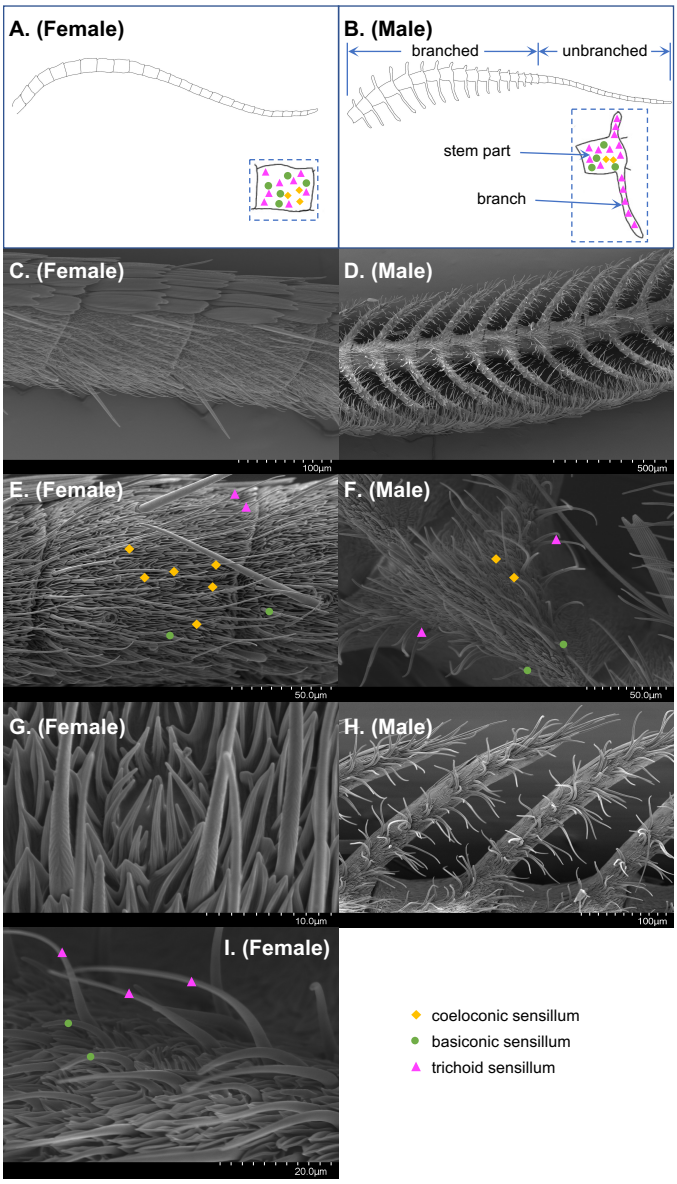
833 the tree, with the light blue color indicating “Lepidoptera-specific IR groups”. The large IR7d,
834 IR75p and IR75q expansions are indicated by red branches, and the putative “expanded acid-
835 sensing cluster” is highlighted in yellow with the exception of IR75d which responded to an
836 amine in *Drosophila*. The five IR75p and IR75q receptors functionally assayed in this study are
837 marked with asterisks. Bootstrap support (100 replicates) values over 0.7 are shown at
838 corresponding nodes.



Female1	Female2	Female3	Female4	Male1	Male2	Male3	Male4	Ovipositor1	Ovipositor2	
51.143	58.644	56.51	54.463	58.877	66.535	64.788	63.645	1.086	0.796	AsegIR25a
30.569	40.547	33.389	30.115	17.33	20.031	23.145	19.889	0.121	0.199	AsegIR8a
27.174	30.978	29.139	30.975	25.812	24.342	24.862	22.769	0.581	0.45	AsegIR76b
1.251	2.139	1.934	2.959	0.762	1.129	2.28	0.783	0.79	0.513	AsegIR93a
2.987	4.089	3.805	3.868	3.312	2.871	3.215	2.674	0	0	AsegIR75p
1.902	1.893	1.437	1.159	0.965	0.964	0.954	0.75	0.548	1.172	AsegIR75p.1
4.012	6.521	4.167	3.378	2.174	3.154	5.133	6.608	1.525	4.208	AsegIR75p.2
14.251	18.352	16.028	15.452	15.552	8.643	14.053	17.15	0.022	0.01	AsegIR75q
25.194	9.55	25.045	23.538	12.901	19.641	16.199	16.248	10.662	6.04	AsegIR75d
6.661	9.067	8.655	9.535	6.136	7.173	6.258	4.717	0.329	0.335	AsegIR31a
4.62	5.859	6.835	5.237	5.485	4.205	5.266	7.26	0.044	0.031	AsegIR1.1
7.851	10.875	7.962	10.055	5.607	4.107	4.98	6.564	0	0	AsegIR1.2
8.294	11.689	8.758	9.805	6.694	7.631	6.592	6.586	0	0.031	AsegIR21a
0.808	1.618	1.479	2.519	1.016	1.217	1.107	0.902	0.033	0.052	AsegIR40a
1.303	1.363	1.892	1.229	1.006	0.759	0.754	1	0	0	AsegIR68a
5.419	6.076	5.708	4.708	3.789	3.737	4.875	3.228	0.055	0.031	AsegIR41a
0.703	0.805	0.6	0.48	0.427	0.633	0.649	0.511	0.175	0.188	AsegIR64a
0.782	0.776	1.065	1.049	1.138	0.74	0.477	0.641	0.274	0.304	AsegIR7d.1
0.608	0.795	0.6	0.69	0.366	0.36	0.401	0.283	0.055	0.126	AsegIR7d.2
118.812	128.418	114.169	125.129	134.86	111.093	121.209	132.973	320.638	396.105	AsegIR7d.3
1.138	0.861	1.644	0.98	0.63	0.837	0.82	0.837	0.044	0	AsegIR60a
8.198	7.326	8.924	9.505	6.247	4.506	4.78	5.13	0	0	AsegIR87a
0	1.855	0	0	0	0	0	0.717	0	0	AsegIR100d
0.834	2.47	1.106	1.069	0.843	1.324	1.183	0.967	0	0.063	AsegIR100i
0.504	0.89	0.507	0.96	0.528	0.603	0.773	0.652	0	0	AsegIR100j

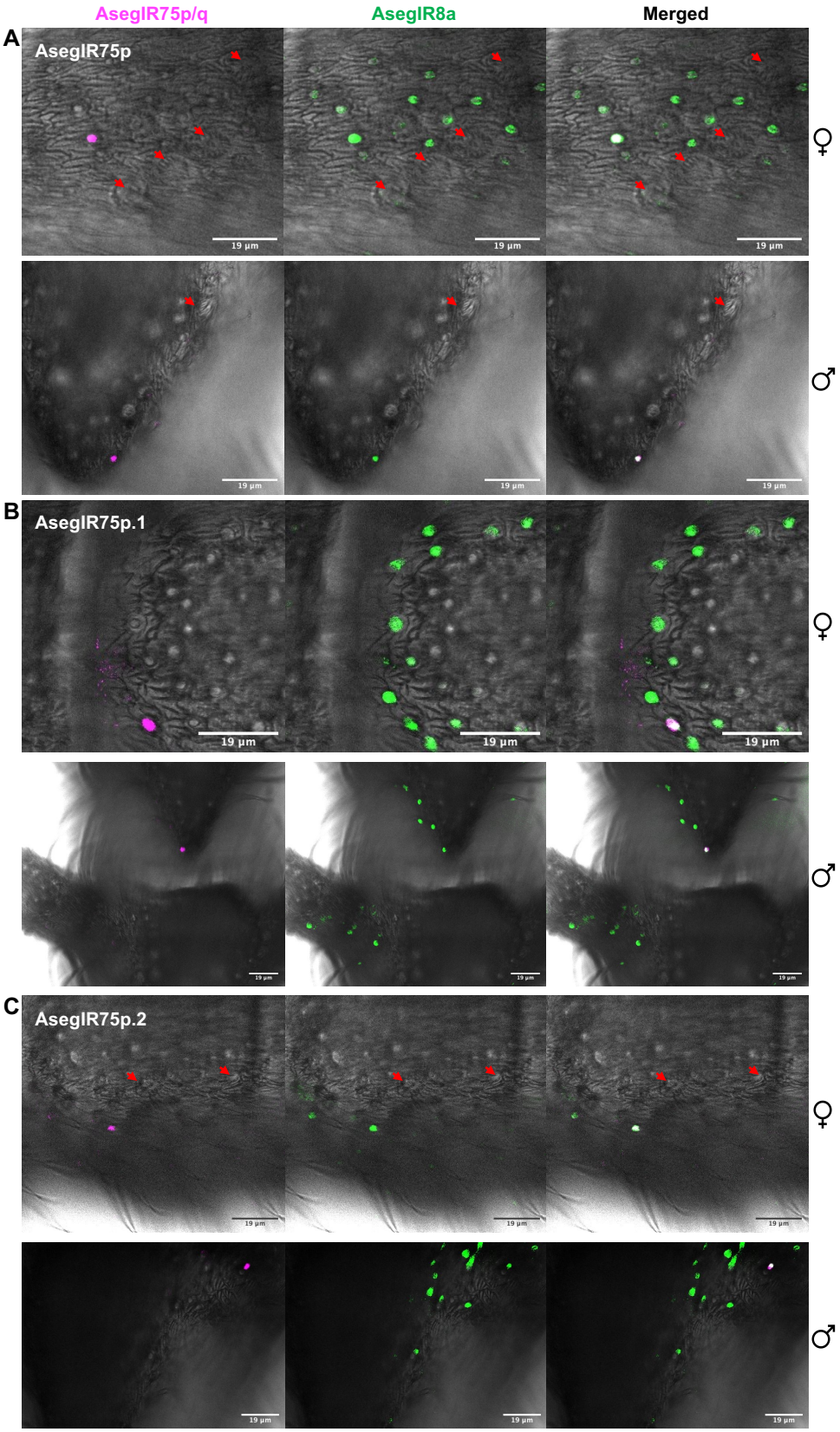
Fig. 2. The expression profiles of AsegIRs. (A) Heatmap with TPM value (Transcripts Per Million) showing the expression levels of the AsegIRs in different female and male antennal

842 samples, as well as ovipositor samples. **(B)** The expression of AsegIRs from IR75p and IR75q
843 lineages and the co-receptor IR8a in different tissues and sexes. M, male; F female; ant,
844 antennae; prob, proboscis; ovi, ovipositors; ab, abdomens; h+t, head and thorax (without
845 antennae); w, wings. As AsegIR75q.1 and AsegIR75q.2 are located on the same transcript, it
846 is not possible to distinguish the TPM value for each of them from the transcriptome data,
847 whereas the qPCR results demonstrated that both were more abundant than the other three
848 AsegIRs in the IR75p/q expansion.



849

850 **Fig. 3. Scanning electron micrographs of *A. segetum* antennae. (A)-(B)** Schematic diagrams
851 and **(C)-(D)** overall SEM images showing the sexually dimorphic structures of female and male
852 antennae of *A. segetum* moths. The distribution of coeloconic sensilla (yellow diamonds),
853 trichoid sensilla (magenta triangles) and basiconic sensilla (green circles) are illustrated in the
854 enlarged view of segments in the dashed frame. The stem part of male antennae, the branches
855 and unbranched part are labelled in the figure. **(E)-(F)** Antennal segments showing
856 representative coeloconic, trichoid, and basiconic sensilla. **(G)** Close-up view of a coeloconic
857 sensillum. **(H)** Coeloconic sensilla are absent on the branches of male antennae. **(I)** Close-up
858 view of trichoid sensilla and basiconic sensilla. The labelled number in the scales represents
859 the length between the first and last marks, and the distance between two marks represents a
860 tenth of the labelled number.

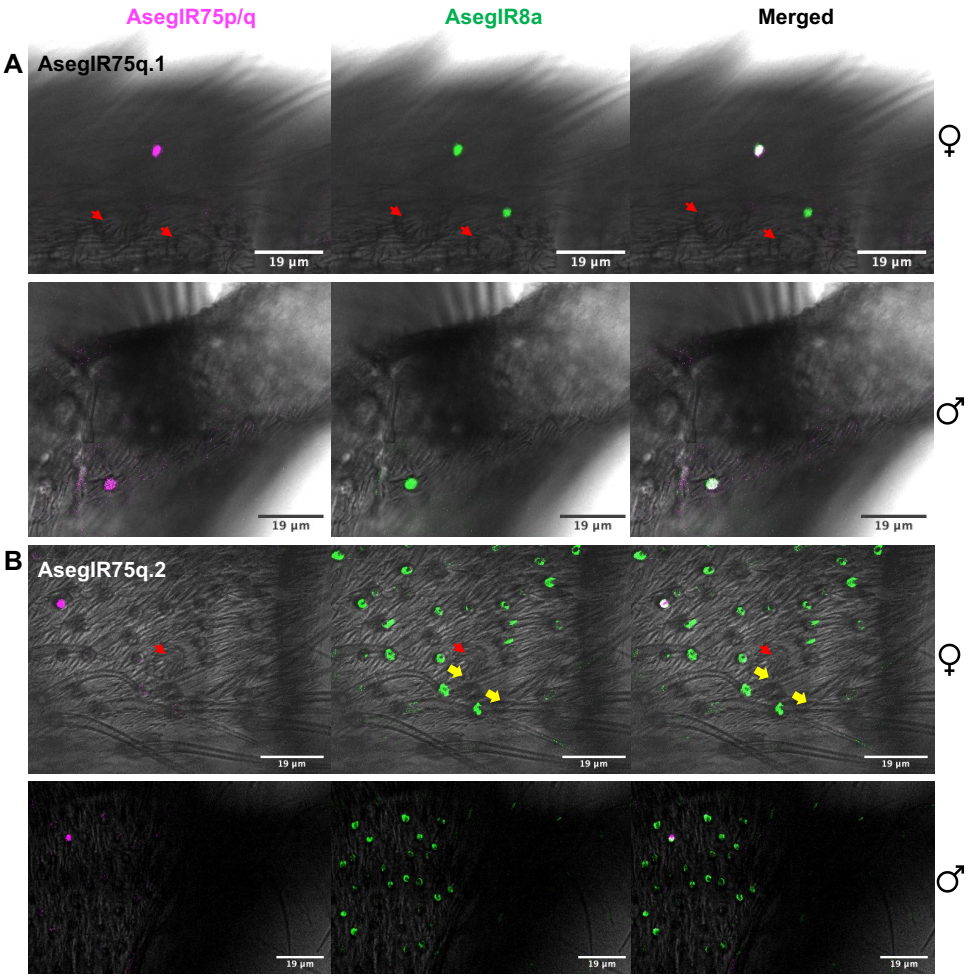


861

862 **Fig. 4. Whole-mount fluorescence *in situ* hybridization (WM-FISH) showing the co-**

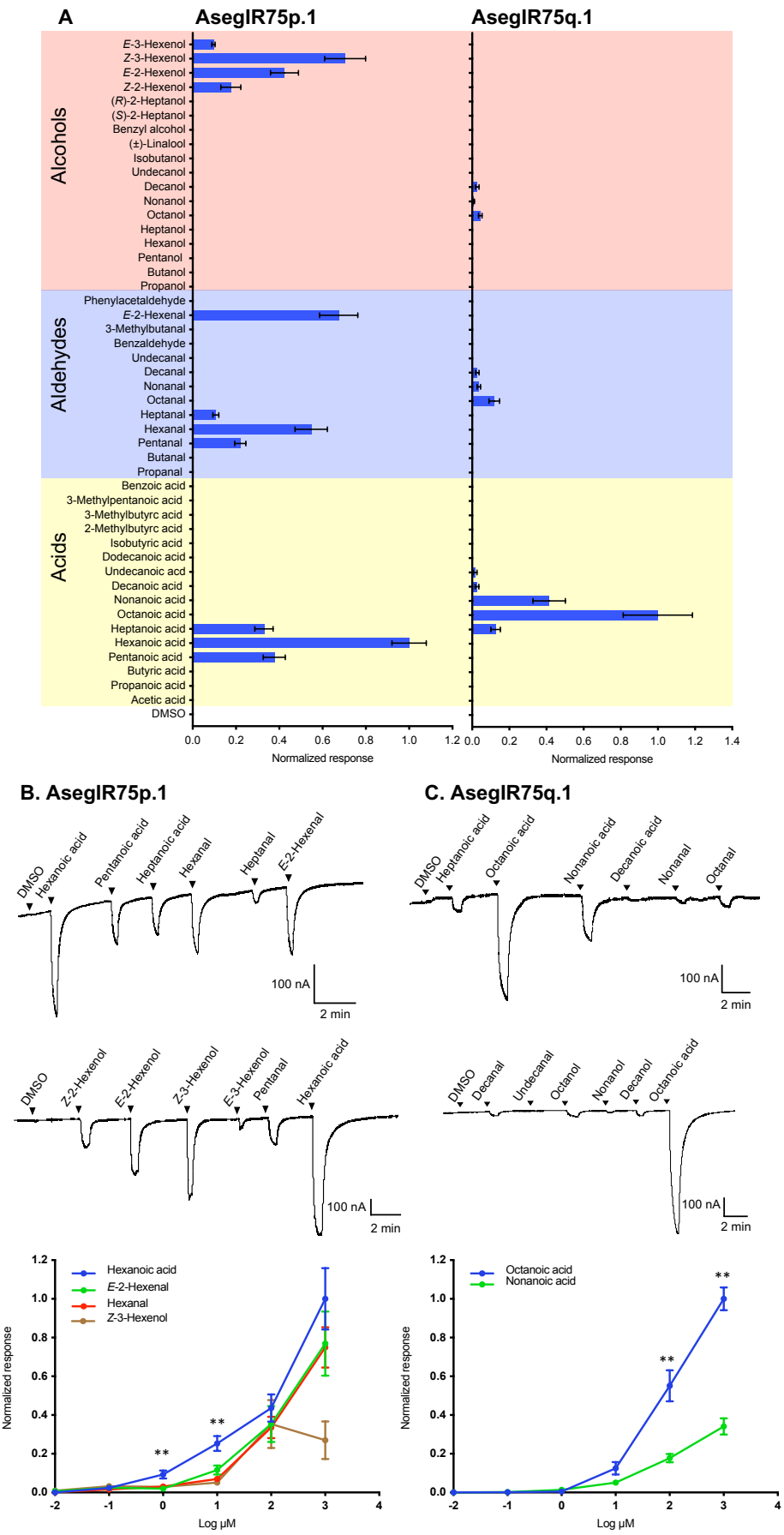
863 **expression of AsegIR8a with AsegIR75p (A), AsegIR75p.1 (B) and AsegIR75p.2 (C) on**

864 **adult female and male antennae**. Neurons expressing AsegIR8a are indicated in green,
865 AsegIR75p/p.1/p.2 signals are indicated in magenta, and the merged signals are shown in white.
866 Red arrowheads point to the location of coeloconic sensilla. The moths used in WM-FISH were
867 3-5 days old after eclosion and unmated.



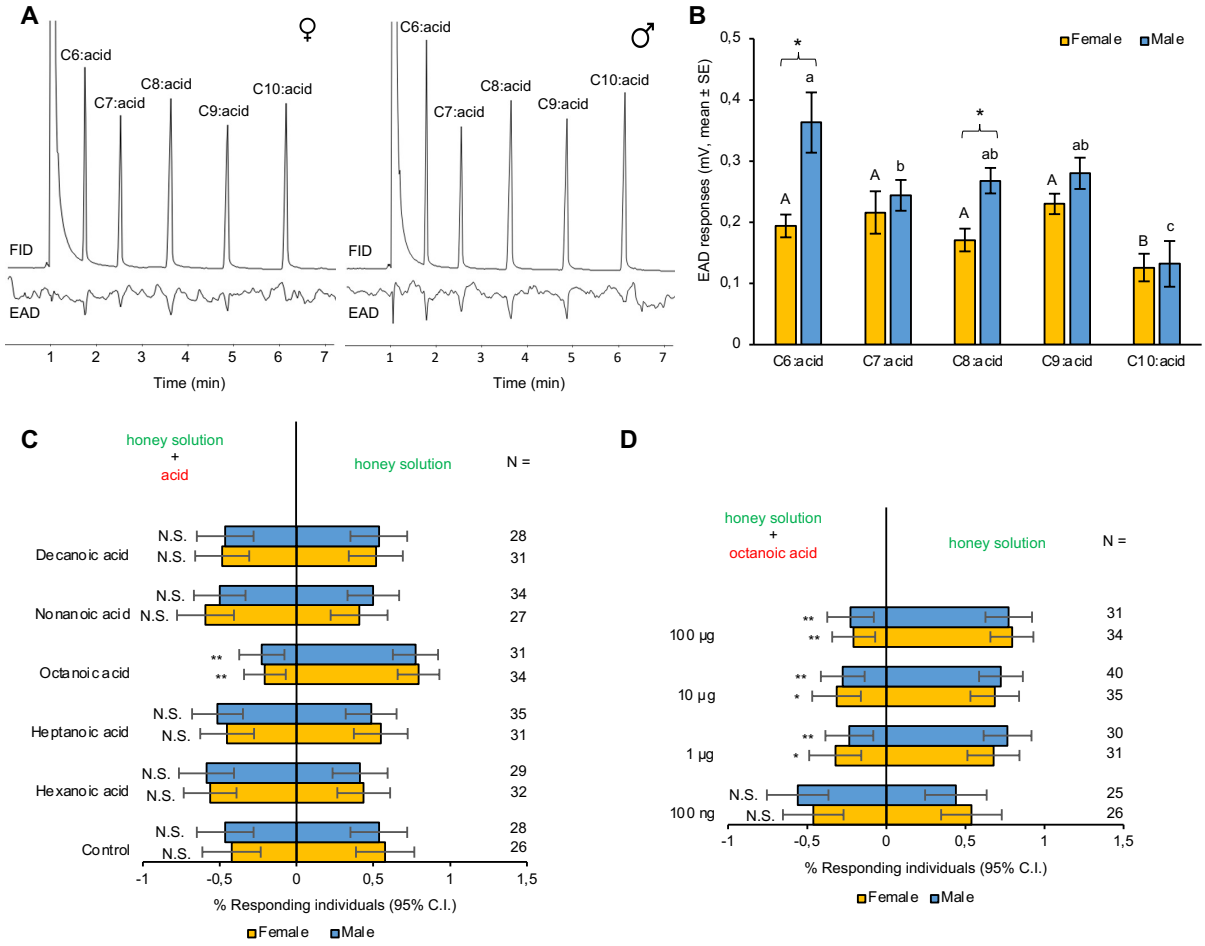
868

869 **Fig. 5. Whole-mount fluorescence *in situ* hybridization (WM-FISH) showing the co-**
870 **expression of AsegIR8a with AsegIR75q.1 (A) and AsegIR75q.2 (B) on adult female and**
871 **male antennae**. Cell bodies of neurons expressing AsegIR8a are indicated in green,
872 AsegIR75q.1/q.2 signals are indicated in magenta, and the merged signals are shown in white.
873 Red arrowheads point to the location of coeloconic sensilla, while the yellow arrows point to
874 the basiconic or trichoid sensilla with AsegIR expressing cell bodies below. The moths used in
875 WM-FISH were 3-5 days old after eclosion and unmated.


A
AseglR75p.1
AseglR75q.1
Alcohols
Aldehydes
Acids
E-3-Hexenol
Z-3-Hexenol
E-2-Hexenol
Z-2-Hexenol
(R)-2-Heptanol
(S)-2-Heptanol
Benzyl alcohol
(±)-Linalool
Isobutanol
Undecanol
Decanol
Nonanol
Octanol
Heptanol
Hexanol
Pentanol
Butanol
Propanol
Phenylacetaldehyde
E-2-Hexenal
3-Methylbutanal
Benzaldehyde
Undecanal
Decanal
Nonanal
Octanal
Heptanal
Hexanal
Pentanal
Butanal
Propanal
Benzoic acid
3-Methylpentanoic acid
3-Methylbutyrc acid
2-Methylbutyrc acid
Isobutyric acid
Dodecanoic acid
Undecanoic acd
Decanoic acid
Nonanoic acid
Octanoic acid
Heptanoic acid
Hexanoic acid
Pentanoic acid
Butyric acid
Propanoic acid
Acetic acid
DMSO
0.0 0.2 0.4 0.6 0.8 1.0 1.2
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4
Normalized response
Normalized response
B. AseglR75p.1
C. AseglR75q.1
DMSO
Hexanoic acid
Pentanoic acid
Heptanoic acid
Hexanal
Heptanal
E-2-Hexenal
Octanoic acid
Nonanoic acid
Decanoic acid
Nonanal
Octanal
Z-2-Hexenol
Z-3-Hexenol
E-3-Hexenol
Decanal
Undecanal
Octanol
Nonanol
100 nA
2 min
Hexanoic acid
E-2-Hexenal
Hexanal
Z-3-Hexenol
Octanoic acid
Nonanoic acid
**
Normalized response
Log μM
-2 -1 0 1 2 3 4

876

877 **Fig. 6. AsegIR75p.1 and AsegIR75q.1 respond to medium-chain fatty acids in *Xenopus***
878 **oocytes. (A)** Response profiles of AsegIR75p.1 (left) and AsegIR75q.1 (right) to the full odor
879 panel. Response magnitudes were normalized to the average response of primary ligand (N ≥4).
880 Error bars indicate the SE. **(B)** and **(C)** Upper panel: Representative current traces of oocytes
881 upon successive exposures to 100 µM stimuli. Each compound was applied at the time
882 indicated by the arrowheads for 20 s. Lower panel: Dose-dependent responses, showing values
883 normalized to the average response of the most active compound at 100 µM (N ≥4 for each
884 ligand).



885

886 **Fig. 7. Antennal and behavioral responses of *A. segetum* to medium-chain fatty acids. (A)**
887 Representative electrophysiological responses of male and female *A. segetum* antennae to
888 medium-chain fatty acids. Top: GC trace of the saturated C_6-C_{10} acids abbreviated as 6:0, 7:0,

889 8:0, 9:0 and 10:0 (FID). Bottom: antennal responses to corresponding compounds (EAD). **(B)**
890 GC-EAD responses of male and female *A. segetum* to C_6-C_{10} acids. Values are amplitude of
891 EAD responses (mV, mean ± SE) after being normalized by corresponding FID peak
892 abundance. Statistically significant differences between stimuli within each sex are indicated
893 by letters (one-way-ANOVA followed by an LSD test at 0.05 level). Differences between males
894 and females responding to the same stimulus are indicated by asterisks after Student´s t-Test at
895 $p < 0.05$ level. N=6 for female, N=4 for male. **(C)** Behavioral responses of female and male
896 turnip moths in a Y-tube olfactometer to saturated fatty acids (with 6-10 carbon atoms) at the
897 100 µg dose, and **(D)** to octanoic acid at a series of doses from 100 ng to 100 µg. N value for
898 each dual choice is shown on the right. Error bars indicate 95% confidence interval. P-values
899 are based on chi-square tests: N.S., P>0.05; *, P<0.05; **, P<0.01.
900 **Additional file 1: Table S1.** Compounds used for characterization of *Agrotis segetum*
901 ionotropic receptors (IRs), including their purities and source information. **Table S2.** Primers
902 used in qPCR. **Table S3.** Primers used in gene cloning. **Fig. S1.** Additional representative FISH
903 images.
904