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Knockdown of a metathoracic scent gland desaturase enhances the production of (E)-4-oxo-2-hexenal and suppresses female sexual attractiveness in the plant bug *Adelphocoris suturalis*

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Abstract

Insect sex pheromones (SPs) are central to mate-finding behaviour, and play an essential role in the survival and reproduction of organisms. Understanding the roles, biosynthetic pathways and evolution of insect chemical communication systems has been an exciting challenge for biologists. Compared with Lepidoptera, little is known about the mechanisms underlying pheromone biosynthesis in Hemiptera. In this study, we isolated and characterized two new desaturase-like genes, termed *Asutdes1* and *Asutdes2*, from *Adelphocoris suturalis*, an important agricultural pest in China. Although the two genes encode an identical protein, Southern blot analysis revealed that they are duplicated genes. The *Asutdes2* transcript is more abundant than *Asutdes1* in the tissues tested, in particular the metathoracic scent gland and fat body. Silencing *Asutdes* expression in females by injecting double-stranded RNA (*dsAsutdes*) against a portion of the coding sequence shared by the two genes enhanced the production of (E)-4-oxo-2-hexenal, a component of the *A. suturalis* SP blend, and dramatically suppressed the sexual attractiveness of *A. suturalis* females. We conclude that *dsAsutdes* is associated with the SP biosynthetic pathway in *A. suturalis*.

Keywords: *Adelphocoris suturalis*, *desaturase*, sex pheromones biosynthesis, (E)-4-oxo-2-hexenal, RNA interference (RNAi).

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Introduction

Despite years of study, a complete understanding of the roles, biosynthetic pathways and evolution of insect chemical communication systems remains an ongoing challenge. In insects, sex pheromones (SPs) comprise a diverse group of chemical compounds that are central to mate-finding behaviour (Jurenka, 2004; Johansson & Jones, 2007). The chemical composition of the SP blends is highly specific even amongst closely related species (Symonds & Elgar, 2008), and in numerous species, changes in the SP profile that affect male attractiveness more broadly or differentially have been implicated as potential mechanisms underlying reproductive isolation and speciation (Phelan, 1992; Symonds & Elgar, 2008). Allelic variations in genes encoding SP biosynthetic enzymes that affect the composition of SPs and/or assortative mating have been well demonstrated in *Drosophila* (Coyne & Charlesworth, 1997; Chung *et al.*, 2014) and Lepidoptera (Roelofs *et al.*, 2002; Lassance *et al.*, 2010; Buček *et al.*, 2015). However, how SP chemical diversity arises is a long-standing problem.

Studies in Lepidoptera, Diptera, Coleoptera and Hymenoptera that focused on elucidating the chemical composition of SPs have revealed a diversity of chemical structures (Symonds & Elgar, 2008). SPs are typically blends of alcohols, aldehydes, ketones, acetates

and long-chain hydrocarbons (10–30C), many of which contain carbon–carbon double bonds in varied positions and spatial orientations (El-Sayed, 2016). Although the chemical backbone of most pheromones arises from general biosynthetic pathways, the observed diversity is generated by a series of enzyme-based substrate modifications that include chain shortening and elongation, desaturation, reduction, oxidation, acetylation, aromatization, decarboxylation, and hydrolysis (Tillman *et al*., 1999; Roelofs & Rooney, 2003). In moths, SPs are typically derived from fatty acid biosynthesis with pheromone biosynthetic pathway enzymes introducing various modifications including: fatty acid chain-shortening enzymes (Jurenka *et al*., 1994; Tabata & Ishikawa, 2005) that use β-oxidation to shorten the pheromone precursors, fatty acid desaturases (Roelofs *et al*., 2002; Moto *et al*., 2004; Xue *et al*., 2007; Wang *et al*., 2010; Fujii *et al*., 2011; Albre *et al*., 2012; Groot *et al*., 2014; Hagström *et al*., 2014) that insert carbon–carbon double bonds into the fatty acyl chains, and fatty acid reductases (Moto *et al*., 2003; Lassance *et al*., 2013; Antony *et al*., 2016) that convert fatty acyl precursors to their corresponding alcohols. The oxygenated functional groups, however, can also be further modified by oxidases and acetyltransferases (Knipple *et al*., 2002; Jurenka, 2004). The molecular basis of lepidopteran pheromone biosynthesis has been effectively elucidated (Groot *et al*., 2016), and numerous studies have been conducted with the goal of similarly understanding the pheromone biosynthetic pathways in Diptera (Chertemps *et al*., 2006, 2007; González-Caballero *et al*., 2014; Ng *et al*., 2015) and Coleoptera (Sandstrom *et al*., 2006; Rubi *et al*., 2012; Keeling *et al*., 2013; Song *et al*., 2014). In contrast, despite numerous studies identifying hemipteran pheromone components and how different compounds affect insect behaviour, little is known about the underlying pheromone biosynthetic mechanisms.

Adelphocoris suturalis (Hemiptera: Miridae) is a polyphagous pest that was originally a secondary pest of cotton, but has since become a significant problem for cotton growing regions in China owing to a reduction in broad-spectrum insecticides following the widespread adoption of transgenic *Bacillus thuringiensis* (Bt) cotton (Lu *et al*., 2008a,; Li *et al*., 2010). Recently, two female metathoracic scent gland (MTG)-derived SP components, (E)-4-oxo-2-hexenal (E4O2H) and hexyl hexanoate (HH) were identified in *A. suturalis* (Zhang *et al*., 2014, 2016). Both compounds are synthesized and released by females around 7–10 days posteclosion (PE; Zhang *et al*., 2011). The structure of these semiochemicals is similar to moth SPs (ie carbon–carbon double bonds and oxygenated functional groups comprising aldehydes and acetates). Thus, it can be hypothesized that pheromone biosynthesis in *A. suturalis* is catalysed by homologues of moth pheromone biosynthetic enzymes. To identify potential *A. suturalis* SP biosynthesis-related genes, we mined previous *A. suturalis* MTG Illumina sequencing data (Luo *et al*., 2014) for transcripts with high sequence similarity to key moth pheromone biosynthesis enzymes. We identified four candidate genes that showed higher expression in female *A. suturalis* MTGs during the calling period: *fatty acyl-coenzyme A (CoA) reductase*, *desaturase* (*des*), *acetyltransferase* and *alcohol oxidase*. RNA interference (RNAi)-mediated silencing of *A. suturalis desaturase* (*Asutdes*) expression potently suppressed the sexual attractiveness of females to males in the field; we thus hypothesized that *Asutdes* functions in *A. suturalis* SP biosynthesis. To test this hypothesis, Y-tube bioassays, field trapping experiments and gas chromatography mass spectrometry (GC-MS) analyses were performed to investigate the effects of RNAi-mediated gene silencing. We showed that suppressing *Asutdes* expression enhanced E4O2H production, changed the ratio of the *A. suturalis* SP blend, and clearly suppressed the sexual attractiveness of *A. suturalis* females to males.

Results

cDNA cloning and sequence analysis

Based on the *A. suturalis* transcriptome data (Luo *et al*., 2014), we isolated two cDNAs encoding putative *Asutdes* sequences and designated them as *Asutdes1* and *Asutdes2*. Although the 5′ untranslated region of the 1863-bp (*Asutdes1*) and 1780-bp (*Asutdes2)* transcripts differed in size and sequence composition (Fig. 1A), both genes contained the same 1077-bp open reading frame (ORF) encoding the same 358 amino acid residue protein. Southern blot analysis was performed to determine if the two *Asutdes* transcripts were derived from two independent genes or a single gene that underwent alternative splicing. The presence of two distinct bands on the blot indicated that *Asutdes1* and *Asutdes2* are two independent genes (Fig. 1B).

The deduced Asutdes amino acid sequence shares 61% sequence similarity with the *Helicoverpa zea* acyl-CoA Δ9-desaturase (Fig. 2A) that catalyses formation of two minor but essential components of the *H. zea* pheromone blend (Rosenfield *et al*., 2001). High similarity was also observed with a *Choristoneura rosaceana* metabolic Z9-desaturase (60% similarity) that acts on palmitic acid (Hao *et al*., 2002), a *Trichoplusia ni* Δ9-desaturase (58% similarity) with high specificity for stearic acid (Liu *et al*., 1999), and the *Drosophila melanogaster* desaturase1 (58% similarity) responsible for the first desaturation step in fruit fly pheromone biosynthesis (Dallerac *et al*., 2000). Asutdes also exhibited 50% sequence similarity with the bifunctional *Bombyx mori* Z11/10,12 fatty-acyl

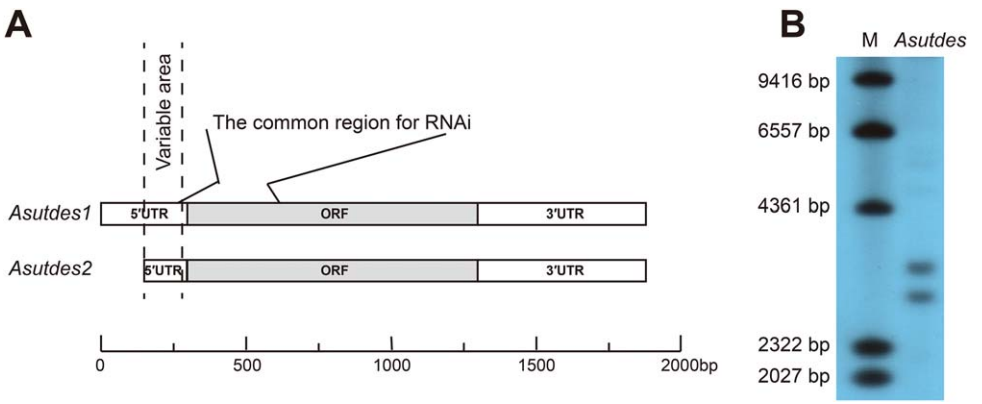

Figure 1. Gene structure of *Adelphocoris suturalis desaturase 1* (*Asutdes1*) and *Asutdes2*. (A) Schematic depiction of the gene structure for *Asutdes1* and *Asutdes2* with the regions selected for double-stranded RNA indicated. (B) A common region of the two *Asutdes* genes was labelled with digoxigenin and used as a probe for Southern blot analysis, in which *A. suturalis* genomic DNA was digested with *Hin*dIII-HF. The Southern blot analysis results showed that *Asutdes1* and *Asutdes2* are two independent genes. M, marker; ORF, open reading frame; RNAi, RNA interference; UTR, untranslated region. [Colour figure can be viewed at wileyonlinelibrary.com]

desaturase that functions in bombykol biosynthesis (Moto *et al*., 2004) (Fig. 2A).

The predicted Asutdes protein shares several key features with other known insect desaturases, including three conserved histamine motifs (H1–H3), which are crucial for spanning the membrane bilayer and catalytic activity (Shanklin *et al*., 1994; Haritos *et al*., 2014), and four transmembrane regions (Fig. 2A). To assess the phylogenetic relationship of Asutdes with other insect desaturases, a maximum-likelihood tree was generated using a representative set of desaturase sequences from 39 additional species. Asutdes sorted to a monophyletic clade with two plant bug desaturase sequences (Fig. 2B) away from the multiple desaturase subfamilies (ie Δ5, Δ6, Δ9, Δ10, Δ11, Δ12, Δ14) reported in moths, beetles and flies.

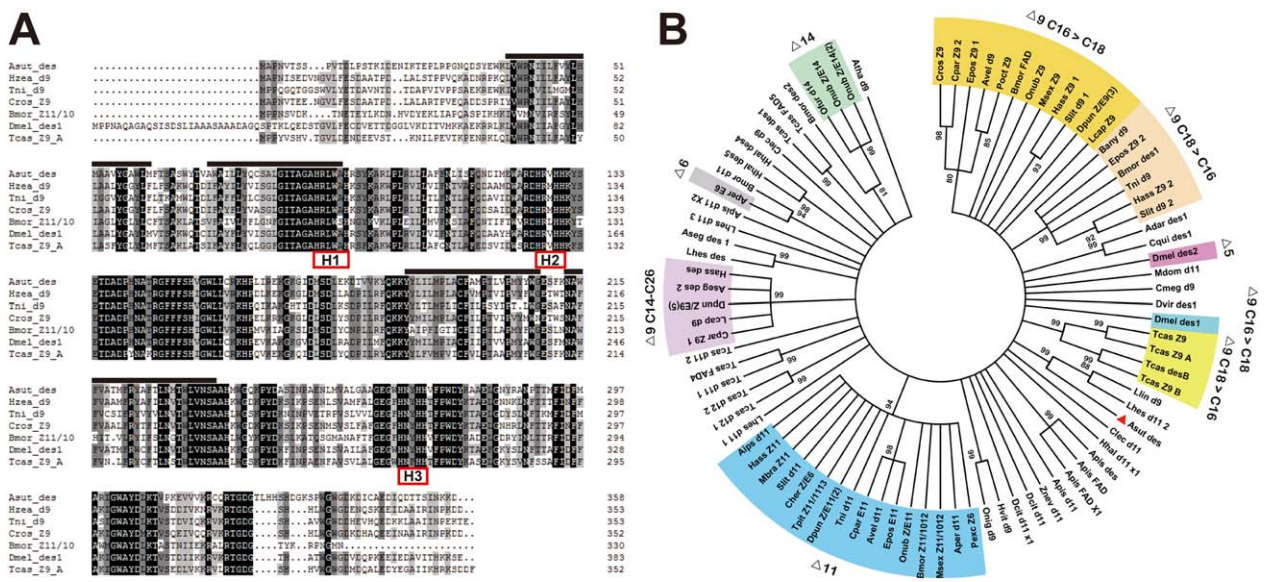

Figure 2. Sequence analysis of *Adelphocoris suturalis* desaturase (Asutdes). (A) Alignment of the predicted Asutdes protein and functional desaturase proteins from other insects. Black, grey and white backgrounds indicate amino acid conservation. The transmembrane domains are indicated by black lines above the sequence, and the three His domains are labelled H1–H3. Abbreviated species names correspond to: Asut, *A. suturalis* [Accession no. KX765194]; Hzea, *Helicoverpa zea* (Accession no. AF272343). Tni, *Trichoplusia ni* (Accession no. AAB92583); Cros, *Choristoneura rosaceana* (Accession no. AAN39697) Bmor, *Bombyx mori* (Accession no. BAD18122); Dmel, *Drosophila melanogaster* (Accession no. NP_731711); Tcas, *Tribolium castaneum* (Accession no. ADK13054). (B) Phylogeny of *Asutdes* with other insect desaturases. The unrooted maximum-likelihood tree was obtained using 86 sequences from 40 species. The confidence values at the edges are derived from 1000 rapid bootstrap replicates. Bootstrap values > 80% are shown on branches. Sequence abbreviations correspond to species names (see Table S1) with Atha (*Arabidopsis thaliana*, Accession no. NP_172098) used as the outgroup. The red triangle denotes *Asutdes*. Accession numbers can be found in Table S1. [Colour figure can be viewed at wileyonlinelibrary.com]

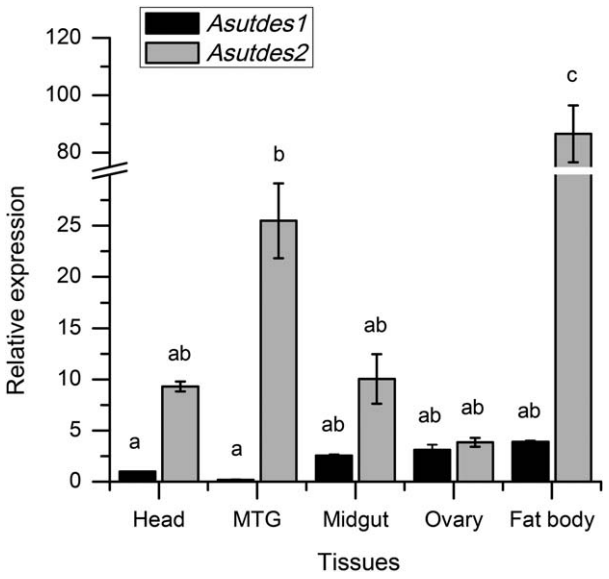

Figure 3. Tissue-dependent transcription pattern analysis of *Adelphocoris suturalis desaturase 1* (*Asutdes1*) and *Asutdes2*. Relative expression levels of *Asutdes1* and *Asutdes2* transcripts in the head, metathoracic scent gland (MTG), midgut, ovary and fat body of females 10 days posteclosion. The values are expressed as the means ± SEM based on three independent biological replicates. Different letters indicate significant differences ($P < 0.05$, one-way analysis of variance followed by Tukey's Honestly Significant Difference (HSD) multiple comparison).

Spatial transcript profiles

The spatial distribution of *Asutdes1* and *Asutdes2* in different tissues (head, MTG, midgut, ovary and fat body) of 10-day-old PE females (calling period) was examined to determine if a biased expression pattern might suggest potential functional roles for the enzyme. Despite encoding the same protein, the two *Asutdes* genes exhibited differential expression with *Asutdes1* expressed in midgut, ovary and fat body albeit at relatively low levels (Fig. 3). In contrast, *Asutdes2* was expressed at varying levels in all tissues examined with maximal expression in the fat body and MTG (Fig. 3). The nearly two orders of magnitude lower expression of the *Asutdes1* transcript in the MTG, the site of *A. suturalis* SP biosynthesis and release (Zhang *et al*., 2016), suggests that the *Asutdes2* gene may play a more important role in *A. suturalis* SP biosynthesis.

The relative expression of Asutdes *after RNAi-mediated knockdown*

To examine the *in vivo* role of *Asutdes* on SP biosynthesis, double-stranded RNA (dsRNA) corresponding to a fragment of the coding sequence shared between the two genes (Fig. 1A) was synthesized and injected into day 0 PE females. The impact of RNAi knockdown was studied at the transcriptional level by quantitative real time PCR (qRT-PCR), using RNA samples from the MTG and the fat body. These templates were chosen because the MTG is the site of SP synthesis and release (Zhang *et al*., 2014) and *Asutdes* was most abundant in the fat body, which plays an essential role in energy storage, metabolism and regulation in insects (Arrese & Soulages, 2010). Compared with the control females injected with dsRNA for green fluorescent protein (ds*GFP*), the dsRNA *Asutdes* (*dsAsutdes*) females had significantly reduced levels of mRNA encoding *Asutdes1*, *Asutdes2* and global *Asutdes* (both *Asutdes1* and *Asutdes2*) in the fat body at 3, 5, 7 and 10 days postinjection (PI) ($P < 0.05$), with transcript reduction ranging from 71–92% (Fig. 4C–E). In the MTG, the relative expression levels of *Asutdes2* and global *Asutdes* were significantly reduced by 45–68% at 3, 7 and 10 days PI ($P < 0.05$), but reduced by 27 and 15%, respectively, at 5 days PI relative to the *dsGFP* control (Fig. 4A, B). As *Asutdes1* transcript levels in control MTG are barely detectable, its RNAi-mediated silencing was essentially negligible. Gene knockdown in females at 7 days PI showed the best gene silencing efficiency, which coincides well with the reported calling period, 7–10 days PE, of *A. suturalis* females (Zhang *et al*., 2011). Based on these results, 7 days PI was selected as a suitable period for subsequent RNAi studies.

Knockdown of Asutdes *clearly suppressed the sexual attractiveness of* A. suturalis *females*

Mate-finding behaviour in many insects is largely influenced by pheromones. Consequently, to examine the effect of *Asutdes* silencing on the sexual attractiveness of *A. suturalis* females, both Y-tube olfactometer bioassays and field trapping experiments were performed. For the Y-tube olfactometer bioassay, a total of 174 virgin males (7 days PE) were tested. Given a choice between control females injected with *dsGFP* and females injected with *dsAsutdes*, males exhibited a statistically significant higher preference for the control females (66.7%, $\chi^2 = 16.67$, $\mathrm{df} = 1$, $P < 0.01$; Fig. 5A). The impact of *dsAsutdes* knockdown on the sexual attractiveness of females was also tested in field trapping experiments in both 2015 and 2016. All of the plant bugs that were attracted by the *dsAsutdes*-injected and control females were males, with the number of males attracted significantly reduced in the *dsAsutdes*-injected females relative to the *dsGFP*-injected and untreated females by 34–53 and 46–59%, respectively ($P < 0.05$; Fig. 5B). The differences between *dsGFP*-injected and untreated groups were not significant (Fig. 5B). Taken together, knockdown of *Asutdes* had clear suppression effects on the sexual attractiveness of *A. suturalis* females in both laboratory and field test experiments.

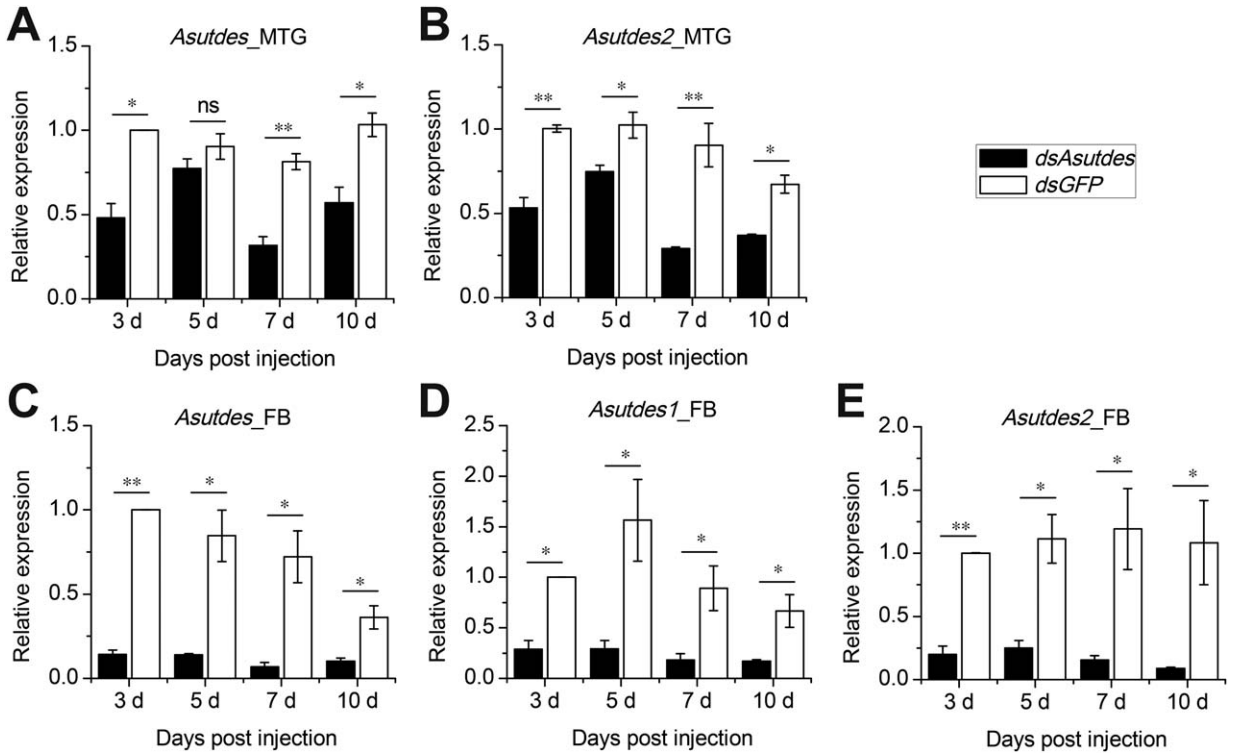

Figure 4. Relative mRNA abundance of *Adelphocoris suturalis desaturase* (*Asutdes*) following double-stranded RNA (dsRNA) injection. Virgin females at day 0 posteclosion (PE) were microinjected with either dsRNA for *green fluorescent protein* (*dsGFP*) or *dsAsutdes*, which corresponds to a fragment shared by the two *dsAsutdes* transcripts. After *dsAsutdes* injection, transcript levels in the metathoracic scent gland (MTG) (A, B) and fat body (FB) (C, D and E) were detected at 3, 5, 7 and 10 days postinjection to determine the knockdown efficiency of *Asutdes1* (D), *Asutdes2* (B, E) and both *Asutdes* transcripts, defined as global *des* (A, C). The values are expressed as the means ± SEM based on three independent biological replicates. The asterisks indicate statistical significance (*, $P < 0.05$; ** $P < 0.01$, Student's *t*-test).

Knockdown of Asutdes *altered the* A. suturalis *female pheromone titre*

Given the reduced female sexual attractiveness following *Asutdes* knockdown, we next examined the effect of *Asutdes* RNAi-mediated silencing on MTG pheromone components. GC-MS analysis of *dsAsutdes*-injected females revealed a notable increase in E4O2H compared with the *dsGFP*-injected controls ($P < 0.05$; Fig. 6C, D). In addition, there was also a slight but nonsignificant increase in HH levels in *dsAsutdes*-injected females ($P = 0.426$; Fig. 6C). The relative ratio between E4O2H and HH in *dsAsutdes*-injected females was 0.90:1 compared to 0.28:1 in the *dsGFP*-injected controls with a significant difference between the two groups ($P < 0.01$; Fig. 6C). Taken together, these results demonstrated that knockdown of *Asutdes* enhances the production of E4O2H and changes the ratio of the *A. suturalis* SP blend.

Discussion

The molecular mechanisms of pheromone biosynthesis have been studied extensively in Lepidoptera (Lassance *et al.*, 2010), Coleoptera (Keeling *et al.*, 2013), Diptera (Chertemps *et al.*, 2006) and Hymenoptera (Buček *et al.*, 2013). In contrast, little is known about the mechanisms underlying pheromone biosynthesis in Hemiptera. In this study, we isolated and characterized two new *desaturase*-like genes, *Asutdes1* and *Asutdes2*, from *A. suturalis*. RNAi-mediated suppression of *Asutdes* significantly enhanced the production of E4O2H, altered the *A. suturalis* SP blend ratio, and suppressed male attractiveness to *A. suturalis* females. These data strongly support a role for *Asutdes* function in *A. suturalis* SP biosynthesis.

Desaturases are the primary group of proteins responsible for introducing carbon–carbon double bonds at specific positions in fatty acyl substrates. They are found in various eukaryotes including animals, plants, yeast, fungi and many bacteria (Los & Murata, 1998; Sperling *et al.*, 2003). They are important for basic biological processes, including cell signalling, lipid metabolism and regulation of cell membrane fluidity (Hazel & Williams, 1990; Vigh *et al.*, 1993; Pyne & Pyne, 2000; Miyazaki & Ntambi, 2003). In insects, desaturases have been extensively studied based on their role in chemical communication and potential to contribute to speciation. To date, a number of desaturase genes have been shown to be

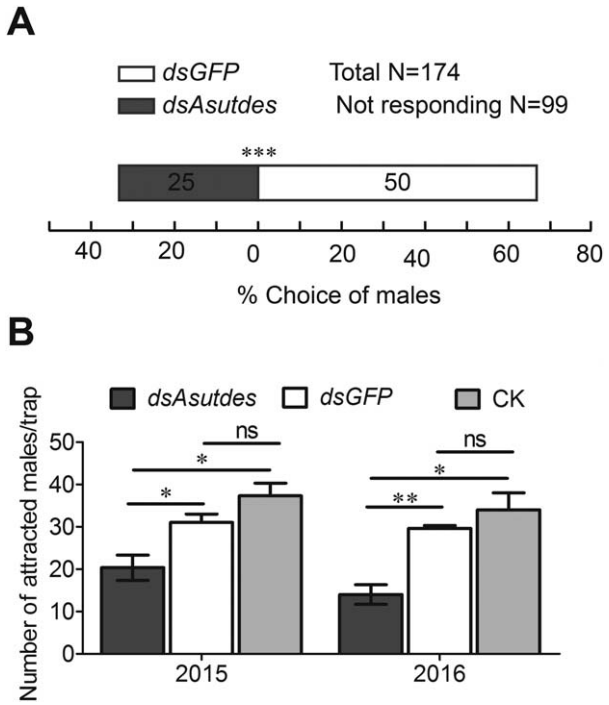

Figure 5. Effects of RNA interference (RNAi) treatment on the sexual attractiveness of *Adelphocoris suturalis* females. (A) Y-tube olfactometer behavioural response of *A. suturalis* males (7 days posteclosion) to females 7 days postinjection (PI) with either double-stranded RNA (dsRNA) for *A. suturalis desaturase* (*dsAsutdes*) or for *green fluorescent protein* (*dsGFP*). The number of males that did not make a choice within the 10-min time period is shown as 'not responding'. The total number of tested males was 174. The data were analysed using a chi-square test (***, $P < 0.001$); males that did not respond were not included in the statistical analysis. (B) The number of field-collected males attracted to dsRNA-injected *A. suturalis* females 7–10 days PI. For the RNAi treatment, females were injected with *dsAsutdes*, whereas control females were injected with *dsGFP* or not injected. The values are expressed as the means ± SEM based on three independent biological replicates. The asterisks indicate statistical significance (*, $P < 0.05$; **, $P < 0.01$, Student's *t*-test). CK, not injected.

involved in pheromone biosynthesis in many insects including moths (Roelofs & Rooney, 2003; Buček *et al*., 2015) and flies (Knipple *et al*., 2002; Buček *et al*., 2013). In these systems, the introduction of carbon–carbon double bonds at specific positions in pheromone precursors to generate pheromone components that vary in chain length, double-bond number, double-bond position and double-bond orientation contributes to the structural diversity in pheromone structures that drive species specificity (Knipple *et al*., 2002; Roelofs & Rooney, 2003). The two putative desaturase genes *Asutdes1* and *Asutdes2* that we identified appear to be the result of gene duplication rather than alternative splicing as Southern blots exhibited two distinct bands corresponding to each gene. Furthermore, the spatial distribution profiles of the two gene products revealed that *Asutdes2* was most abundant in the MTG and fat body. In contrast, the levels of *Asutdes1* transcripts were low in the five tissues assayed, in particular the MTG in which expression was lowest (Fig. 3). Based on this differential expression, we hypothesize that *Asutdes2* may play a more important role in *A. suturalis* SP biosynthesis.

Given the structural similarities between moth SPs and those described in *A. suturalis*, we hypothesized that the pheromone biosynthetic pathways might also be similar. We initially identified four genes in *A. suturalis* as candidate pheromone biosynthesis genes based on sequence homology with key enzymes in moth SP biosynthesis (Luo *et al*., 2014). In this study, we found that silencing *Asutdes* expression potently suppressed the sexual attractiveness of males to females in both laboratory (Y-tube olfactometer) and field conditions. Further analysis revealed that *Asutdes* knockdown significantly increased levels of E4O2H in the MTG and by doing so altered the *in vivo* ratio of the SP blend (ie E4O2H:HH) by threefold. In insects, the ratios of components in SP blends are highly species specific such that even slight changes have significant effects on male attractiveness (Phelan, 1992; Symonds & Elgar, 2008).

The pheromone biosynthetic pathways of many insects are derived from conventional metabolic pathways (Jurenka, 2004). For example, in moths, the backbones of most pheromone components are generated via fatty acid biosynthesis and then undergo various modifications prior to release (Knipple *et al*., 2002; Jurenka, 2004; Groot *et al*., 2016). In *D. melanogaster*, Z9-hexadecenoic is used as a precursor for both ω7 fatty acid biosynthesis and unsaturated hydrocarbon sex pheromones (Ueyama *et al*., 2005). Consequently, disruption of one part of the pathway typically results in the accumulation of compounds upstream of the affected site. Furthermore, SP biosynthetic pathways are controlled both by the flux of precursor products through the pathway and enzyme regulation (Lassance *et al*., 2010; Blaul *et al*., 2014). We thus speculate that the increase in E4O2H content might be an indication that *Asutdes* functions downstream of E4O2H. In this scenario, *Asutdes* would modulate the rate of substrate movement through the SP biosynthetic pathway by using either E4O2H or a compound downstream of E4O2H as a substrate. The biologically relevant ratio of E4O2H in the MTG would thus be maintained via the controlled flux of substrates, and conversely disruption of *Asutdes* function would impede the flux and trigger an accumulation of E4O2H and the associated disequilibrium of the E4O2H:HH ratio. This proposed function is similar to that of ipsdienol dehydrogenase in *Ips pini*, which oxidizes ipsdienol (a component of the SP in *I. pini*) to ipsdienone, which is then consumed to maintain the biologically active enantiomeric ratio of ipsenol (Rubi *et al*., 2012). A second potential mechanism for the observed biological effects is that *Asutdes* could function

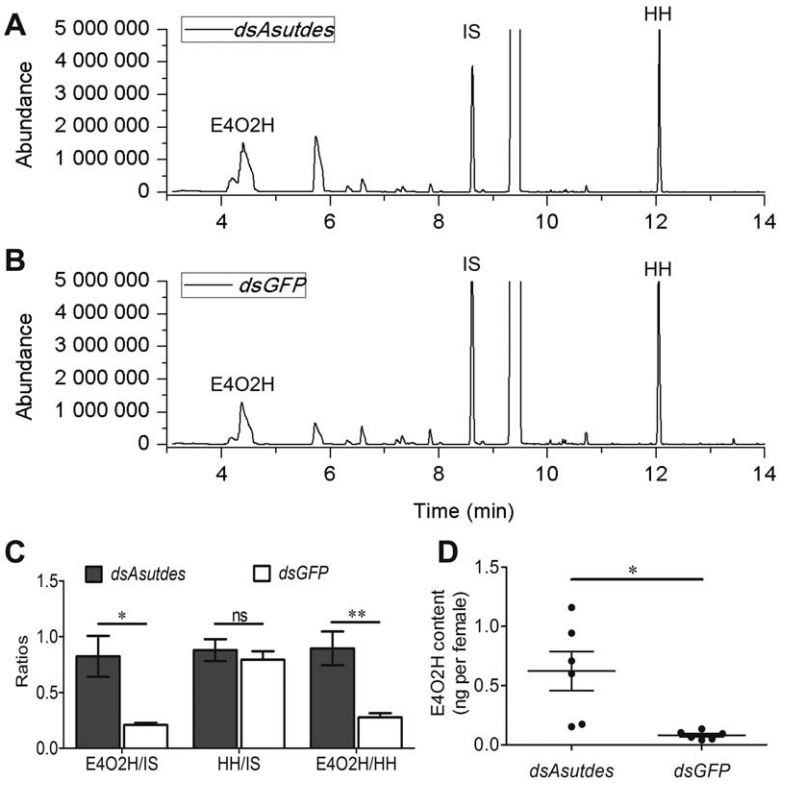

Figure 6. Effect of *Adelphocoris suturalis desaturase* (*Asutdes*) knockdown on *A. suturalis* female sex pheromone titres. Graphs represent gas chromatogram traces for total ion currents of *n*-hexane extracted metathoracic scent glands from *A. suturalis* females injected with double-stranded RNA for *Asutdes* (*dsAsutdes*; A) or for *green fluorescent protein* (*dsGFP*; B). The internal standard (IS) was cis-3-hexenyl isobutyrate. (C) Compared with females injected with *dsGFP*, there was a notable increase in the levels of (E)-4-oxo-2-hexenal (E4O2H) in extracts from *dsAsutdes*-injected females ($P < 0.05$). There was a significant difference in the relative E4O2H : hexyl hexanoate (HH) ratio between *dsAsutdes*-injected females and the *dsGFP* controls. The *y*-axis represents the relative abundance. (D) The total amounts of E4O2H were calculated with the internal standard method in ChemStation. All values are expressed as the means ± SEM based on six independent biological replicates. A Shapiro–Wilk test was used to test for normality ($P > 0.05$) with significant differences determined using Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$).

in a non-SP pathway that utilizes the same pool of precursors as SP biosynthesis such that *Asutdes* knockdown would result in an accumulation of precursors that would then be shunted exclusively into the SP pathway and converted at a greater rate than normal into E4O2H. Although these hypotheses are potentially verifiable by GC-MS, we did not observe any significant changes in the MTG chemical profile aside from the increase in E4O2H.

Alternatively, silencing *Asutdes* expression might lead to an overexpression of other genes that contribute to E4O2H production. Although the specific involvement of desaturases in this type of regulatory mechanism has not been reported, feedback loops are common. For example, in the migratory brown planthopper, *Nilaparvata lugens*, high *Nilaparvata lugens insulin receptor1 (NlInR1)* signalling activity inhibits *Nilaparvata lugens forkhead transcription factor Foxo (NlFoxo)* activity, leading to long-winged morphs. However, the binding of *NlInR1* to *NlInR2* compromises *NlInR1* signalling activity, which activates *NlFoxo* and results in short-winged morphs (Xu *et al*., 2015).

Although the precise mechanism underlying the role of *Asutdes* in *A. suturalis* remains to be clarified, our findings provide the first molecular evidence for specific gene function in plant bug SP biosynthesis. Although further exploration of how gene functionality impacts this critical pathway and complex communication systems in insects is challenging, ongoing advances in molecular techniques are increasing the feasibility. Consequently, future work will focus on exploring the potential molecular processes affected in response to *Asutdes* and elucidating the molecular mechanisms driving the *A. suturalis* SP biosynthetic pathway. Knowledge of these systems can then be applied to further our understanding of similar processes in other hemipteran pests.

Experimental procedures

Insect rearing

A. suturalis were originally collected from a Bt cotton field located in Wuhan (Hubei Province, China) in July 2014. Nymphs and adults were maintained in plastic cages (22.5 × 15 × 11 cm) and reared on a 5% sugar solution and green beans, which also served as oviposition substrates (Lu *et al*., 2008b). All insects were placed in environmental chambers maintained at 75 ± 5% relative humidity, 26 ± 2 °C temperature and a 16:8 h light : dark (L : D) cycle. The newly emerged adults were separated by sex daily and designated as 0 days PE. All of the experiments performed in this study used virgin females and males.

cDNA cloning and sequence analysis

Total RNA was isolated from *A. suturalis* females 7–10 days PE using a SV total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's protocol. RNA integrity was evaluated on 1.5% agarose gels and quantified using a

Table 1. PCR primers used in this study

Primers	Primer sequence	Product size (bp)	PCR efficiency (%)	Standard curve R^2
For cDNA cloning				
Asutdes1-F	CGGGCATCCGAGATTTCAC	1863	n.a.	n.a.
Asutdes1-R	ACAGAGCAAGAACGGTGGTG			
Asutdes2-F	GCGTCTCGCGTTTAAACTTTTC	1780	n.a.	n.a.
Asutdes2-R	ACAGAGCAAGAACGGTGGTG			
For real-time PCR				
Q-*Asutdes1*-F	TCCGAGATTTCACGTGTCGCA	127	99.5	0.997
Q-*Asutdes1*-R	AGCAAAATGCTGAAGTGACTCGAC			
Q-*Asutdes2*-F	CTTTTCACGCGCCCTGACCTG	145	94.0	0.997
Q-*Asutdes2*-R	GCAGGGGCTCCGTCTTGATG			
Q-*Asutdes*-F	GCTCCCTCTGAGATTGCTGC	130	97.6	0.999
Q-*Asutdes*-R	TGGTAGCGTTGTGAGGGTCG			
Q-*RPS15*-F	TGGTTGGCAGCATTGTCGGTG	137	92.6	0.997
Q-*RPS15*-R	ACCAATACCGGGCCTTCCGT			
Q-*EF1*γ-F	TTGGCCCTTGCTGCAGAACC	170	91.0	0.998
Q-*EF1*γ-R	TCTCCGAGCCAGATGGAGTAGTT			
For dsRNA synthesis				
dsAsutdes-F	gcgtaatacgactcactatagg (T7 promoter) CTATAGCGATGGCCCCCAAC	324	n.a.	n.a.
dsAsutdes-R	gcgtaatacgactcactatagg (T7 promoter) GCAATCTCAGAGGGAGCCTG			
dsGFP-F	gcgtaatacgactcactatagg (T7 promoter) TGGTCCCAATTCTCGTGGAAC	467	n.a.	n.a.
dsGFP-R	gcgtaatacgactcactatagg (T7 promoter) CTTGAAGTTGACCTTGATGCC			
For *Asutdes* probe synthesis				
p-*Asutdes*-F	CTCCCTCTGAGATTGCTGCT	599	n.a.	n.a.
p-*Asutdes*-R	ATTCTGGCCATGAAGTCGAT			

Asutdes, *Adelphocoris suturalis desaturase*; dsRNA, double-stranded RNA; *EF1*γ, *elongation factor-1*γ; *GFP*, *green fluorescent protein*; n.a., not applicable; *RPS15*, *ribosomal protein 15*.

Nano-Drop 2000 (Thermo Scientific, Wilmington, DE, USA). For first-strand cDNA synthesis, 1 μg RNA was reverse transcribed using PrimeScript™ RT Master Mix (perfect real time) (Takara, Kyoto, Japan). Full-length *Asutdes1* and *Asutdes2* were amplified using primers (Table 1) designed based upon *A. suturalis* transcriptome data (Luo *et al*., 2014), cloned into a pEASY-T1 Simple Cloning Kit (TransGen, Beijing, China) and sequenced. The ExPASy translate tool (http://web.expasy.org/translate/) was used to deduce the amino acid sequence of the protein encoded by the *Asutdes* ORF. The full-length *Asutdes* sequence and desaturase sequences from 40 insect species (accession numbers in Table S1) were used to construct a rooted phylogenetic tree with the maximum-likelihood method in MEGA 7.1 (Kumar *et al*., 2016). Statistical support for the tree was carried out using bootstrap analysis of 1000 replicates. The protein sequence alignments were formatted in DNAMAN 6.0 using the ClustalX colour scheme (Lynnon, San Ramon, CA, USA). The SMART webserver tool (http://smart.embl.de/) was used to predict functional domains. The *A. suturalis* cDNA sequences have been deposited with GenBank under the following accession numbers: *Asutdes1*, KX765193; *Asutdes2*, KX765194.

Southern blot

Genomic DNA was extracted from *A. suturalis* females 7–10 days PE using Insect DNA Kit (Omega Bio-tek, Norcross, GA, USA) and a 30 μg aliquot was digested for 48 h with *Hin*dIII-HF and electrophoresed on a 0.8% agarose gel. The separated DNA fragments were blotted onto a Hybond N+ nylon membrane (Millipore, Darmstadt, Germany) and a common region of the two *Asutdes* genes, labelled with digoxigenin, was used as a probe to hybridize with the digested genomic DNA. Southern hybridization was performed using a DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche, Mannheim, Germany), according to the manufacturer's instructions. The primers used to generate the *Asutdes* probe are listed in Table 1.

qRT-PCR

We performed qRT-PCR according to the Minimum Information for publication of Quantitative real time PCR Experiments guidelines (Bustin *et al*., 2010). All qRT-PCR reactions were performed in a 20 μl reaction volume using a 20-fold dilution of cDNA with qRT-PCR primers (Table 1) and SYBR® Premix ExTaq™ II (Takara) on a Bio-Rad Detection iQ2 System (Bio-Rad, Hercules, CA, USA). PCR conditions consisted of: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60–64 °C for 35 s. The qRT-PCR primers were designed using an online tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the specificity of the PCR products was verified by direct sequencing. PCR efficiencies (Table 1) were determined using standard curves generated from serially diluted cDNAs and the specificity of the reaction confirmed by melt curve analysis (Liu *et al*., 2016). Each reaction was performed in triplicate using at least three biological replicates per individual treatment. The qRT-

PCR data were analysed via the $2^{-\triangle\triangle Ct}$ method (Schmittgen & Livak, 2008).

Spatial expression pattern analyses

To investigate the spatial expression profile of the *Asutdes* transcripts, total RNAs were collected from the head, MTG, midgut, ovary and fat body of females 10 days PE (calling period; Zhang *et al.*, 2011). cDNA synthesis and mRNA quantification were performed as described above. *Ribosomal protein S15* was used as the reference gene for gene expression normalization (unpubl. data). The values are expressed as the means ± SEM. The data were analysed using one-way analysis of variance followed by Tukey's Honestly Significant Difference (HSD) multiple comparison test with SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Statistical differences are indicated by different letters in the figures.

RNAi in A. suturalis

dsRNAs corresponding to a 324-bp fragment of the coding region shared by the *Asutdes1* and *Asutdes2* genes were synthetized as described (Liu *et al.*, 2016). Similarly, *dsGFP* was synthesized and used as the control. dsRNA integrity was evaluated on 1.5% agarose gels and quantified on a Nano-Drop 2000 (Thermo Scientific). *A. suturalis* females at 0 day PE were microinjected with 2 μg dsRNA per 200 nl. Total RNAs from the MTG and fat body were extracted 3, 5, 7 and 10 days PI to determine the RNAi efficiency via qRT-PCR. *Elongation factor-1γ* was used as the reference gene for gene expression normalization (unpubl. data). Significant differences were determined using Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Based on these results, females 7 days PI were used in all subsequent bioassays.

Y-tube olfactometer bioassay

To evaluate the biological effect of *Asutdes* knockdown, the sexual attractiveness of *A. suturalis* females was assayed in a Y-tube olfactometer system. Mature males (7 days PE) were assayed for behavioural responses to odours from two experimental groups that consisted of five females injected 7 days prior with either *dsAsutdes* or *dsGFP*. The olfactometer set-up consisted of a Y-shaped Pyrex tube (Wuhan, China) (internal diameter 30 mm), with a 220 mm central stem and two 160 mm side arms set at a 90° angle. Air was moved through the system using silicon tubing attached to an air pump (Beijing Municipal Institute of Labor Protection, Beijing, China). An activated charcoal filter was used to purify the air, which was humidified by bubbling through distilled water prior to being split with a glass Y-connector. The air flow rate of each branch was set to 150 ml/min with a flow meter (Huangming, Zhejiang, China) and then passed through two modified jars (100 ml volume capacity), each containing the odour source, before being passed through the arms of the Y-tube. In each test, a single mature male was released at the entrance of the central stem of the Y-tube. All insects were reared under a photoperiod of 16:8 h (L : D) and maintained in the dark for at least 1–2 h after lights out. In the olfactometer set-up, movement of the tested males beyond the decision line (2 cm up either side arm) for a minimum of 20 s was recorded as a response, whereas males that failed to move within 10 min were recorded as 'no response' (Bodino *et al.*, 2016). Each male was tested only once to avoid any conditioned behaviour and the test odour sources were changed after every 10 tested males. To compensate for spatial asymmetry in the set-up, we switched the odour sources after testing four males. The Y-tube was cleaned thoroughly with hexane and baked before each individual test. All trials were performed at the same time (from 8 pm to 2 am). Olfactory bioassays were conducted in the dark (with a red light) at 26 ± 1 °C and 45 ± 10% relative humidity (Zhang *et al.*, 2011). The data were analysed using a chi-square test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$); males that did not respond were not included in the statistical analysis.

Field trapping experiments

Field trapping experiments were carried out in transgenic cotton fields in Wuhan (Hubei Province, China) during August of both 2015 and 2016. The number of adults assayed per 100 plants per year was 21 and 19, respectively. An equilateral-triangular-prism trap (16 cm on each side and 19 cm in height) was used for this study. Adhesive glue was painted on the inner surface of the traps to catch insects that entered. Three females injected with *dsAsutdes* (7 days PI) were placed in a small nylon cage (3.5 × 3.5 × 3.5 cm) and used as lures, with one young bud of cotton inside for female physical activity. The lures were hung in the middle of the traps, 2 cm above the base. Traps were placed randomly in the cotton field, 1.5 m above the ground and a minimum of 15 m apart (Zhang *et al.*, 2011). Females injected with *dsGFP* or that were untreated were used as controls. All traps were placed in the cotton field at 5 pm and removed at 7 am the next morning, at which time the number of trapped adults was recorded. Each female was tested only once. The field trapping experiments were performed for at least 1 week with three biological replicates performed for each individual treatment in the field. A Student's *t*-test was used to test for significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Pheromone extraction and GC-MS analysis

Extracts were prepared by placing 10 MTGs from dsRNA-injected females (7 days PI) in 100 μl *n*-hexane (Aladdin, Shanghai, China purity > 98%) for 30 min at room temperature with an internal standard, which consisted of 50 ng/ml cis-3-hexenyl isobutyrate (Sigma, St Louis, MO, USA, purity > 98%). MTG extracts were then stored at −20 °C until needed. GC-MS analyses were performed on an Agilent 7890B GC (Agilent Technologies, Palo Alto, CA, USA) coupled to a mass detector (Agilent 5977C) equipped with a HP-5 capillary column (30 m × 0.25 mm). Six independent biological replicates were performed for each treatment. GC-MS conditions consisted of: He as the carrier gas at 1 ml/min; 1 μl splitless injection; 220 °C injector temperature; thermal gradient of 50 °C for 1 min then 50 to 250 °C at 10 °C/min with termination at 280 °C for 2 min. Data were analysed using CHEMSTATION software (Agilent Technologies). HH was purchased from Sigma (purity > 98%) and E4O2H (purity > 95%) was donated from Tao Zhang (State Key Laboratory for Biology of Plant Diseases and

Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China). Chromatograms shown are representative of the extract analyses. A Shapiro–Wilk test was used to test for normality ($P > 0.05$) and a Student's *t*-test was used to test for significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. List of species names and corresponding published accession numbers for sequences used in the desaturase phylogeny in Fig. 2B.