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Identification and characterization of a fatty acyl reductase from a *Spodoptera littoralis* female gland involved in pheromone biosynthesis

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Abstract

Fatty acyl-CoA reductases (FARs), the enzymes that catalyse reduction of a fatty acyl-CoA to the corresponding alcohol in insect pheromone biosynthesis, are postulated to play an important role in determining the proportion of each component in the pheromone blend. For the first time, we have isolated and characterized from the Egyptian cotton leaf worm *Spodoptera littoralis* (Lepidoptera: Noctuidae) a FAR cDNA (Slit-FAR1), which appeared to be expressed only in the pheromone gland and was undetectable in other female tissues, such as fat body, ovaries, wings, legs or thorax. The encoded protein has been successfully expressed in a recombinant system, and the recombinant enzyme is able to produce the intermediate fatty acid alcohols of the pheromone biosynthesis of *S. littoralis* from the corresponding acyl-CoA precursors. The kinetic variables Km and Vmax, which have been calculated for each acyl-CoA pheromone precursor, suggest that in *S. littoralis* pheromone biosynthesis other biosynthetic enzymes (e.g. desaturases, acetyl transferase) should also contribute to the final ratio of components of the pheromone blend. In a phylogenetic analysis, Slit-FAR1 appeared grouped in a cluster of other FARs involved in the pheromone biosynthesis of other insects, with little or non-specificity for the natural pheromone precursors.

Keywords: fatty acyl-CoA reductase, *Spodoptera littoralis*, Egyptian cotton leaf worm, pheromone biosynthesis, enzyme kinetics, cDNA identification.

Abbreviations: 14:OH, 1-Tetradecanol; 15:OH, pentadecanol; 16:OH, hexadecanol; (Z)-11–14:OH, (Z)-11-tetradecenol; (E)-11–14:OH, (E)-11-tetradecenol; (Z)-9–14:OH, (Z)-9-tetradecenol; (Z,E)-9,11–14:OH, (Z,E)-9,11-tetradecadienol; THF, tetrahydrofuran; (Z,E)-9,11–14:SCoA, (Z,E)-9,11-tetradecadienoyl coenzyme A; (Z)-9–14:SCoA, (Z)-9-tetradecenoyl coenzyme A; (E)-11–14:SCoA, (E)-11-tetradecenoyl coenzyme A; (Z)-11–14:SCoA, (Z)-11-tetradecenoyl coenzyme A; FAR, fatty acyl-CoA reductase; pgFAR, pheromone gland fatty acyl reductase; GC, gas chromatography; MS, mass spectrometry; RT, reverse transcriptase; Km^{app}, apparent Michaelis constant; $Vmax^{app}$, apparent maximum rate.

Introduction

Pheromones are mixtures of long-chain aliphatic compounds with different degrees of unsaturations, and mostly comprise alcohols, aldehydes, acetate esters and hydrocarbons. Because of the long-chain nature of pheromone compounds, their biosynthesis has been described as closely related to fatty acid metabolism (Vogt, 2005). Indeed, many enzymes needed for pheromone biosynthesis, such as acetyl-CoA carboxylases, fatty acid synthase complex (including elongases) and fatty acid desaturases, are common in both fatty acid metabolism and pheromone biosynthesis pathways, although other enzymes appear to be specific in pheromone biosynthesis (Jurenka, 2003). The nature of these enzymes depends on the pheromone components, and includes desaturases, chain-shortening enzymes and functionalization enzymes, such as reductases, acetyl transferases and

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alcohol oxidases. These pheromone-specific enzymes have been suggested to be responsible for the final ratio of the different components of the pheromone blend (Roelofs & Jurenka, 1996). According to this hypothesis, the differences in affinity (represented by the kinetic variable Km) and the turnover (represented by the kinetic variable Kcat) of the enzyme to each pheromone precursor will lead to a different proportion of the components in the final pheromone blend.

Although very few data have been published on acetyl transferases and alcohol oxidases involved in pheromone biosynthesis, information regarding fatty acyl-CoA reductases (FARs) has been increasing in the last few years (Moto *et al*., 2003; Antony *et al*., 2009; Lassance *et al*., 2010; Liénard *et al*., 2010; Vogel *et al*., 2010; Hagström *et al*., 2013). Reduction of fatty acids into their corresponding alcohols is widespread in nature, and animals and plants use this reaction as a step in wax synthesis (Riendeau & Meighen, 1985). Although the mechanism of the FAR reaction is still unclear, different studies suggest that it occurs in a single step, in contrast to other fatty acid reductions that are performed in a two-step reaction, transferring two electrons in each step and producing an aldehyde as intermediate (Kolattukudy & Rogers, 1986). In addition to these mechanistic differences, FARs differ from other reductases in their subcellular localization. Thus, while enzymes responsible for the reduction of fatty acids to aldehydes have been identified as soluble proteins, FARs have been typically localized into the microsome fraction in animals and plants (Kolattukudy & Rogers, 1986; Metz *et al*., 2000). Recently, a study on the *Heliothis virescens* pheromone biosynthesis has demonstrated the endoplasmic reticulum localization of the pheromone gland FAR (pgFAR; Hagström *et al*., 2013).

The first FAR involved in the pheromone biosynthesis of a moth was identified in the silk moth *Bombyx mori* (Moto *et al*., 2003). The cDNA of *B. mori* FAR was amplified using a degenerated primer strategy, picking the human HMG-CoA reductase as a reference sequence. This allowed isolation of other cDNAs potentially involved in fatty acid reduction in the pheromone biosynthesis of other insects, such as *Ostrinia scapulalis* (Antony *et al*., 2009), *Ostrinia nubilalis* (Lassance *et al*., 2010), *Yponomeuta sp.* (Liénard *et al*., 2010) and *H. virescens* (Vogel *et al*., 2010). Despite the existence of ubiquitous FARs, some of the identified sequences exhibited a specific expression pattern, detected only in the pheromone gland. Few data have been published regarding the enzymatic characterization of insect FARs; however, some of the identified enzymes (including *B. mori* FAR) have been heterologously expressed and their activity has been studied *in vivo* (Antony *et al*., 2009; Lassance *et al*., 2010; Liénard *et al*., 2010; Hagström *et al*., 2013). A recombinant yeast expressing the *B. mori* FAR was able to produce pentadecanol (15:OH) and hexadecanol (16:OH) when the corresponding fatty acid precursors were added to the growth media; however, 17:Acid and 18:Acid were reduced to the corresponding alcohols in a very small percentage and 19:Acid and 20:Acid failed to be reduced, suggesting a chain-length specificity of the pgFAR (Moto *et al*., 2003). Substrate specificity was also observed in the orthologue of the European corn borer *O. nubilalis*, which showed a strong preference for either *Z* or *E* isomer, depending on the strain (Lassance *et al*., 2010). In contrast, other studies on *Yponomeuta evonymellus* FAR (YevoFAR) showed a multi-substrate activity profile of the pgFAR producing tetradecanol and 16:OH, saturated and/or mono-unsaturated, when the corresponding fatty acids were added to the growth media (Liénard *et al*., 2010).

The pheromone biosynthesis of the Egyptian cotton leaf worm *Spodoptera littoralis* (Lepidoptera: Noctuidae) has been studied (Muñoz *et al*., 2008). Although composition of the final pheromone blend depends strongly on the origin of each strain, all the biosynthetic pathways reported agree to consider palmitic acid as the first precursor of the pheromone biosynthesis. After chain-shortening by β-oxidation enzymes and unsaturation by different fatty acyl-CoA desaturases, the corresponding 14-carbon fatty acyl-CoA precursors are reduced to alcohols by a FAR, which are finally esterified by an alcohol acetyl transferase to the corresponding acetates of the pheromone blend. The final composition of the pheromone blend in our strain was (*Z,E*)-9,11–14:OAc (57%), (*E,E*)-10,12–14:OAc (14%), (*Z*)-9–14:OAc (11%), (*E*)-11–14:OAc (11%), (*Z*)-11–14:OAc (6%) and 14:OAc (1%) (Muñoz *et al*., 2008). As in many other moths, the possible candidates to modulate the final ratio of components in the *S. littoralis* pheromone blend are desaturases, a FAR, and an acetyl transferase. In the present study, we report the characterization and recombinant expression of a new specific FAR of *S. littoralis* pheromone biosynthesis, as well as *in vitro* studies to determine the kinetic parameters of the reactions leading to the alcohol precursors of the pheromone acetates. In addition, a phylogenetic analysis of the new FAR has been undertaken.

Results

Spodoptera littoralis *fatty acyl-CoA reductase identification*

We performed PCR using the degenerated primers FAR-for and FAR-rev, which yielded a DNA fragment of 294 bp. The amino acid sequence of this fragment pointed to a 97-residue protein with 51% identity to the corresponding region of the *B. mori* FAR sequence. The corresponding 5′ and 3′ extension yielded PCR bands of 849 and 812 bp

respectively, turning into a construction of 1602 bp that encompassed an open reading frame (ORF) of 1362 bp. The entire Pfu amplified ORF was named Slit-FAR1 and submitted to GenBank with the accession number HG423128. The translated amino acid sequence of Slit-FAR1 corresponded to a protein of 454 residues with a theoretical molecular weight of 51.4 kDa and isoelectric point of 8.9 (Fig. 1). The sequence displayed 42% identity with the *B. mori* FAR (Moto *et al.*, 2003) and contained the consensus NAD(P)H-binding motif VFITGGTGFLG (Aarts *et al.*, 1997) at the N-terminus between residues 23 and 33 (both included). Considering the accepted idea that FARs are membrane-associated enzymes (Metz *et al.*, 2000; Hagström *et al.*, 2013), the sequence was analysed using the transmembrane domain prediction software TMHMM (Krogh *et al.*, 2001) and TMPRED (Hofmann & Stoffel, 1993). Both algorithms predicted three putative transmembrane domains between residues 24 to 44, 256 to 275 and 360 to 376; however, only the TMPRED results rated them as significant, whereas TMHMM scored only the region between positions 360 and 376 close to the threshold of significance.

Tissue distribution and phylogenetic analysis

The presence of the transcript in different tissues of the insect was evaluated by semi-quantitative PCR using total cDNA of different parts of the animal (Fig. 2). The amplification level of the *rpL8* gene, used as a control gene, was visibly constant in all analysed tissues, as expected. Under these conditions, Slit-FAR1 appeared to be expressed only in the pheromone gland and was undetectable in other female tissues such as the fat body, ovaries, wings, legs or thorax. The transcript also appeared to be sex-specific as no amplification band was found in the entire male abdomen.

Neighbour-joining analysis of all lepidopteran FAR sequences published to date (including Slit-FAR1) resulted in a phylogenetic tree (Fig. 3) with a big cluster (in red) containing FARs involved in the biosynthesis of the corresponding pheromone components. It is noteworthy that while most of the clusters of the phylogenetic tree consisted of mixtures of orthologues from different species, the groups encompassed in the big cluster with the FARs involved in pheromone biosynthesis were rather homogeneous with respect to the species. Slit-FAR1 appeared to be encompassed in a small cluster, supported by a bootstrap value of 100%, containing sequences of FARs of *Helicoverpa assulta*, *H. virescens* and *Heliothis subflexa*, with 72–74% pairwise amino acid identity.

Chemical synthesis of the acyl-CoA thioesters

After purification by high-performance liquid chromatography, the acyl-CoA thioesters were obtained with the following yields and purity ≥99%: (*Z,E*)-9,11-tetradecadienoyl coenzyme A [(*Z,E*)-9,11–14:SCoA]: 39% yield. HRMS Calcd for $C_{35}H_{57}N_7O_{17}P_3S$ (M^+): 972.2745; Found: 972.2742; (Z)-9-tetradecenoyl coenzyme A [(*Z*)-9–14:SCoA]: 45% yield. HRMS Calcd for $C_{35}H_{59}N_7O_{17}P_3S$ (M^+): 974.2901; Found: 974.2894; (*E*)-11-tetradecenoyl coenzyme A [(*E*)-11–14:SCoA]: 42% yield. HRMS Calcd for $C_{35}H_{59}N_7O_{17}P_3S$ (M^+): 974.2901; Found: 974.2922; (*Z*)-11-tetradecenoyl coenzyme A [(*Z*)-11–14:SCoA]: 40% yield. HRMS Calcd for $C_{35}H_{59}N_7O_{17}P_3S$ (M^+): 974.2901; Found: 974.2889.

Protein expression and kinetic assays

The construction pESC-SlitFAR1 was successfully obtained by cloning SlitFAR1 into a pESC-URA vector under the control of a GAL1 promoter. After transforming pESC-SlitFAR1 into the YPH501 strain of *Saccharomyces cerevisiae*, both the presence of the vector and the integrity of the sequence were verified by PCR. The recovery of total protein was higher when the culture was harvested and disrupted under an exponential grow phase, yielding 18 mg of protein per 100 ml of culture. It is noteworthy that the obtained protein extract included the microsomal fraction, as the centrifugation force used during the clearance step (18 000 *g*) was far below that required for microsome separation (Schneider & Hogeboom, 1950).

In a preliminary step for the kinetic characterization, the activity of the recombinant protein Slit-FAR1 was explored by incubating (*Z,E*)-9,11–14:SCoA; (*Z*)-9–14:SCoA and 14:SCoA in the presence of either the recombinant yeast or the same strain of *S. cerevisiae* transformed with the vector pESC-URA. Once the absence of activity was confirmed in the control extract, all the pheromone precursors were evaluated as substrates. As shown in Fig. 4, in all cases the corresponding alcohol was detected in the reaction performed with the recombinant yeast; however, in addition to the expected products, other compounds identified by gas chromatography (GC)-mass spectrometry (MS) as isomers (*E,E*)-9,11-tetradecanol (14:OH), (*E*)-9–14:OH, (*E*)-11-tetradecenol [(*E*)-11–14:OH], and (*Z*)-9-tetradecenol [(*Z*)-9–14:OH] were also detected from incubations with (*Z,E*)-9,11–14:SCoA, (*Z*)-9–14:SCoA, (*Z*)-11–14:SCoA and (*E*)-11-tetradecenoyl coenzyme A [(*E*)-11–14:SCoA], respectively (see Fig. S1 for chromatogram of the incubation with the dienyl CoA). These minor components were not present in the original stereomerically pure acyl-CoA substrates, suggesting that some isomerization took place in the incubation process.

The integrity of the protein extract after freezing was verified by evaluating the activity of a fresh extract in comparison to that of a frozen one. To this end, 200 μM of

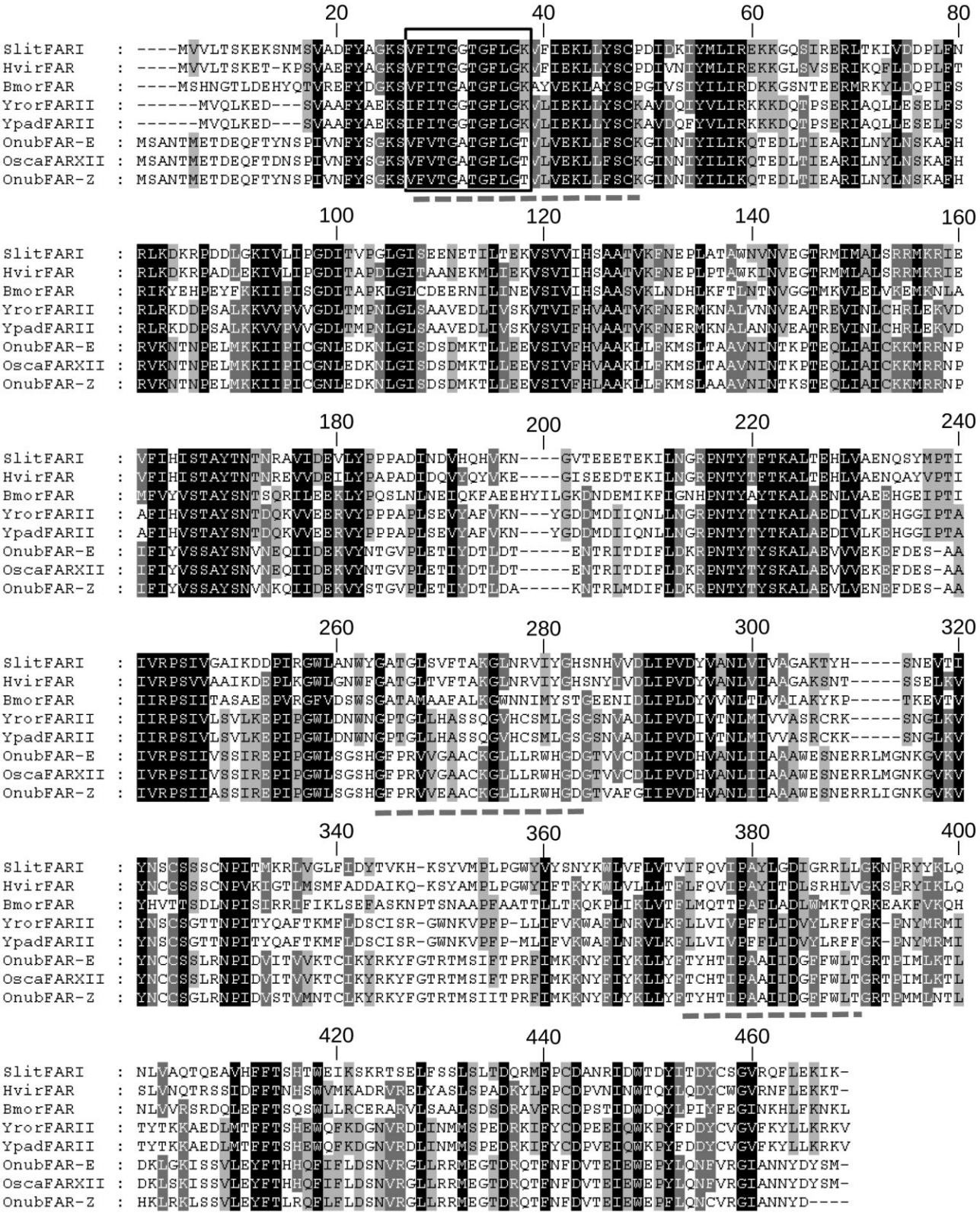

Figure 1. Alignment of SlitFAR1 with other pheromone gland fatty acyl reductases from Lepidoptera: HvirFAR from *Heliothis virescens* (GenBank accession no. ACX53790); BmorFAR from *Bombyx mori* (NP_001036967); YrorFARII from *Yponomeuta rorellus* (ADD62441); YpadFARII from *Y. padellus* (ADD62442); OnubFAR-E from *Ostrinia nubilalis E* strain (FJ807735); OscaFARXIII from *Ostrinia scapulalis* (ACJ06520); and OnubFAR-Z from *O. nubilalis Z* strain (FJ807736). The consensus NAD(P)H-binding motif is boxed and the predicted transmembrane domains are underlined.

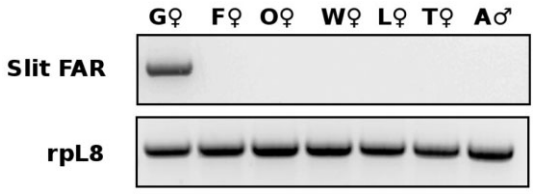

Figure 2. Detection by semi-quantitative PCR of the Slit-FAR1 transcript in different parts of the body of *S. littoralis*. G: pheromone gland, F: fat body, O: ovaries, W: wings, L: legs, T: thorax and A: abdomen. The ribosomal protein rpL8 was used as a control gene to verify the homogeneity of the cDNA samples.

(*Z,E*)-9,11–14:SCoA was incubated in the presence of either fresh or frozen extract under initial velocity conditions, yielding 21.1 ± 1.7 μM and 20.4 ± 1.5 μM of the corresponding dienol, respectively.

The kinetic values of the enzyme were determined at different concentrations of the substrate (0–80 μM), after optimization of the incubation time (10 min). The enzyme displayed a Michaelis–Menten profile for all pheromone precursors, showing, however, a depletion of the enzyme activity at high concentration of substrate (Fig. 5). The inhibition was observed with all precursors at a substrate

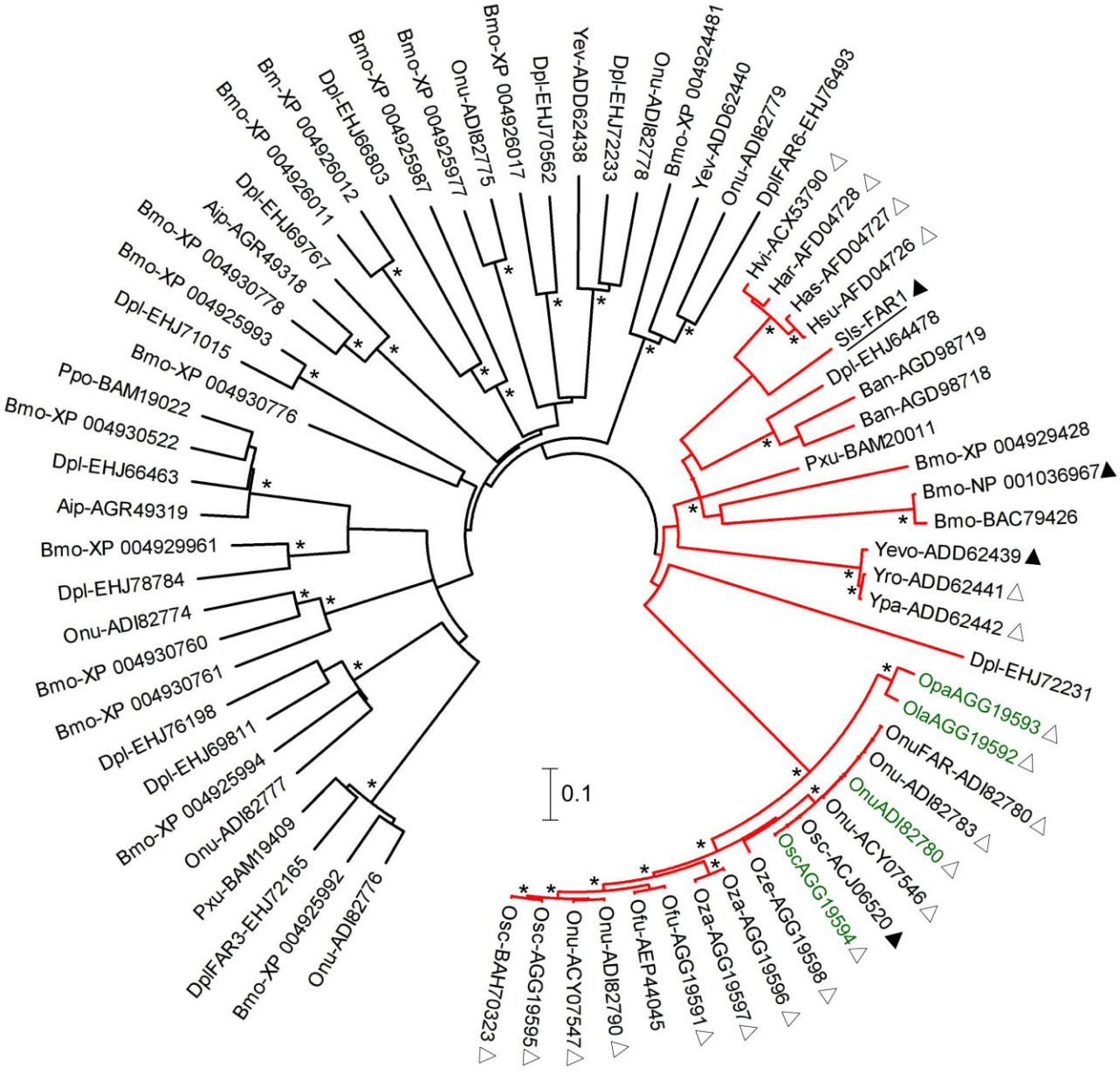

Figure 3. Phylogenetic analysis of lepidoptera FARs. Branches corresponding to the group of Slit-FAR1 (underlined) are in red. Functionally verified proteins are marked with a triangle (full triangle indicate also proven pheromone gland specificity) and FARs with substrate specificity are in green. Asterisks indicate a bootstrap value ≥90%. See Table S2, Supporting Information for the species abbreviations.

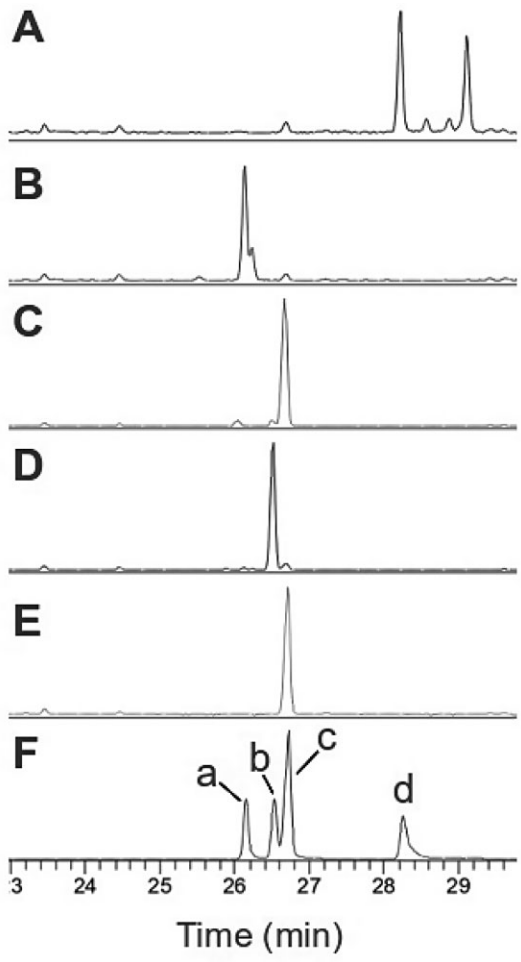

Figure 4. A–E: Total ion chromatograms of the extracts obtained after incubation of SlitFAR1 in the presence of different pheromone precursors: A: (*Z,E*)-9,11-tetradecadienoyl coenzyme A; B: (Z)-9-tetradecenoyl coenzyme A; C: (*Z*)-11-tetradecenoyl coenzyme A; D: (*E*)-11-tetradecenoyl coenzyme A; E: 14:SCoA. F: Chromatogram corresponding to the synthetic N,O-bis-(trimethylsilyl)trifluoroacetamide-silanized standard alcohols used as reference: A: (*Z*)-9-tetradecenol; B: (*E*)-11-tetradecenol; C: (*Z*)-11-tetradecenol + 14:OH; D: (*Z,E*)-9,11-tetradecadienol.

concentration > 40 μM for (*Z,E*)-9,11–14:SCoA, 20 μM for (*Z*)-9–14:SCoA and (*Z*)-11–14:SCoA, and 10 μM for (*E*)-11–14:SCoA and 14:SCoA. The apparent Michaelis constant (Km^{app}), apparent maximum rate ($Vmax^{app}$) and catalytic efficiency of the enzyme, calculated by nonlinear regression, were in the ranges 2.5–7.7 μM, 4.0–7.8 mM/s and 6.9–17.7 $\times$ 10^{-4}/s, respectively (Table 1).

Discussion

Since the identification of the *B. mori* FAR (Moto *et al.*, 2003), the cDNA of other FARs has been isolated from pheromone glands of different Lepidoptera. The analysis of pheromone gland expressed sequence tag libraries in some species revealed the presence of several FARs (Antony *et al.*, 2009; Vogel *et al.*, 2010; Gu *et al.*, 2013); however, in those species wherein FAR expression was studied in different tissues, only few sequences turned out to be specific or mostly expressed in the pheromone gland (Antony *et al.*, 2009; Gu *et al.*, 2013). In *S. littoralis*, the deduced amino acid sequence of Slit-FAR1 contained 454 residues, consistent with the length of other FARs identified in pheromone glands and functionally characterized (from 449 residues for *Ypenomeuta sp.* FARs to 462 for those of *Ostrinia sp.*), and included the NAD(P)H cofactor-binding motif observed in FARs.

When compared with other lepidopteran FARs, Slit-FAR1 appeared to be encompassed in a big cluster containing other FARs with proven capacity to reduce the pheromone acyl-CoA precursors to the corresponding pheromone alcohols. Substrate specificity did not seem to have a strong influence in phylogenetic grouping, as the four sequences with proven specificity for a particular substrate appear grouped in separate sub-clusters, and the average divergence of those sequences (10.6%) was close to that found among all *Ostrinia* FARs (8.1%) (Lassance *et al.*, 2013). Substrate preferences also seemed to have little influence in phylogenetic grouping. Thus, the amino acid sequence of Slit-FAR1 appeared to be closer to the FARs of *Heliothis* and *Helicoverpa sp.* (belonging to the Noctuidae family as *S. littoralis*), although the pheromone precursors of *S. littoralis* are structurally closer to those of *Ostrinia sp.* (mostly monounsaturated 14-carbon fatty acyl-CoAs), than those of *Heliothis* and *Helicoverpa sp.* (mostly 16-carbon fatty acyl-CoAs).

Overall, the results obtained in our phylogenetic analysis suggest that sequences with proven implication in pheromone biosynthesis are grouped according to the evolutionary distance between species, rather than to the FAR characteristics. Considering the lack of functional information available in most of the analysed sequences, however, any interpretation of the phylogenetic analysis must be taken cautiously.

Very little structural information of reductases is available, but subcellular localization studies of the *H. virescens* FAR have shown that this enzyme is located in the endoplasmic reticulum (Hagström *et al.*, 2013), in agreement with the general accepted hypothesis that FARs are membrane-associated proteins (Kolattukudy & Rogers, 1986; Metz *et al.*, 2000). To study the presumed membrane localization of Slit-FAR1, the obtained amino acid sequence was analysed by two transmembrane domain prediction software systems. As cited, three regions were identified by both algorithms as potential transmembrane helices; however, only the region comprised between positions 360 and 376 was scored over the significant threshold. Remarkably, the first domain to be predicted as a possible transmembrane helix (residues

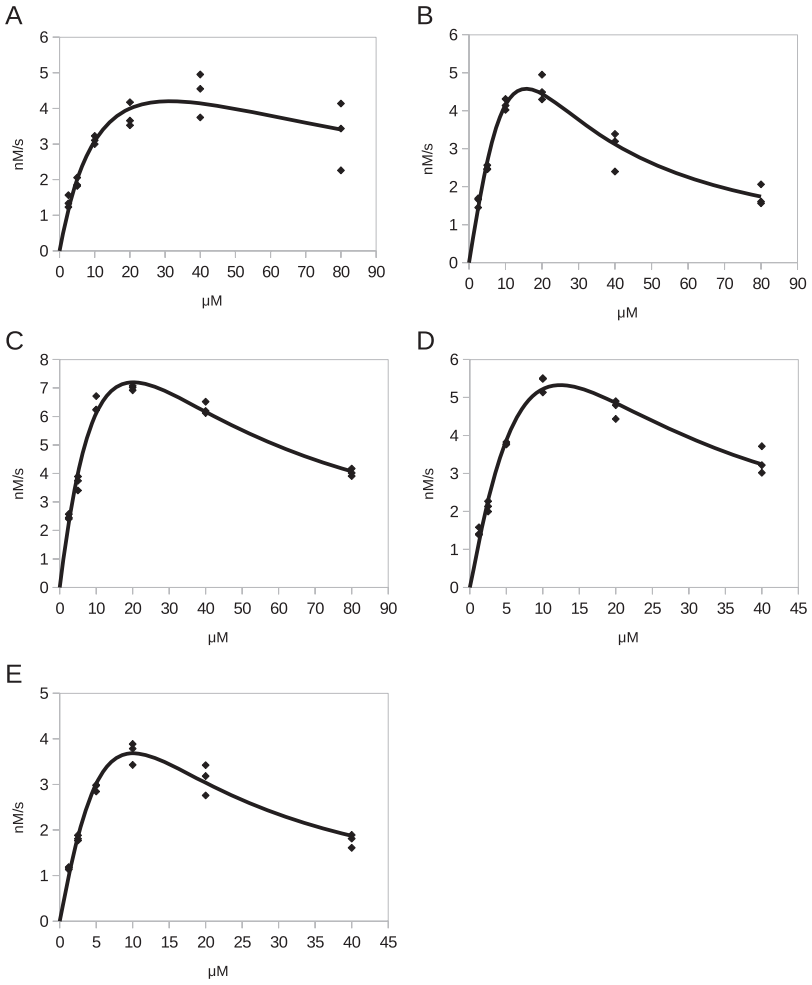

Figure 5. Michaelis Menten representation of Slit-FAR1 activity on different acyl-CoA precursors of *S. littoralis* pheromone: A: (*Z,E*)-9,11-tetradecadienoyl coenzyme A; B: (*Z*)-9-tetradecenoyl coenzyme A; C: (*Z*)-11-tetradecenoyl coenzyme A; D: (*E*)-11-tetradecenoyl coenzyme A; E: 14:SCoA.

24 to 44) encompasses part of the NAD(P)H-binding motif, in contrast to the structural data available for other NAD(P)H-dependent enzymes (Fita & Rossmann, 1985; Hamdane *et al*., 2009), for which the nucleotide binding site is located far from any transmembrane domain.

In addition to pheromone biosynthesis, FARs have been described to participate in wax synthesis to help insects keeping the impermeability of the cuticle (Locke, 1965). To study the specificity of Slit-FAR1 to the pheromone gland, we investigated the presence of the cDNA in cuticle-rich tissues, such as legs, wings and thorax. In addition, considering the fatty acid nature of the FAR substrates we also explored the presence of the transcript in tissues with enhanced fatty acid metabolism or storage, such as fat body and ovaries. Finally, to verify the sex-specificity of the transcript, the male abdomen was also tested. Despite the fact that the sensitivity of the semi-quantitative PCR does not completely exclude the presence of traces of the transcript in other tissues, Slit-FAR1 appeared to be expressed only in the pheromone gland, which agrees with the expression pattern observed in the FARs of other moths, such as *B. mori*, *O. scapulalis* and *Y. evonymellus* (Moto *et al*., 2003; Antony *et al*., 2009; Liénard *et al*., 2010).

	Km^{app} (µM)	$Vmax^{app}$ (nM/s)	Catalytic efficency (s^{-1})*
(*Z,E*)-9,11-tetradecadienoyl coenzyme A	7.7 ± 1.2	5.3 ± 0.3	6.9×10^{-4}
(*Z*)-9-tetradecenoyl coenzyme A	7.0 ± 1.2	6.4 ± 0.5	9.1×10^{-4}
(*Z*)-11-tetradecenoyl coenzyme A	4.4 ± 1.0	7.8 ± 0.5	17.7×10^{-4}
(*E*)-11-tetradecenoyl coenzyme A	3.7 ± 0.8	6.2 ± 0.5	16.8×10^{-4}
Tetradecanoyl coenzyme A	2.5 ± 0.6	4.0 ± 0.3	16.0×10^{-4}

Km^{app} refers to apparent Michaelis constant. $Vmax^{app}$ refers to apparent maximum rate.
*The catalytic efficiency is the quocient $Vmax^{app}/Km^{app}$.

Table 1. Kinetic values of Slit-FAR1 using different pheromone precursors as substrates. The values corresponding to the kinetic constants Km^{app} and $Vmax^{app}$ are expressed ± SE

As mentioned above, FARs are biosynthetic enzymes that have been postulated to be responsible for the relative ratio of components in the final pheromone blend. To investigate this hypothesis, Slit-FAR1 was expressed in a recombinant system and tested using different pheromone precursors as a substrate. Under *in vitro* conditions, the enzyme was on all pheromone precursors, yielding the corresponding pheromone alcohols, and did not show any trace of activity in the control extract; however, analysis by GC-MS revealed some isomerization in the reduction process, particularly when the dienoyl-CoA was used as substrate (Fig. 4). Although no conjugated double bond isomerase has been observed so far in yeast metabolism, the presence of a Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase in *S. cerevisiae* has been suggested (Gurvitz *et al*., 1998). Moreover, the capacity of desaturases to promote double bond isomerization has also been noticed (Serra *et al*., 2006). This suggests that the isomerization observed in our case could proceed from enzymatic activity in the yeast metabolism.

When plotting substrate concentrations over reaction velocity, the enzyme displayed a Michaelis–Menten profile for all substrates, but in all cases a depletion of reaction velocity was observed at high substrate concentrations. This phenomenon has been described as substrate inhibition (Copeland, 2000), and occurs at high concentrations when a second molecule of substrate binds the catalytic pocket, leading to a dead-end ternary complex. However, the non-productive ternary complex is unlikely to occur in the natural scenario, where the substrate molecules proceed from one enzymatic process to the next in the pheromone biosynthetic pathway (Ohnishi *et al*., 2006).

When we compared the affinity of the substrates for the enzyme, only slight differences in the corresponding affinity values (Km^{app} and catalytic efficiency) were found. Moreover, these differences did not correlate well with the ratio of the components in the pheromone blend, where (*Z,E*)-9,11–14:OAc appears as the major pheromone compound (55–95%) in almost all strains (Muñoz *et al*., 2008). The catalytic efficiency of the biosynthetic enzymes, particularly FARs, has been hypothesized to be related to the final ratio of the pheromone components (Roelofs & Jurenka, 1996; Lassance *et al*., 2010; Liénard *et al*., 2010); however, when the specificity of the FARs for different pheromone precursors was analysed, the results were contradictory. Thus, a single pgFAR of *Yponomeuta* sp. reduced a broad range of saturated and unsaturated C14 and C16 acyl precursors including pheromone precursors (Liénard *et al*., 2010). Similarly, pgFARs of different *Heliothis* and *Helicoverpa sp.* were active on a broad set of C8 to C16 fatty acyl substrates, including key pheromone precursors (Hagström *et al*., 2012). In contrast, yeasts expressing the FAR-*Z* from the pheromone gland of the *Z* strain of *O. nubilalis* converted almost exclusively the (*Z*)-11-tetradecenoyl precursor into the corresponding *Z* alcohol, whereas the FAR-*E* from the *E* strain transformed the *E* precursor with only a minute amount of the *Z* isomer being reduced (Lassance *et al*., 2010). The observed ratios of the fatty alcohols were identical to the final ratios of the acetates present in females of either strain. By site-specific mutagenesis on FAR orthologues of several *Ostrinia sp*., the authors later demonstrated that the enzymatic step catalysed by the FAR is a candidate source of variation in moth pheromone signals of the genus (Lassance *et al*., 2013). It is noteworthy, however, that both strains of *O. nubilalis* have only two components in the pheromone blend, released in a very different ratio (97–98% of the major *vs* 3–2% of the minor component), whereas the pheromone of other species, such as *Y. evonymelus* or *S. littoralis*, consists of a multicomponent mixture with less extreme proportions in the pheromone blend. This scenario could lead in *S. littoralis* to more subtle differences in the activity of the biosynthetic enzymes, which might be difficult to identify in an *in vitro* assay where the enzymatic activity can be influenced by different factors, such as protein extraction or chemical environment.

Notwithstanding the limitations of the assays performed on FARs, it appears that Slit-FAR1 does not display a significant preference for any acyl pheromone precursor, and that modulation of the final ratio of pheromone components could be induced by a possible combined action of other biosynthetic enzymes. The Δ-9 and Δ-11 desaturases (Muñoz *et al*., 2008 and references cited therein) and the acetyl transferase, identified as a key biosynthetic enzyme in the pheromone gland of *Argyrotaenia velutinana* (Jurenka & Roelofs, 1989), are good candidates to play a complementary role in the modulation of the final ratio of the pheromone components; however, a full characterization of the *S. littoralis* acetyl transferase, and a deep analysis of the *S. littoralis* desaturases are necessary to test this assumption.

In summary, we have isolated and characterized for the first time a FAR cDNA from the pheromone gland of *S. littoralis* (Slit-FAR1). This enzyme, whose amino acid sequence is close to the FARs of *Heliothis sp.* and *Helicoverpa sp*, appears to be expressed specifically in the pheromone gland. The recombinant expression of Slit-FAR1 reveals the capacity of the enzyme to reduce all acyl-CoA precursors into the corresponding pheromone alcohols. The kinetic variables Km^{app} and $Vmax^{app}$ displayed for each pheromone precursor, however, suggest that the ratio of the pheromone components may be also modulated by other biosynthetic enzymes. Phylogenetically, Slit-FAR1 appeared to be close to other FARs involved in the pheromone biosynthesis with little or nonspecificity for the natural pheromone precursors.

Experimental procedures

Insects and tissue collection

Spodoptera littoralis were reared in the laboratory, sexed as pupae and maintained at 25 °C and 60% humidity until emergence (Bellés *et al*., 1985). Pheromone gland, ovaries, fat body, wings, legs, thorax and abdomen were dissected from 2-day-old adults and stored at −80 °C until use.

Chemicals

(*Z*)-11-tetradecenol [(*Z*)-11–14:OH]; (*E*)-11–14:OH, (*Z*)-9–14:OH and (*Z,E*)-9,11-tetradecadienol [(*Z,E*)-9,11–14:OH], used as standards to confirm the reductase activity, were obtained by hydrolysis of the corresponding commercial acetates (Bedoukian; Danbury, CT, USA) in 95–99% yields with a purity ≥95%. 14:OH was purchased from Fluka (Madrid, Spain). Acyl coenzyme A derivatives of fatty acids were obtained from the corresponding acids according to the general procedure described below (Kawaguchi *et al*., 1981).

General procedure to obtain acyl-CoA thioesters

A solution of the fatty acid (10 μmol) in tetrahydrofuran (THF; 200 μl) was added to a solution of carbonyldiimidazole (12 μmol) in THF (200 μl). After 30 min of stirring at room temperature, the solvent was evaporated off and the residue was dissolved in a mix 2:1 THF:H_2O (400 μl). This new solution was allowed to react with 10 μmol of CoASH, dissolved in 2:1THF:H_2O (1 ml). The pH of the reaction mixture was adjusted to 8.0 with 1N NaOH. The stirring was maintained at room temperature for 4 h. Then, the solvent was evaporated off and the residual solution was acidified to pH 3–4 by adding small amounts of Dowex 50 (H^+) resin. The resin was removed by filtration, the filtrate was extracted with ethyl ether to remove the unreacted fatty acid and the aqueous layer was lyophilized. Purification of the acyl-CoA thioesters was conducted by semipreparative high-performance liquid chromatography on a Gemini 5 μm C_{18} column (250 × 10.0 mm) using a mixture of H_2O ($HCOONH_4$ 400 mM soln. + HCOOH, pH 4.5):MeOH in gradient mode from 30:70 to 5:95.

cDNA amplification

Total RNA was extracted from the female pheromone gland with TRIzol reagent (Gibco, Paisley, UK) according to the manufacturer's instructions and quantified in a spectrophotometer ND-1000 (Nanodrop Technologies, Wilmington, DE, USA). For cDNA synthesis, total RNA (8 μg) from the dissected tissues was treated with 1.5 units of RQ1 RNase-free DNaseI (Promega, Madison, WI, USA) and then reverse transcripted for 1 h at 42 °C, using Oligo(dT) as primer for first strand cDNA synthesis and SuperScriptII (Invitrogen, Carlsbad, CA, USA) as reverse transcriptase. The sequences where completed with a 3′- and 5′-rapid amplifications of cDNA ends (RACE), using 1 μg of total RNA and the FirstChoice® RLM-RACE Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions.

After an accurate alignment of the amino acid sequence of different FARs, two conserved regions were selected for degenerated primer design. The obtained primers FARfor and FARrev (Table S1) were used at a final concentration of 2 μM in a PCR under the following conditions: 3 min of denaturation at 94 °C followed by 40 cycles of 30 s at 94 °C, 30 s at 45 °C and 2 min at 72 °C, with a final extension of 10 min at 72 °C. The nucleotide sequence obtained was used as a template for 3′ and 5′ specific primers design.

For the 5′ and 3′ RACE-PCR, the primers 5′-Outer, 3′-Outer and 3′-Inner (FirstChoice RLM-RACE kit; Ambion) were used in combination with the specific primers 5′SlsFAR and 3′SlsFAR (Table S1) at the indicated annealing temperatures. The amplification of the 5′-end was achieved in a single PCR with 5′-Outer and 5′SlsFAR primers, whereas the 3′-end sequence was obtained using a nested PCR strategy. To this end, the 3′SlsFAR primer was used in a two consecutive PCRs, first in combination with 3′-Outer primer, and then with 3′-Inner primer. Finally, the entire ORF sequence was amplified using 1.2 units of a *Pfu* DNA polymerase (Promega) with the specific primers SlsFAR1-For and SlsFAR1-Rev (Table S1). The PCR was performed as follows: 3 min of denaturation at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 48 °C and 4 min at 72 °C. The PCR product was finally treated with 2 units of Taq polymerase (BioTools, Jupiter, FL, USA), without cycling, at 72 °C for 10 min to transfer a terminal deoxyadenosine to the 3′ end.

For sequence analysis, all PCR products were separated by agarose gel electrophoresis, extracted with GenElute™ Minus EtBr Spin Columns (Sigma-Aldrich, St Louis, MO, USA), cloned into a pCRII-TOPO vector (Invitrogen) and finally transformed into TOP10 chemically competent cells (Invitrogen). Recombinant plasmids were then purified using the High Pure Plasmid Isolation Kit (Roche, Indianapolis, IN, USA) and sequenced (Institute of Molecular Biology of Barcelona, CSIC).

RNA expression

The presence of the transcript in different tissues of *S. littoralis* was studied by semi-quantitative PCR, using the pair of primers SlitFAR1for2 and SlitFAR1rev2 (Table S1), and the ribosomal control gene *rpL8* to check the quality and the homogeneity of the cDNAs (Maïbèche-Coisne *et al*., 2004). Using the cDNA of the pheromone gland as a template, the PCR conditions were first set up to fit the linear range of amplification. The obtained conditions (26 cycles and 54 °C of annealing temperature) where then used for the corresponding PCRs on the cDNA of different tissues of *S. littoralis*, and the resulting amplification products were loaded into a 1.5% agarose gel and visualized with Sybr-safe (Invitrogen).

Phylogenetic analysis

The collection sequences used in the phylogenetic analysis was obtained from a BLAST search (TBLASTN and BLASTP) (Altschul *et al*., 1997), using the amino acid sequence of Slit-FAR1 as a query sequence, and restricting search to Lepidoptera taxa. A FAR-like sequence isolated from the pheromone gland of *Heliothis virescens* (accession number. ACX53790) (Vogel *et al*., 2010) was added to the collection, even though it did not appear in the BLAST search. Sequences shorter than 316 amino acids or named 'partial' in the GenBank were excluded, and so 10 sequences from *O. nubilalis* (ADI82791, ADI82792, ADI82794, ADI82796, ADI82797, ADI82798 and ADI82799, with high amino acid identity to ADI82790; and ADI827984, ADI827985 and ADI827987, with high amino acid identity to ADI827983) were

also removed. The obtained amino acid sequences (66) were aligned using CLUSTALW2 (EMBL-EBI), and computed for phylogenetic analysis and graphical edition with MEGA 4 (Tamura *et al*., 2007) under the neighbour-joining algorithm (Saitou & Nei, 1987) and 10 000 bootstrap replicates.

Protein expression and extraction

The *S. littoralis* FAR was amplified with primers SlitFARpESCfor and SlitFARpESCrev to introduce the restriction sites BamHI and XhoI, and ligated into the MCS 2 of the yeast expression vector pESC-URA (Stratagene, La Jolla, CA, USA). After verification of the sequence integrity, the construction pESC-SlitFAR1 was transformed into LiOAc competent *S. cerevisiae* cells of strain YPH501, according to manufacturer's instructions. The expression was induced by inoculating the recombinant yeast into a synthetic medium containing galactose as carbon source. The culture was grown at 30 °C and 250 rpm (orbital agitator) until the optical density at 600 nm reached the range 1.2–1.5. Cells were then harvested by centrifugation at 10 000 *g* and 4 °C for 15 min, and the pellet was washed with 10 mM Tris-HCl pH 7.3 and stored at −80 °C until purification.

For protein extraction, the cell pellet obtained from 250 ml of culture was resuspended in 35 ml of 10 mM Tris-HCl pH 7.3 containing 1% of protease inhibitor cocktail (Sigma-Aldrich) and disrupted for one cycle at 1.86 kbar using a cell disruptor (Constant Systems Ltd, Daventry, UK). Non-soluble proteins and membrane debris were separated by centrifugation at 18 000 *g* and 4 °C for 1 h. Glycerol was added to the supernatant for a final concentration of 10%, and the solution was distributed in aliquot fractions and rapidly frozen in liquid nitrogen to prevent possible unfolding (Bhatnagar *et al*., 2007). Aliquots were stored at −80 °C until needed.

Activity assays

Reaction mixtures (400 µl) were prepared in 10 mM Tris-HCl pH 7.3, and contained 2% of dimethyl sulfoxide, 50 µg of protein extract, 2.5 mM of NADPH, and the corresponding fatty acyl-CoA at six final concentrations in the range of 1.25 to 80 µM, depending on the precursors. To minimize differences in activity from one batch to another, a single protein extract was used for all reactions. After establishing the initial velocity conditions, all reaction mixtures were incubated at 30 °C for 10 min and immediately quenched by dipping into a CH_3OH/dry ice bath. The samples were thawed on ice and immediately extracted with 800 µl of hexane containing 375 ng of 1-undecanol as internal standard. The organic phases were concentrated, silanized with N,O-bis-(trimethylsilyl)trifluoroacetamide for 1 h at 70 °C, and diluted with 30 µl of n-heptane for analysis. After confirmation of the presence of all reaction products (trimethylsilyl derivatives of the expected pheromone alcohols) by mass spectrometry (Thermo Finnigan Trace GC-MS; Thermo Finnigan, San Jose, CA, USA), samples were quantified by GC on a FID detector (Thermo Finnigan Trace GC) and an HP-5 capillary column (30 m × 250 µm i.d.; Agilent Technologies). The chromatographic conditions were injection at 60 °C for 1 min, then a programme of 10 °C/min to 120 °C, followed by 2 °C/min to 180 °C and then 10 °C/min until 280 °C, and hold at 280 °C for an additional 5 min. Experiments were replicated three times for each concentration, and statistical analysis and calculation of the kinetic variables were performed with GRAPHPAD PRISM software version 4.0.

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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:

Figure S1. Identification of a fatty acyl reductase from *Spodoptera littoralis* female gland involved in pheromone biosynthesis.

Table S1. Nucleotide sequence of the primers used in Slit-FAR1 cDNA isolation and characterisation.

Table S2. Abreviations used to designate species in the phylogenetic analysis.