Insect Molecular Biology (2007) **16**(1), 73–81 doi: 10.1111/j.1365-2583.2006.00702.x

Antennal esterase cDNAs from two pest moths, *Spodoptera littoralis* and *Sesamia nonagrioides*, potentially involved in odourant degradation

C. Merlin*, G. Rosell†, G. Carot-Sans‡, M.-C. François*, F. Bozzolan*, J. Pelletier*, E. Jacquin-Joly*, A. Guerrero‡ and M. Maïbèche-Coisne*

**Unité 1272, UPMC–INRA–INA.PG, Physiologie de l'Insecte: Signalisation et Communication, Centre INRA, France;* †*Unit of Medicinal Chemistry (Associated with CSIC). Faculty of Pharmacy. University of Barcelona, Barcelona, Spain; and* ‡*Department of Biological Organic Chemistry, IIQAB (CSIC), Barcelona, Spain*

Abstract

Rapid degradation of odours after interaction with olfactory receptors is a critical step of the signal reception process. However, the implied mechanisms are still largely unknown in vertebrates as well as in insects. Involvement of odourant-degrading enzymes in odourant degradation within the antennae has been shown in some insect species and, in particular, esterases could play a key role in degradation of sex pheromones from Lepidoptera. Using a PCR-based strategy, we isolated cDNAs encoding two new esterases from two moths which used acetates as pheromone compounds: the Egyptian armyworm *Spodoptera littoralis* and the Mediterranean corn borer *Sesamia nonagrioides*. In antennae, both transcripts were clearly restricted to olfactory sensilla, suggesting their involvement in the degradation of odourant acetate components.

Keywords: odourant-degrading enzymes, antennal esterases, olfaction, Lepidoptera.

Introduction

Sensitive and specific detection of volatile chemical cues is essential for insects to interpret their environment and communicate with conspecifics. Detection of these chemical signals at the peripheral level takes place in the antennae, where several steps occur successively. While odourant solubilization and delivery by odourant-binding proteins (OBPs) and detection by membrane-bound odourant-receptors (ORs) were intensely studied and, in a large part, deciphered (review in Jacquin-Joly & Lucas, 2005), the last step of odourant degradation is still largely unknown. Efficient degradation of the signal is, however, an integral part of the signal reception and transduction processes. Among the different mechanisms potentially involved in this termination step, enzymatic degradation is certainly the most studied and some odourant-degrading enzymes (ODEs) have been identified in insects, particularly in Lepidoptera (Vogt, 2005). These insects are good models for this purpose because males possess a highly specific and sensitive olfactory system, allowing them to detect small amounts of the sex pheromone released by females. During their flight through the discontinuous pheromone trail, rapid deactivation of the pheromone appears to be crucial for males to obtain a spatial and temporal resolution of this intermittent signal (Vickers, 2006), and to allow the olfactory neurones to respond to frequent periodic stimulations (Vogt & Riddiford, 1981; Vogt, 2003). However, involvement of ODEs in fast pheromone deactivation is still debated, because some authors favour a rapid inactivation by OBPs, followed by enzymatic processes (review in Kaissling, 2001).

Nevertheless, biochemical approaches have led in the past to the identification of ODEs belonging to different families, such as esterases, aldehyde-oxidases, alcohol-oxidases/dehydrogenases or cytochromes P450 (review in Vogt, 2005), a diversity that could be correlated with the variety of chemical structures of pheromones. In pioneering works, esterases from the silkmoth *Antheraea polyphemus* were studied two decades ago, and four esterase isozymes were isolated, one from the sensillum lymph (Apol-SE), two from its antennal integument and one from its scales (Vogt & Riddiford, 1981, 1986; Klein, 1987). As the four esterases were able to degrade the ester components of the pheromone blend, it was proposed that Apol-SE removes the

Received 13 June 2006; accepted after revision 1 August 2006; first published online 19 December 2006. Correspondence: Dr Martine Maïbèche-Coisne, Unité 1272, UPMC–INRA–INA.PG, Physiologie de l'Insecte: Signalisation et Communication, Route de Saint-Cyr, Bat A, 78026 Versailles Cedex, France. Tel.: +33 1 30 83 32 12; fax: +33 1 30 83 31 19; e-mail: maibeche@versailles.inra.fr

pheromone inside the sensillum lymph, thus maintaining the ability of the olfactory neurones to respond to new stimuli. The other esterases should participate in removing the hydrophobic pheromone from the integument and the cuticle of the insects, in order to reduce background noise (Vogt & Riddiford, 1981; Vogt, 2005).

Although the catabolic properties of ODEs towards pheromones were demonstrated biochemically many years ago, few antennal esterase genes have been characterized to date. Indeed, molecular data are available in only three species, two Lepidoptera (*A. polyphemus* and the cabbage armyworm *Mamestra brassicae*), and one Hymenoptera (*Apis mellifera*). Three esterase cDNAs have been characterized from *A. polyphemus* antennae: a putative integumental esterase (Apol-IE) expressed in the antennae and legs of both sexes, and two male antennae specific esterases: a putative odourant-degrading enzyme (Apol-ODE; Ishida & Leal, 2002) and a pheromone-degrading enzyme (Apol-PDE; Ishida & Leal, 2005). Interestingly, purified Apol-PDE enzyme was shown to inactivate the pheromone with kinetic parameters compatible with male moth behaviour during its flight toward the pheromone plume, suggesting that it could thus correspond to the Apol-SE formerly characterized (Vogt *et al.*, 1985). In the noctuid moth *Mamestra brassicae*, we have cloned an esterase expressed in the antennae of both sexes (Maïbèche-Coisne *et al.*, 2004) with an expression restricted to olfactory sensilla, including pheromone-sensitive ones. This localization is compatible with a function in the degradation of sex pheromone acetate components. Finally, in the bee *A. mellifera*, an antennal esterase overexpressed in drones, and potentially involved in the blockage of acetate pheromone signalling in this caste, has been also identified (Kamikouchi *et al.*, 2004). All of the antennal esterases characterized to date belong to the carboxyl/cholinesterase family, whose members present a great structural and functional diversity (Oakeshott *et al.*, 1999).

Despite the few biochemical and molecular data available, as many of the components identified to date in sex pheromone blends or in plant scents are esters (Vogt, 2005), we can suppose that olfactory esterases are widely present in insects, and particularly in moths. In order to improve our knowledge of this kind of enzyme, and to obtain a larger repertoire of candidate genes, we started a comparative study of the antennal esterases of two moths, *Spodoptera littoralis* (*Slit*) and *Sesamia nonagrioides* (*Snon*), which use two different types of acetates as main sex pheromone components. Esterase activities have been found previously in their antennae (Durán *et al.*, 1993; Quero *et al.*, 2003), and studies with esterase inhibitors revealed an inhibition of olfactory neurone responses to the pheromone blend (Quero *et al.*, 2004). We thus focused our work on the characterization of their antennal esterases using biochemical and molecular approaches. We first investigated their diversity in the antennae and their distribution throughout the body by native PAGE and naphthyl acetate staining. In each species, a PCR-based strategy using degenerate primers led us to characterize two full-length cDNAs encoding esterases from male antennae, called *Slit-EST* and *Snon-EST*. Their expression patterns were studied by Northern-blot and *in situ* hybridization. Although the two transcripts were expressed in all of the tissues tested, their cellular localizations in the antennae at the base of olfactory sensilla, including the pheromone-sensitive ones, suggested that they could be involved in the degradation of acetate volatiles, including the components of the sex pheromones of these two species.

Results and Discussion

Esterase activities in various tissues of S. littoralis *and* S. nonagrioides

Because global esterase activity has been detected in *in vitro* experiments of hydrolysis of the pheromone acetates in male antennae of both species (Quero *et al.*, 2003), we investigated the presence of esterases responsible for this catabolism in the antennae of the two moths. After separation of male antennal homogenates by 10% native PAGE and staining with α-/β-naphthyl acetate, at least seven bands were detected in the antennae of both species (Fig. 1A). These patterns are more complex than those observed in the antennae of *M. brassicae* and *A. polyphemus*, with five (Maïbèche-Coisne *et al.*, 2004) and two bands (Vogt & Riddiford, 1981; Ishida & Leal, 2002), respectively. However, in this latter species, recent molecular data showed that these two bands could in fact correspond to three cloned esterases, Apol-IE, Apol-ODE (Ishida & Leal, 2002) and Apol-PDE (Ishida & Leal, 2005), leading to the hypothesis of a putative comigration of some of these proteins at the same level in native PAGE gels. If similar comigration of esterases could occur in *S. littoralis* and *S. nonagrioides*, we could expect that more than seven genes would be expressed in the antennae. Comparison of the migration patterns of male antennal esterases in both species on the same gel showed that five bands migrated similarly, suggesting that the corresponding proteins could share close structural features (Fig. 1A). On the other hand, esterase migrations in antennae and other tissues also revealed a great number of bands in non-olfactory tissues, but most of them were also present in the antennae (Fig. 1B). In *S. littoralis*, although one band seemed enriched in antennae and wings, there was no obvious olfactory-specific band, in contrast to *S. nonagrioides*, in which one band appeared antennae-specific (Fig. 1B; arrow and arrowhead, respectively). In the two moths, the esterase profiles of antennal extracts were comparable in both sexes, as previously observed for other noctuids such as *Trichoplusia ni* (Ferkovich *et al.*, 1980) and *M. brassicae* (Maïbèche-Coisne

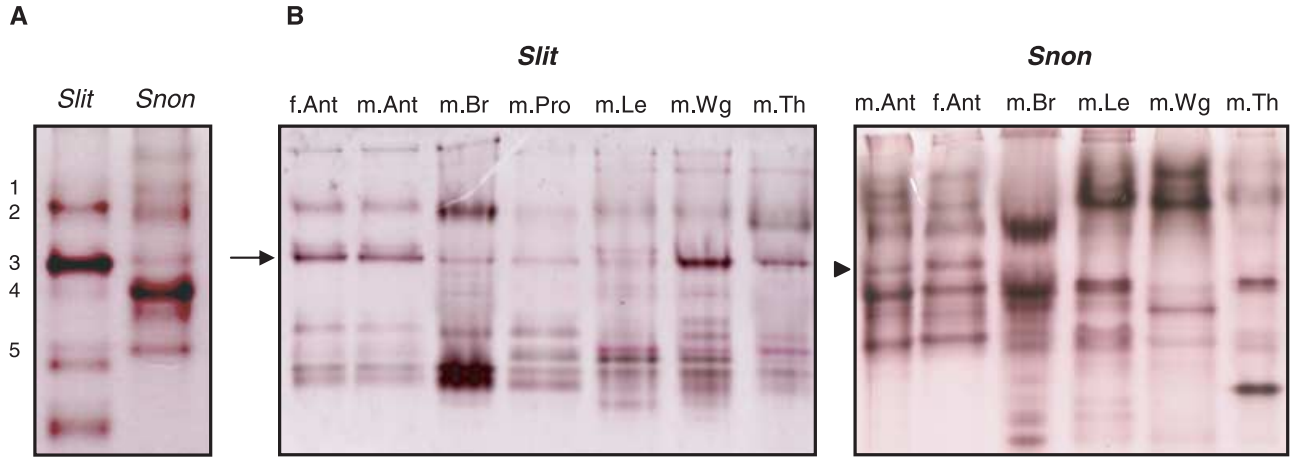

Figure 1. (A) Esterases from male antennae of *S. littoralis* (*Slit*) and *S. nonagrioides* (*Snon*) separated on a 10% native polyacrylamide gel and stained with α-/β-naphthyl acetate. Numbers on left indicate bands with similar migration patterns between the two species. (B) Esterase patterns in different tissues of *S. littoralis* and *S. nonagrioides*, f: females; m: males; Ant: antennae; Br: brains; Pro: Proboscis; Le: Legs; Wg: Wings; Th: Thorax; Abd: Abdomens. Arrow indicates a band overexpressed in *Slit* antennae and wings, whereas arrowhead represents an antennal specific esterase in *Snon*.

et al., 2004). This lack of sex differences is in contradiction with the data from the saturniid moth *A. polyphemus*, where Apol-PDE and Apol-ODE are clearly male-specific (Ishida & Leal, 2002, 2005). As found in other moth species (reviewed in Callahan *et al*., 2000), *S. littoralis* females are able to detect various acetate compounds, including their own pheromone (Ljungberg *et al*., 1993). This auto-detection remains to be established in *S. nonagrioides*, but this, as shared by various Lepidoptera females, could explain the apparent lack of sex differences observed in esterase patterns. Contrary to the situation observed in *A. polyphemus*, the complex patterns of esterase bands in the antennae of these moths that use acetate compounds in their pheromonal and general odourant communication strongly suggests that the degradation of esters in the antennae could be performed by the intervention of numerous esterases.

Characterization of two antennal esterase cDNAs from both species

To further investigate the antennal esterases in *S. littoralis* and *S. nonagrioides*, we have undertaken the cDNA cloning of these genes in the two species. Two cDNA products were amplified by RT-PCR from male antennae using different strategies. In *S. littoralis*, a 950-bp cDNA product was amplified using two degenerate primers deduced from highly conserved regions of various insect esterase sequences. After cloning and sequencing, the deduced amino acid sequence appeared to belong to the esterase family. It was then extended to the 5′ and 3′ region by 5′ and 3′ RACE-PCR. In *S. nonagrioides*, a 1340-bp cDNA product was directly amplified by 3′ RACE using a degenerate primer deduced from a conserved region of insect esterases, and then extended to the 5′ region. This led us to obtain two full-length cDNAs of 2835 and 1854 bp, called *Slit-EST* and *Snon-EST*, respectively. *Slit-EST* sequence analysis revealed that it contained a 1683-bp ORF, with a 119-bp 5′ untranslated region (UTR) and a 1033-bp 3′ UTR. *Snon-EST* was composed of a 1593-bp ORF, with a 41-bp 5′ UTR and a 220-bp 3′ UTR. The complete ORFs of the two putative esterases were amplified with a high fidelity Taq DNA polymerase and sequenced, confirming that the sequences obtained did not correspond to chimeras. *Slit-EST* and *Snon-EST* sequences were deposited into the GenBank database under accession numbers DQ680828 and DQ680829, respectively. *Slit-EST* and *Snon-EST* encode proteins of 558 and 530 amino acids (Fig. 2) with calculated molecular masses of 63 kDa and 60.0 kDa and isoelectric points of 8.4 and 7.0 (MWCALC; Infobiogen). Signal sequence cleavage sites were predicted between Gln-16 and Gln-17 for Slit-EST and between Ala-19 and Val-20 for Snon-EST (SignalP 3.0; Bendtsen *et al*., 2004), adding support to the theory that they could be secreted proteins, as known for other antennal esterases. Slit-EST showed five potential N-glycosylation sites predicted by NetNGlyc 1.0 Server, as Apol-ODE (Ishida & Leal, 2002), whereas Snon-EST exhibited only four potential N-glycosylation sites. Moreover, the two deduced polypeptides Slit-EST and Snon-EST displayed the Ser-active site (Ser-195 in both sequences) included in the conserved pentapeptide Gly-X-Ser-X-Gly common in enzymes of the α-/β-hydrolase family (Oakeshott *et al*., 1999) (Fig. 2). Comparison of Slit-EST and Snon-EST sequences with known antennal esterases revealed that Slit-EST was very close to Apol-ODE, with 63.5% of sequence identity, whereas Snon-EST was quite different from other known antennal esterases, with a maximum of 25% of identity with Slit-EST. As shown in the phylogenetic analysis, these three esterases clustered

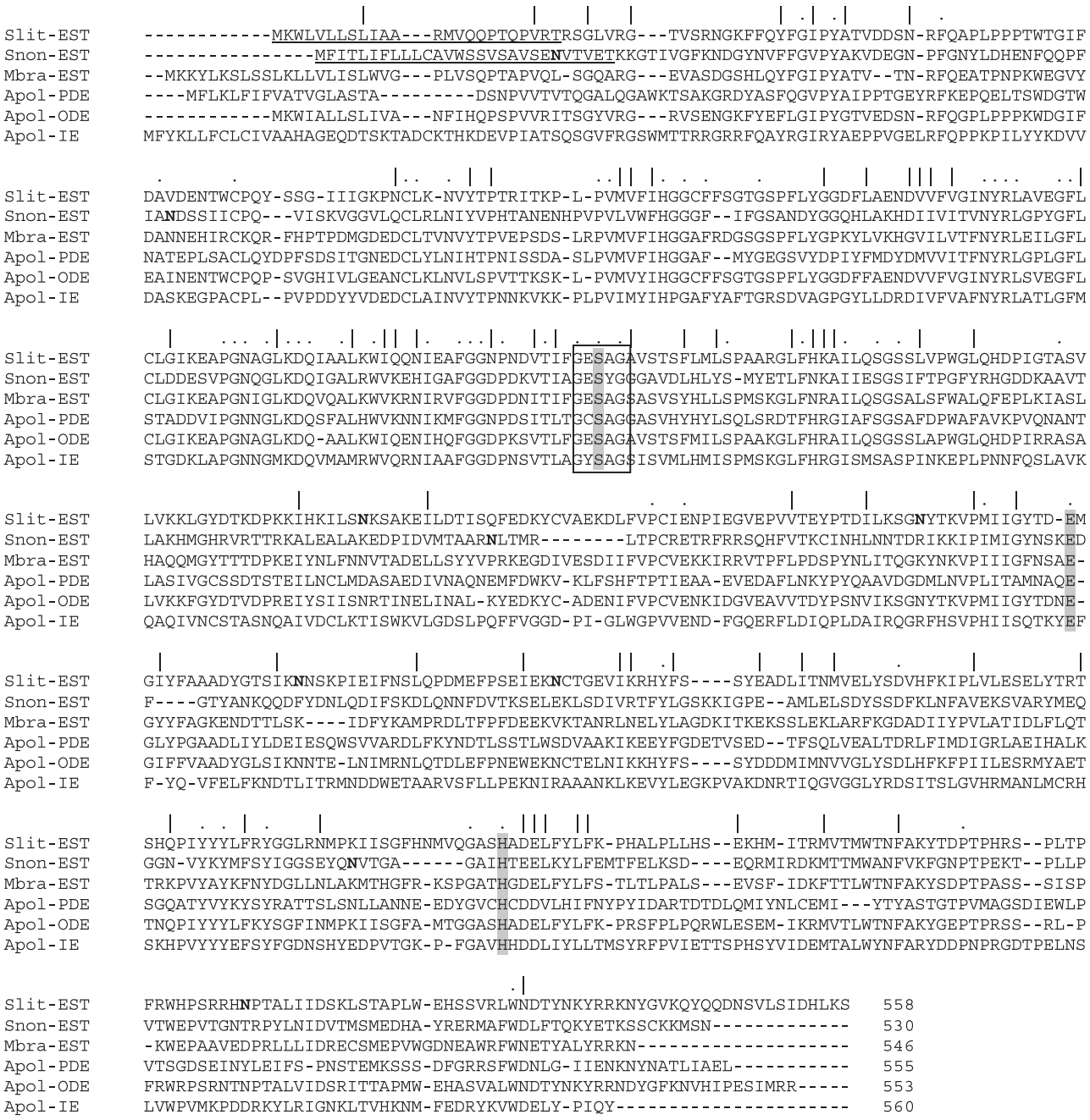

Figure 2. Alignment of Slit-EST and Snon-EST predicted amino acid sequences with *M. brassicae* antennal esterase (Mbra-EST; GenBank accession no. AY390258), *A. polyphemus* pheromone-degrading enzyme (Apol-PDE; AY866480), odourant-degrading enzyme (Apol-ODE; AAM14415) and integumental esterase (Apol-IE; AAM14416). The residues of the catalytic triad (Ser^{195}, Glu^{327} and His^{450}) are highlighted in grey and the Ser catalytic sites boxed. The putative signal peptides are underlined and the potential glycosylation sites are in bold. Identical and similar amino acids are indicated by dots and vertical lines, respectively.

with the antennal esterase from *M. brassicae* (Fig. 3). This group of antennal esterases was separated from another one in which Apol-PDE clustered with insect β-esterases and Apol-IE with a dipteran homologue. Carboxyl/cholinesterase members are known to show a great diversity in their substrate recognition as well as in their catalytic parameters (Satoh *et al.*, 2002). Consistent with their functional diversity, their sequences are also highly divergent (Cygler *et al.*, 1993), and the olfactory members of the family do not appear to be an exception to the rule.

Tissue distribution of Slit-Est and Snon-Est

Slit-Est and *Snon-Est* transcriptions in various tissues were examined by Northern blot using *rpL8*, a gene encoding an

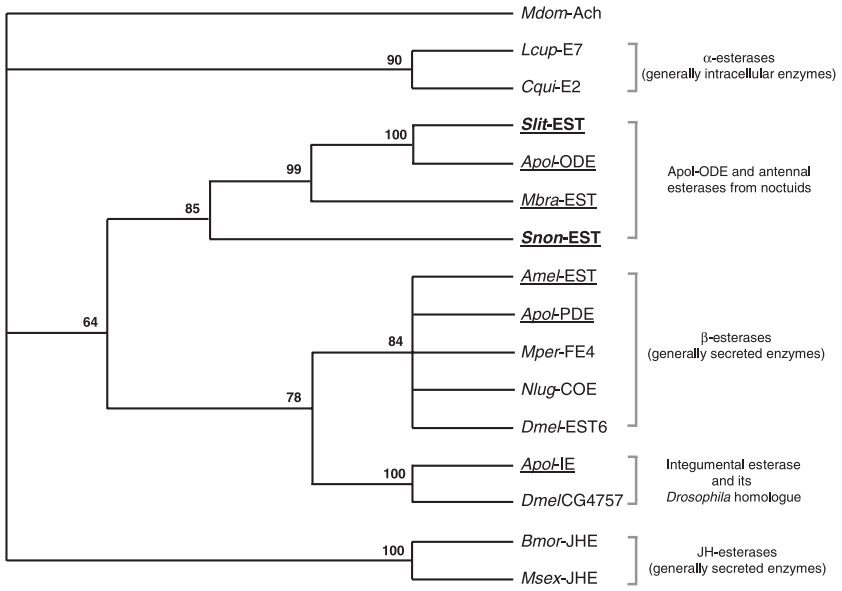

Figure 3. Phylogenetic analysis of Slit-EST and Snon-EST with various insect esterases, aligned using Multalign program (Corpet, 1988). GenBank accession numbers are as follows: *Amel*-EST, *Apis mellifera* antennal esterase D-AP1, AB083009; *Bmor*-JHE, *Bombyx mori* juvenile hormone esterase, AF287267; *Cqui*-E2, *Culex quinquefasciatus* esterase α-2, CAA88030; *Dmel*CG4757, *Drosophila melanogaster* esterase, CG4757; *Dmel*-EST6, *D. melanogaster* esterase-6, CG6917; *Lcup*-E7, *Lucilia cuprina* esterase, AAB67728; *Msex*-JHE, *Manduca sexta* JHE, AF327882; *Mper*-FE4, *Myzus persicae* E4 esterase, P35502; *Nlug*-COE, *Nilaparvata lugens* carboxylesterase, AF302777. The consensus tree was built in Paup 4.0b10 software (Sinauer associates, USA) using maximum parsimony. Bootstrap support values (in percent) based on 1000 replicates are indicated. Antennal esterases are underlined.

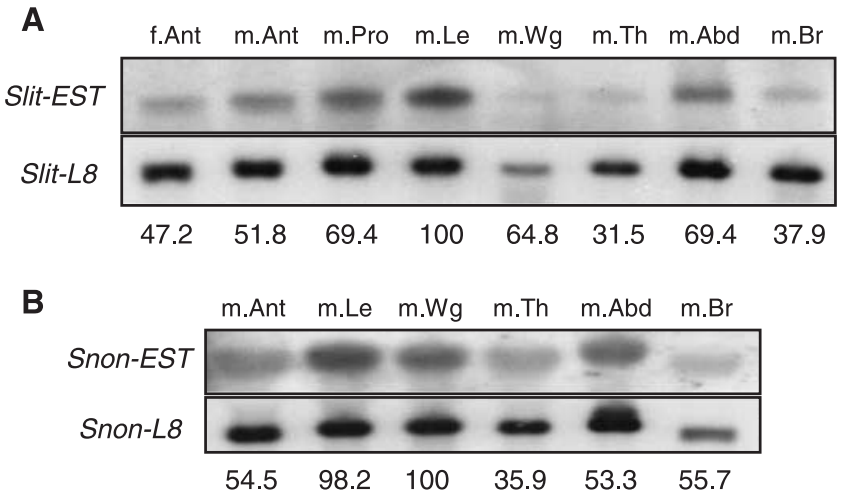

Figure 4. Analysis of *Slit-EST* (A) and *Snon-EST* (B) expression throughout the body by Northern-blot with templates from female antennae (f.Ant), male antennae (m.Ant), proboscis (m.Pro), legs (m.Le), wings (m.Wg), thorax (m.Th), abdomen (m.Abd) and brains (m.Br). The numbers under figures represent the relative expression level referring to ratios of *EST* vs. *rpL8* intensities then converted to percentages of maximal level.

ubiquitous ribosomal protein, as positive control (Fig. 4). The sizes observed for the two transcripts (~2800 bp for *Slit-EST* and ~1900 bp for *Snon-EST*) were in agreement with the corresponding cDNA lengths. The two genes were expressed in male antennae but also in all other tissues tested. In particular, *Slit-EST* and *Snon-EST* presented higher expression in legs (Fig. 4A) and legs and wings (Fig 4B), respectively. In both species, the two genes were expressed in female antennae. This was demonstrated by RT-PCR in *S. nonagrioides* (data not shown). In *S. littoralis*, the ratio of *Slit-EST* to *rpL8* was quite the same between male and female antennae, confirming the lack of sex differences previously observed after native-PAGE. The ubiquitous expression pattern of these two esterases was not incompatible with a putative function in odourant molecule degradation, as they were also expressed in olfactory organs. Indeed, in spite of its expression in all tissues of *A. polyphemus*, including the antennae (Ishida & Leal, 2002, 2005), integument esterase was demonstrated also to efficiently degrade the acetate components of the pheromone (Vogt & Riddiford, 1981).

Expression of Slit-Est *and* Snon-Est *within the male antennae*

The cellular localizations of *Slit-EST* and *Snon-EST* in antennae have been specified by *in situ* hybridization (Fig. 5). The male antennae of the two species present different morphologies, filiform in *S. littoralis* (Ljungberg *et al.*, 1993) and comb-like (bipectinate) in *S. nonagrioides* (Solinas & Trona, 2002). They are dorsally covered with scales and

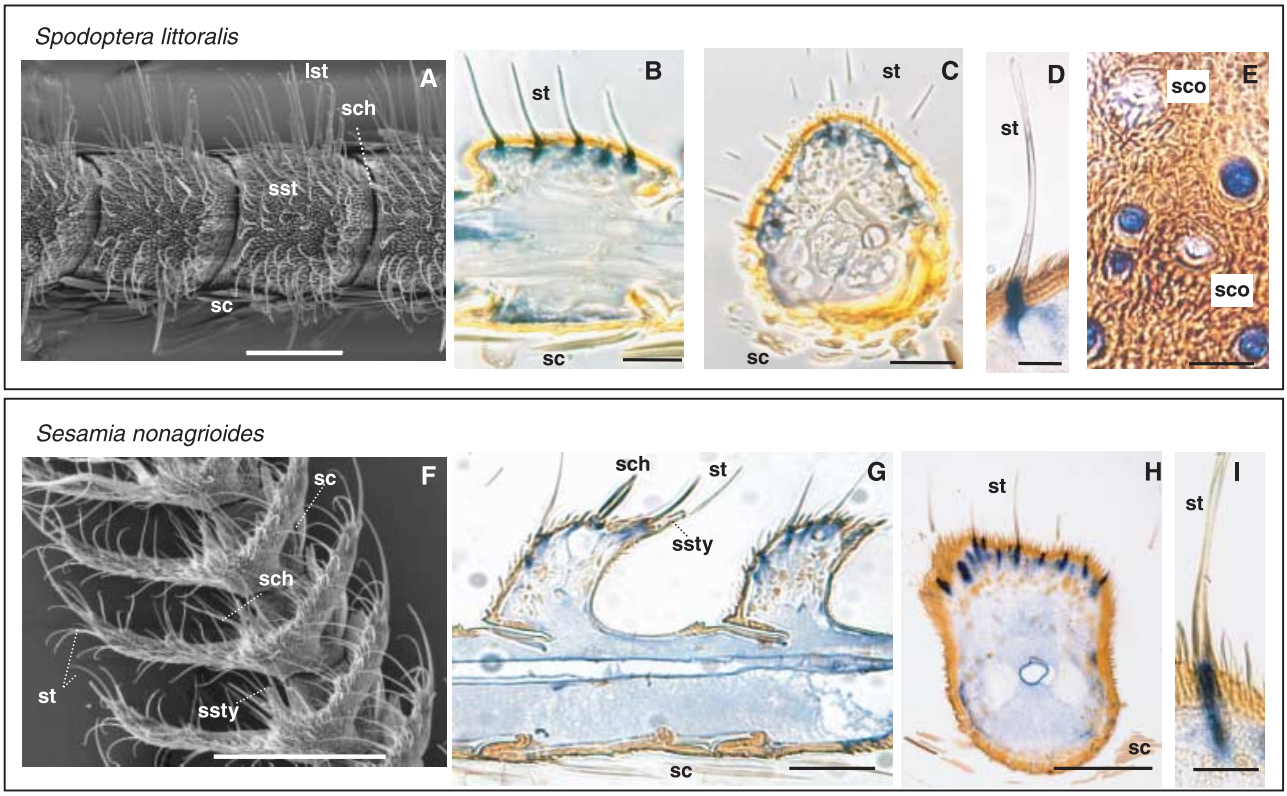

Figure 5. Expression patterns of *Slit-EST* (B, C, D, E) and *Snon-EST* (G, H, I) after *in situ* hybridization on sections of male antennae. SEM ventral views of *S. littoralis* (A) and *S. nonagrioides* (F) male antennal segments showing the localization of scales (sc), long (lst) and short (sst) sensilla trichodea, sensilla chaetica (sch) and sensilla styloconica (ssty). Longitudinal (B, G) and transversal (C, H) sections with labelling restricted to the ventral side carrying olfactory sensilla. (D, I) Details of sensilla trichodea showing intense labelling at the base. (E) Section through the cuticle with unlabelled sensilla coeloconica (sco) that resemble flowers. Bars: 200 μm in F; 100 μm in A; 50 μm in B, C, G, H; 10 μm in D, E, I.

carry olfactory sensilla on the ventral side. *S. littoralis* male antennae support long and short sensilla trichodea (Ljungberg *et al.*, 1993), with the larger situated more laterally and the smaller more ventrally (Fig. 5A). In *S. non-agrioides*, olfactory hairs are distributed across both portions of the lateral and the medial processes; the former carrying a great number of sensilla trichodea (Fig. 5F). In both species, the responses of these different olfactory sensilla to the components of the pheromone blend have been characterized by single cell recordings. In *S. littoralis*, the most lateral long sensilla respond to (Z,E)-9,12-tetradecadienyl acetate (Z9E12–14Ac) and (Z)-9-tetradecenyl alcohol (Z9–14OH), whereas the other long and the short sensilla trichodea respond to Z9E11–14:Ac (Ljungberg *et al.*, 1993). In *S. nonagrioides*, two functional types of sensilla trichodea have been described: type A responds to (Z)-11-hexadecenylacetate (Z11–16Ac) and to the corresponding aldehyde (Z11–16Ald), and type B to Z11–16Ac and to Z11–16OH (Quero *et al.*, 2004). After *in situ* hybridization, *Slit-EST* and *Snon-EST* signals were restricted to the sensilla side of the antennae, with no labelling on the scale side (Fig. 5B,C,G,H). For the two genes, signals were located just below the cuticle, at the base of the sensilla trichodea (Fig. 5B,C,D,G–I), in cells that could be accessory cells surrounding sensory neurones, or the neurones themselves. On the longitudinal sections, the distinction between long and short sensilla trichodea was not possible. However, in transversal sections of *S. littoralis* male antennae, the labelling was observed all over the ventral and lateral surface suggesting an expression of *Slit-EST* in both long and short olfactory sensilla (Fig. 5C). Sensilla coeloconica, typical structures that resemble flowers in scanning microscopy and known as olfactory in some species (Pophof, 1997), but whose function is still unknown in *S. littoralis*, were not labelled (Fig. 5E). In male antennae of *S. nonagrioides*, olfactory sensilla were labelled but no labelling was observed in sensilla chaetica, which are thought to function in gustation in other Lepidoptera (Jørgensen *et al.*, 2006), nor in sensilla styloconica, a thermo-hygro receptor (Fig. 5G). In transversal sections of antennae, a great number of spots were observed, probably corresponding to labelling of long and short sensilla trichodea (Fig. 5H). Our results suggested that *Slit-EST* and *Snon-EST* were expressed in sensilla types involved in responses to different acetate components of the pheromone blend, and could consequently be implicated in

Table 1. Primers used for the molecular cloning of two antennal esterase cDNAs from *S. littoralis* and *S. nonagrioides*

Primer name	Primer sequence	Amino acid motif
Spodoptera littoralis		
SlESTUp1	5′-AACTTTGGMCTNAARGAYCA-3′	NAGLKDQ
SlESTDo1	5′-AAAGTTRGYCCACAKWGT-3′	TMWTNF
3′ESTSlUp	5′-GAGCCCGTGGTGACGGAATACCCGACC-3′	EPVVTEYPT
5′ESTSlDo	5′-GAAGACCCCACGGGACCAATGAAGAGCC-3′	GSSLVPWGL
SlESTorfUp	5′-ATGAAGTGGTTAGTGCTGCTGTCGC-3′	MKWLVLLS
SlESTorfDo	5′-TTATGACTTTAAGTGATCTATAGAGAGTACGCT-3′	MHARAA
SlESTUpsp	5′-ATACTACAAAGTGGCTCTTCATTGGTC-3′	ILQSGSSLV
SlESTDosp	5′-CTTGAACAGGTAAAACAGCTCGTC-3′	DELFYLFK
Sesamia nonagrioides		
3′ESTSnUp	5′-AACTTTGGMCTNAARGAYCA-3′	NQGLKDQI
5′ESTSnDo	5′-TGCCCCACCACCATAGCTTTCGCCAG-3′	GESYGGGA
SnESTorfUp	5′-ATGCATGCTCGAGCGGCC-3′	SVLSIDHLKS
SnESTorfDo	5′-TTAATTGTACATCTTCTTACATGAACTCTTTGT-3′	TKSSCKKMYN
SnESTUp	5′- ATGATTGGCTACAATAGTAAAGAAGACTTT-3′	MIGYNSKEDF
SnESTDo	5′-TGTCTTTTCAGGCGTTGGGTTCCC-3′	GNPTPEKT

degradation of acetate pheromone components. In addition, we can not exclude a role for these enzymes in the degradation of plant volatiles with an ester functional group.

In conclusion, we have highlighted in the present work the occurrence, in the male moths *S. littoralis* and *S. nonagrioides*, of a greater number of antennal esterases than previously observed in other Lepidoptera. In each species, one cDNA encoding a putative extracellular esterase expressed in all tissues tested has been characterized. As in other noctuid moths, no sexual dimorphism was observed in the expression patterns of the two genes. In male antennae, the expression of *Slit-EST* and *Snon-EST* were precisely localized at the base of olfactory sensilla, compatible with a function in the degradation of volatile acetate compounds, either pheromones or plant volatiles. The screening of an antennal cDNA library from male *S. littoralis,* in progress in our laboratory, will certainly allow us to identify other olfactory esterases in this species, as revealed by the biochemical approach used in the present study. All of these esterases could contribute together to the degradation of acetate odourants, in agreement with their substrate specificities.

Experimental procedures

Animals and tissue collection

The two species were reared in the laboratory, sexed as pupae and maintained at 20 °C and 60% humidity until emergence. Dissected tissues from sexually mature males (antennae, brain, proboscis, wings, legs, thorax and abdomen) and females (antennae) were frozen in liquid nitrogen and stored at −80 °C until use. For *in situ* hybridization, male antennae were removed from adults, cut into pieces and fixed overnight in 4% paraformaldehyde (PFA) at 4 °C.

RNA isolation and cDNA synthesis

Total RNAs were extracted with TRIzol reagent (Gibco, BRL, USA) and treated with DNase (Promega, France). Single-stranded cDNAs were synthesized from 1 μg of total RNAs from various tissues using 200 U of M-MLV reverse transcriptase (Advantage™ RT-for-PCR kit, Clontech, USA). For 3′ and 5′ RACE-PCR, antennal cDNAs were synthesized from 1 μg of male antennal total RNAs at 42 °C for 1.5 h using the SMART™ RACE cDNA Amplification kit (Clontech) with 200 U of Superscript II (Gibco, BRL, USA), 5′-CDS-primer and SMART II oligonucleotide.

Cloning of a putative esterase antennal cDNA in male antennae of the two moths

For *S. littoralis* esterase amplification, two degenerate primers, SlESTUp1 and SlESTDo1 (Table 1) were designed according to highly conserved regions of various insect esterase sequences. PCR was carried out with 1 U Taq DNA polymerase (Invitrogen, USA) using an annealing temperature of 50 °C and 40 cycles of amplification. The 950-bp PCR product obtained was gel purified (GenElute™ Minus EtBr Spin Columns, Sigma, USA) and cloned into pCR II-Topo plasmid (Invitrogen). Recombinant plasmids were isolated by mini preparation (QIAprep® Spin Miniprep Kit, Qiagen, France) and sequenced (GenomeExpress). For *S. nonagrioides*, 3′ RACE-PCR was achieved with Universal Primer Mix (UPM; Clontech) and a degenerate primer (3′ESTSnUp, Table 1) corresponding to a consensus sequence conserved in various insect esterases. Touchdown PCR was performed as follow: after 5 min at 94 °C, three cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C, three cycles of 30 s at 94 °C, 30 s at 57 °C and 30 s at 72 °C, then three cycles of 30 s at 94 °C, 30 s at 54 °C and 30 s at 72 °C, followed by 25 cycles of 30 s at 94 °C, 30 s at 50 °C and 30 s at 72 °C, with a final elongation step of 5 min at 72 °C. The 1340-bp PCR fragment generated was purified, cloned and sequenced as described above.

The sequences of the 950 bp and 1340 bp PCR products from *S. littoralis* and *S. nonagrioides*, respectively, were analysed using BLAST (Altschul *et al.*, 1990) and GeneJockey software (Biosoft, Cambridge, UK), leading to the identification of these two fragments as putative esterases. The 3′ and 5′ regions of the corresponding cDNA from *S. littoralis* were obtained by 3′–5′ RACE using two specific primers (3′ESTSlUp and 5′ESTSlDo, respectively). For *S. nonagrioides*, the 5′ part of the corresponding cDNA was obtained by 5′ RACE, using a specific antisense primer, 5′ESTSnDo. Touchdown PCR was performed as described in

Maïbèche-Coisne *et al.* (2002). The different PCR products obtained were purified and cloned as described above.

By merging the overlapping sequences obtained by PCR amplification, 3′ and 5′ RACE, two putative full-length cDNAs of 2835 bp and 1854 bp, called *Slit-EST* and *Snon-EST*, respectively, were generated in each species. In order to confirm the complete sequences, two pairs of specific primers (Table 1) were designed according to the Open Reading Frame (ORF) of the putative esterases of both species: SlESTorf Up/Do for *S. littoralis* and SnESTorf Up/Do for *S. nonagrioides*. Thirty-five cycles of PCR were performed with the high fidelity Taq DNA polymerase PHUSION (1 U; Finnzymes, Finland) and 1 μl of 3′-ready cDNA, with annealing temperatures of 58 °C for *Slit-EST* and 50 °C for *Snon-EST*. cDNA fragments were cloned and sequenced as described above for sequence confirmation.

Tissue distribution of Slit-EST *and* Snon-EST *by Northern blot analysis*

Two specific and two control digoxigenin labelled probes were synthesized by PCR on antennal cDNAs using the Dig-DNA labelling mix (Roche Diagnostics, France) and four specific primers: in *S. littoralis* (1) a 701 bp fragment of the *Slit-EST* cDNA (SlESTUpsp and SlESTDosp) and (2) a ~500 bp fragment of the *rpL8* cDNA (*rpL8* primers described in Maïbèche-Coisne *et al.*, 2004), and in *S. nonagrioides* (3) a 540 bp fragment of the *Snon-EST* cDNA (SnESTUp and SnESTorfDo) and (4) a ~500 bp fragment of the *rpL8* cDNA. Total RNAs (20 μg) from different tissues collected from male and female *S. littoralis* and *S. nonagrioides* were separated on a 1% formaldehyde gel and transferred on to a positively charged nylon membrane. Equal loading was monitored by comparing the density of the ribosomal RNA band under UV on the agarose gel and membrane, before and after transfer. For each species, sequential overnight hybridization at 45 °C of the two genes, *EST* and *rpL8* control, washes and chemiluminescent detection were performed as described previously (Maïbèche-Coisne *et al.*, 2002).

In situ *hybridization*

A cDNA fragment of 471 bp for *S. littoralis* and another one of 483 bp for *S. nonagrioides* were obtained by PCR strategy using, respectively, 3′ESTSlUp/SlESTDosp primers and SnESTUp/SnESTDo, and cloned into pCR-II Topo plasmid. The recombinant plasmids obtained were used for PCR using M13 reverse and forward primers. The amplification products were used as matrix for *in vitro* transcription. RNA sense and antisense probes were performed using T7 and SP6 RNA polymerase (Promega) using the Riboprobe® kit (Promega). Probes were purified with RNA G50 sephadex columns (Quick Spin columns, Roche). The hybridization protocol was performed on whole-mount pieces of antennae, as described in Jacquin-Joly *et al.* (2000). Longitudinal and transversal sections were performed at 6 μm, counter-stained with acridine orange and photographed. Pictures were digitized and processed using Adobe Photoshop 7.0 (Adobe, USA).

Native PAGE separation of extracts from different tissues and esterase staining

Male and female tissues from *S. littoralis* and *S. nonagrioides* were homogenized on liquid nitrogen in 10 mM Tris-HCl buffer at pH 7.4 and pH 8.0, respectively. Homogenates were centrifuged at 18 500 g for 5 min at 4 °C and supernatants were used immediately for electrophoresis. Separation of proteins by native PAGE on a 10% acrylamide gel and esterase's staining using a substrate mixture of α- and β-naphthyl acetate were done as described (Vogt & Riddiford, 1981).

Acknowledgements

This work was supported by Université Paris VI, INRA, an Ile-de-France region graduate fellowship to C. Merlin, and a PICASSO API fellowship. We also thank CICYT (Project AGL2003-06599-C02-01), Generalitat de Catalunya (ACI, 2002) and MEC (HF2004-0272) for financial support. We are also grateful to D. Tauban and A. Popescu (INRA, Versailles) for the SEM on male antennae of *S. littoralis*.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403–410.

Bendtsen, J.D., Nielsen, H., Von Heijne, G. and Brunak, S. (2004) Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* **340**: 783–795.

Callahan, F., Vogt, R.G., Tucker, M., Dickens, J. and Mattoo, A. (2000) High level expression of 'male-specific' pheromone binding proteins in the antennae of female noctuid moths. *Insect Biochem Mol Biol* **30**: 507–514.

Corpet, F. (1988) Multiple sequence alignment with hierarchical clustering. *Nucl Acids Res* **16**: 10881–10890.

Cygler, M., Schrag, J.D., Sussman, J.L., Harel, M., Silman, I., Gentry, M.K. and Doctor, B.P. (1993) Relationship between sequence conservation and three-dimensional structure in a large family of esterases, lipases, and related proteins. *Protein Sci* **2**: 366–382.

Durán, I., Parrilla, A., Feixas, J. and Guerrero, A. (1993) Inhibition of antennal esterases of the Egyptian armyworm *Spodoptera littoralis* by trifluoromethyl ketones. *Bioorg Med Chem Lett* **3**: 2593–2598.

Ferkovich, S., Van Essen, F. and Taylor, T.R. (1980) Hydrolysis of sex pheromone by antennal esterases of the cabbage looper *Trichoplusia ni*. *Chem Senses* **5**: 33–46.

Ishida, Y. and Leal, W.S. (2002) Cloning of putative odorant-degrading enzyme and integumental esterase cDNAs from the wild silkmoth, *Antheraea polyphemus*. *Insect Biochem Mol Biol* **32**: 1775–1780.

Ishida, Y. and Leal, W.S. (2005) Rapid inactivation of a moth pheromone. *Proc Natl Acad Sci USA* **102**: 14075–14079.

Jacquin-Joly, E., Bohbot, J., Francois, M.C., Cain, A.H. and Nagnan-Le Meillour, P. (2000) Characterization of the general odorant-binding protein 2 in the molecular coding of odorants in *Mamestra brassicae*. *Eur J Biochem* **267**: 6708–6714.

Jacquin-Joly, E. and Lucas, P. (2005) Pheromone reception and transduction: common importance and converging mechanisms across phyla. *Cur Top Neurochem* **4**: 75–105.

Jørgensen, K., Kvello, P., Almaas, T.J. and Mustaparta, H. (2006) Two closely located areas in the suboesophageal ganglion and the tritocerebrum receive projections of gustatory receptor neurons located on the antennae and the proboscis in the moth *Heliothis virescens*. *J Comp Neurol* **496**: 121–134.

Kaissling, K.E. (2001) Olfactory perireceptor and receptor events in moths: a kinetic model. *Chem Senses* **26**: 125–150.

Kamikouchi, A., Morioka, M. and Kubo, T. (2004) Identification of honeybee antennal proteins/genes expressed in a sex- and/or caste selective manner. *Zool Sci* **21**: 53–62.

Klein, U. (1987) Sensillum-lymph proteins from antennal olfactory hairs of the moth *Antheraea polyphemus* (Saturniidae). *Insect Biochem* **17**: 1193–1204.

Ljungberg, H., Anderson, P. and Hansson, B.S. (1993) Physiology and morphology of pheromone-specific sensilla on the antennae of male and female *Spodoptera littoralis* (Lepidoptera: Noctuidae). *J Insect Physiol* **39**: 253–260.

Maïbèche-Coisne, M., Jacquin-Joly, E., François, M.C. and Nagnan-Le Meillour, P. (2002) Characterization of biotransformation enzymes belonging to the P450 family in the antennae of the noctuid moth *Mamestra brassicae*. *Insect Mol Biol* **11**: 273–281.

Maïbèche-Coisne, M., Merlin, C., François, M.C., Queguiner, I., Porcheron, P. and Jacquin-Joly, E. (2004) Putative odorant-degrading esterase cDNA from the moth *Mamestra brassicae*: cloning and expression patterns in male and female antennae. *Chem Senses* **29**: 381–390.

Oakeshott, J.G., Claudianos, C., Russell, R.J. and Robin, G.C. (1999) Carboxyl/cholinesterases: a case study of the evolution of a successful multigene family. *Bioessays* **21**: 1031–1042.

Pophof, B. (1997) Olfactory responses recorded from sensilla coeloconica of the silkmoth *Bombyx mori*. *Physiol Entomol* **22**: 239–248.

Quero, C., Bau, J., Guerrero, A. and Renou, M. (2004) Responses of the olfactory receptor neurons of the corn stalk borer *Sesamia nonagrioides* to components of the pheromone blend and their inhibition by a trifluoromethyl ketone analogue of the main component. *Pest Manag Sci* **60**: 719–726.

Quero, C., Rosell, G., Jiménez, O., Rodriguez, S., Pilar Bosch, M. and Guerrero, A. (2003) New fluorinated derivatives as esterase inhibitors. Synthesis, hydration and crossed specificity studies. *Bioorg Med Chem* **11**: 1047–1055.

Satoh, T., Taylor, P., Bosron, W., Sanghani, S., Hosokawa, M. and La Du, B. (2002) Current progress on esterases: from molecular structure to function. *Drug Metab Disp* **30**: 488–493.

Solinas, M. and Trona, F. (2002) Antennal sensilla of *Sesamia nonagrioides* (Lef.) (Lepidoptera: Noctuidae). Morphology and possible behavioural significance. *Bull Zool Agr Bachic, Series II* **34**: 321–345.

Vickers, N.J. (2006) Winging it: moth flight behavior and responses of olfactory neurons are shaped by pheromone plume dynamics. *Chem Senses* **31**: 155–166.

Vogt, R.G. (2003) Biochemical diversity of odor detection: OBPs, ODEs and SNMPs. In: *Insect Pheromone Biochemistry and Molecular Biology* (Blomquist, G.J. and Vogt, R.G., eds), pp. 391–445. Elsevier, Oxford, UK.

Vogt, R.G. (2005) Molecular basis of pheromone detection in insects. In: *Comprehensive Insect Physiology, Biochemistry, Pharmacology and Molecular Biology*, Vol. 3. *Endocrinology*. (Gilbert, L.I., Iatro, K., Gill, S., eds), pp. 753–804. Elsevier, London, UK.

Vogt, R.G. and Riddiford, L.M. (1981) Pheromone binding and inactivation by moth antennae. *Nature* **293**: 161–163.

Vogt, R.G. and Riddiford, L.M. (1986) Scale esterase: a pheromone-degrading enzyme from the scales of the silk moth *Antheraea polyphemus*. *J Chem Ecol* **12**: 469–482.

Vogt, R.G., Riddiford, L.M. and Prestwich, G. (1985) Kinetic properties of a sex pheromone-degrading enzyme: the sensillar esterase of *Antheraea polyphemus*. *Proc Natl Acad Sci USA* **82**: 8827–8831.

