Chem. Senses 29: 381–390, 2004 DOI: 10.1093/chemse/bjh039

Putative Odorant-degrading Esterase cDNA from the Moth *Mamestra brassicae*: Cloning and Expression Patterns in Male and Female Antennae

Martine Maïbèche-Coisne[1,2], Christine Merlin[1,2], Marie-Christine François[1], Isabelle Queguiner[2], Patrick Porcheron[2] and Emmanuelle Jacquin-Joly[1]

[1]INRA, Unité de Phytopharmacie et Médiateurs Chimiques, Bât. A, route de Saint-Cyr, F-78026 Versailles Cedex, France and [2]Université Paris VI, EA3501, Laboratoire Physiologie Cellulaire des Invertébrés, 12 rue Cuvier, F-75005 Paris, France

Correspondence to be sent to: M. Maïbèche-Coisne, INRA Unité de Phytopharmacie et Médiateurs Chimiques, Bat. A, route de Saint-Cyr, F-78026 Versailles Cedex, France. e-mail: maibeche@versailles.inra.fr

Abstract

An esterase cDNA was isolated from the cabbage armyworm *Mamestra brassicae* antennae by PCR strategy. The full-length cDNA, designated as *Mbra-EST*, contains a 1638 bp open reading frame encoding a predicted protein of 546 amino acids. This predicted protein presents the structural characteristics of known insect carboxyl-esterases, in particular the Ser–His–Glu catalytic triad. The expression pattern of the gene was studied by RT–PCR, Northern-blot and *in situ* hybridization. The ribosomal protein *rpL8* gene from *M. brassicae* was also cloned to obtain a normalized tool for the comparative gene expression studies. *Mbra-EST* transcripts are specifically expressed in the antennae of males and females and in the proboscis of males. In antennae of both sexes, expression is restricted to the olfactory sensilla trichodea, suggesting a role in degradation of odorant acetate compounds, such as pheromones as well as plant volatile acetate components.

Key words: antennal esterase, *Mamestra brassicae*, odorant-degrading enzymes, olfaction

Introduction

Olfaction is an important sensory modality in insects, playing a crucial role in reproduction (mate and oviposition site finding) or in nutrition (detection of food sources). Recognition of chemical signals takes place in olfactory sensilla located on the antennae. These sensilla are cuticular structures with an aqueous lumen surrounding the dendrites of the olfactory neurons, and containing high concentrations of odorant-binding proteins (OBPs), as well as extracellular odorant-degrading enzymes (ODEs; Kaissling, 2001). Hydrophobic odorant molecules and pheromones entering the sensilla are supposed to interact with OBPs, which transport them through the aqueous lumen and deliver them to the dendritic olfactory receptors (Vogt *et al.*, 1999). After this interaction with olfactory receptors (ORs), degradation of odorant molecules is a necessity to avoid the continuous stimulation of the receptors. This last step is particularly important in odorant-mediated behaviors in moths, well exemplified in pheromone communication. Males have to know immediately when they leave the pheromone trail emitted by females to obtain a spatial and temporal resolution of the sex pheromone plume. Indeed, in flying moths, the progress upwind toward the source is modulated by 'moment-to-moment' interception of the sinuous and filamentous structure constituting the pheromone plume (Bau *et al.*, 2002).

In contrast to the number of OBPs and ORs known to date in insects, only a few insect ODEs have been identified at the molecular level. The first studies were focused on enzymes able to degrade lepidopteran sex pheromones. These pheromones usually consist of blends of a few chemicals, generally straight chain esters, aldehydes or alcohols, whose presence and relative abundance ensure the specificity of communication and contribute to reproductive isolation between species. In Lepidoptera, two major types of extracellular antennal-specific ODEs were characterized by biochemical studies: a 55 kDa male antennal-specific sensillar esterase (SE) was first identified in *Antheraea polyphemus* (ApolSE) (Vogt and Riddiford, 1981; Vogt *et al.*, 1985; Maida *et al.*, 1993), followed by the aldehyde-oxidases from *Manduca sexta* (Rybczynski *et al.*, 1989), *A. polyphemus* and *Bombyx mori* (Rybczynski *et al.*, 1990). These soluble enzymes are localized within the extracellular sensilla lumen where they are able to degrade pheromone compounds. *A. polyphemus* and *M. sexta* ODEs were shown

to degrade rapidly the sex pheromone with an *in vivo* estimated pheromone half life of, respectively, 15 and 0.6 ms in the presence of these enzymes (Vogt *et al.*, 1985; Prestwich *et al.*, 1986; Rybczynski *et al.*, 1989). However, some other studies have reported slow rates of antennal pheromone degradation (for a review, see Vogt, 2002). The mechanisms of pheromone deactivation are thus still under discussion. Instead of a rapid inactivation by enzymatic processes, some authors favor a first inactivation by OBPs followed by enzymatic degradation (Steinbrecht, 1998; Kaissling, 2001). In addition to extracellular ODEs located in the sensillum lymph, other intracellular ODEs, such as glutathione-*S*-transferase or cytochrome P450, are supposed to degrade odors entering the support cells of the sensilla (Rogers *et al.*, 1999; Wang *et al.*, 1999; Maïbèche-Coisne *et al.*, 2002). A particular case is the cytochrome P450 characterized in the beetle *Phyllopertha diversa*. Indeed, this enzyme is able to degrade the pheromone and may be localized in the plasma membrane of the dendrite (Wojtasek and Leal, 1999).

No molecular data were available on extracellular odorant-degrading enzymes until recently. An esterase cDNA, Apol-ODE, which could correspond to ApolSE, has been cloned using primers designed according to conserved regions of known insect esterase enzymes (Ishida and Leal, 2002). The 60 kDa encoded protein belongs to the carboxyl/cholinesterase family, which is a branch of the α/β hydrolase's fold superfamily that catalyzes the hydrolysis of ester bonds of various substrates, varying in size and complexity (Oakeshott *et al.*, 1999). The members of this superfamily present a high conservation of the tertiary structure and most of these enzymes are believed to use a reaction mechanism based on a Ser–His–Glu serine–protease-like catalytic triad.

In addition to ApolSE, esterase activities were found in the antennae of other moth species that use acetate compounds as pheromone, including the cabbage looper *Trichoplusia ni* (Ferkovich *et al.*, 1980) and the diamondback moth *Plutella xylostella* (Prestwich *et al.*, 1989). In the cabbage armyworm *Mamestra brassicae*, 92% of the sex pheromone blend is composed of 11-*cis*-hexadecenyl acetate (*Z*11–16:Ac; Farine *et al.*, 1981). We thus focused our work on this class of enzymes. We used a PCR-based strategy with a degenerate primer to amplify cDNA fragments from antennal extracts. A full-length cDNA encoding a putative antennal esterase (*Mbra-EST*) was isolated, and its expression pattern was studied by RT–PCR, Northern-blot and *in situ* hybridization. In order to have a normalized tool for the comparative gene expression experiments, we also cloned the ribosomal protein *rpL8* gene from *M. brassicae*.

The Mbra-EST deduced protein shares the common structural features of known insect esterases and the corresponding transcripts are specifically expressed in the antennae of males and females, as well as in the proboscis of males. In antennae of both sexes, expression is restricted to the olfactory trichodea sensilla, which strongly suggests a potential role in odorant clearance.

Material and methods

Animals and tissue collection

Animals were reared in Domaine du Magneraud (INRA, France). Pupae were maintained at 20°C and 60% humidity until emergence. Tissues from sexual mature 3-day-old males (antennae, brain, proboscis, gut, fat body, legs, thorax and abdomen) and females (antennae, brain) were dissected and used directly for total RNA isolation. For *in situ* hybridization, male antennae were immediately fixed in 4% paraformaldehyde (PFA).

RNA isolation and cDNA synthesis

Total RNAs were extracted with TRIzol reagent (Gibco BRL), according to the manufacturer's instructions, and were quantified by spectrophotometry at 260 nm. Single-stranded cDNAs for PCR were synthesized from total RNAs (1 µg) from various tissues with 200 U of M-MLV reverse transcriptase using the Advantage™ RT-for-PCR kit (Clontech). The reaction contained a dNTP mix, Rnasin, Oligo(dT) primer and sterile water to a final volume of 25 µl. The mix was heated at 70°C for 2 min before adding the enzyme and then incubated for 1 h at 42°C. For 5′ and 3′ RACE PCR, antennal cDNAs were synthesized from 1 µg of male antennal total RNA at 42°C for 1.5 h using the SMART™ RACE cDNA Amplification kit (Clontech) with 200 U of SuperscriptII (Gibco BRL) according to the kit instructions.

Cloning of the 3′ part of the putative esterase cDNA by 3′ RACE

A degenerate sense primer (3′EstUp: 5′-AACTTTGGMCT-NAARGAYCA-3′) corresponding to the amino acid sequence NFGLKDQ, which is conserved in various insect esterases, was designated for 3′ RACE amplification. We used 1 µl of 3′-RACE-ready cDNA with Universal Primer Mix (UMP; Clontech) as antisense primer. After 5 min at 94°C, touchdown PCR was performed with hot start as follow: three cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C; then three cycles of 30 s at 94°C, 30 s at 57°C and 1 min at 72°C, followed by three cycles of 30 s at 94°C, 30 s at 54°C and 1 min at 72°C; then 25 cycles of 30 s at 94°C, 30 s at 50°C and 1 min at 72°C, with a final elongation step of 5 min at 72°C. A PCR fragment of ~1200 bp was gel purified (Gene Clean II kit, Bio101, Inc) and cloned into pCR®II-Topo plasmid (Invitrogen). Recombinant plasmids were purified (QIAprep® Spin Miniprep kit; Qiagen) and sequenced (GenomeExpress). Gene sequence analyses and database comparisons were carried out using the BLAST (Altschul *et al.*, 1990) or GeneJockey software (Biosoft, Cambridge, UK). One clone of 1218 bp was identified as a putative new carboxylesterase.

Cloning of the 5′ part of the putative esterase by 5′ RACE

The 5′ region of the corresponding cDNA was obtained by 5′ RACE using a specific antisense primer 5′ EstDo (5′-GATTGTAGGACTGTCAGGCAGGAATGGGGT-3′). We used 2.5 µl of 5′-RACE-ready cDNA with UMP as sense anchor primer. Touchdown PCR was performed as described in Maïbèche-Coisne *et al.* (2002). The PCR product of 980 bp was purified and cloned as described above. By merging the overlapping sequences obtained by 3′ and 5′ RACE, a putative full-length cDNA of 1758 bp, called *Mbra-EST* was generated.

Cloning of the ribosomal *rpL8* cDNA

Two degenerate primers were deduced from consensus motifs from several rpL8 proteins sequences, including *Spodoptera frugiperda* (GenBank accession no. AF429973), *Drosophila melanogaster* (GenBank accession no. AF098520) and *Aedes albopictus* (GenBank accession no. M99055) sequences. The sequence of the sense primer is based on the amino-acid motif RVIRAQRK (rpL8Up: 5′-GAGTCATCCGAGCTCARMGNAARGG-3′) and the antisense primer is based on the amino-acid sequence YKVKRNCW (rpL8Do: 5′-CCAGCAGTTTCGCTTNA-CYTTRTA-3′). A cDNA fragment of 580 bp was obtained by RT–PCR after 35 cycles (94°C for 30 s; 50°C for 30 s; 72°C for 30 s) followed by a 5 min step at 72°C. The 5′ and 3′ regions of the cDNA were obtained by 5′–3′ RACE using 2 specific primers (5′-CGGGGTCGTGGATGATGTCCT-TCACGAC-3′ and 5′-CGGCGGTGGTCGTATT-GACAAGCCCATC-3′) as described above.

Northern-blot and RT–PCR studies

Equal amounts of total RNA from various tissues (20 µg/lane) were separated on a 0.9% formaldehyde gel and transferred onto a positively charged nylon membrane. Equal loading was monitored by comparing under UV the density of the 18S ribosomal RNA band on the agarose gel and membrane, before and after the transfer. Two specific digoxigenin-labeled probes were produced by PCR on antennal cDNAs using the Dig-DNA labeling mixture (Roche) and two specific primers: (i) a 447 bp fragment of the *Mbra-EST* cDNA (EstUp: 5′-ACCCCATTCCTGCCT-GACAGTCCC-3′ and EstDo: 5′-ATCGCCGT-GCGTCGCTCCGGG-3′) and (ii) a 508 bp fragment of the *rpL8* cDNA (rpL8Up and rpL8Do). They were sequentially hybridized to the membrane overnight at 45°C in high SDS buffer (50 mM sodium phosphate pH 7.0; 50% formamide; 7% SDS; 5× SSC; 0.1% *N*-lauroylsarcosine; 2% blocking reagent from Boehringer) as described previously (Maïbèche-Coisne *et al.*, 2002). After stringent washes of the membrane (2× SSC, 0.1% SDS at room temperature followed by 0.5× SSC, 0.1% SDS at 68°C), luminescent detection of the bound probes was performed using a DIG luminescent detection kit (Boehringer).

For RT–PCR, equal amounts of RNA extracted from male antennae, proboscis, thorax and abdomen and from female antennae were reverse-transcripted and simultaneously amplified with *Mbra-EST* and *rpL8* primers (to obtain, respectively, fragments of 447 and 508 bp). Products of amplification were loaded on 1.8% agarose gel and visualized by ethidium bromide. After Northern-blot and RT–PCR experiments, fluorescence intensities of the bands from the film or the gel were quantified using FluorChem™ 800 Advanced Fluorescence Imaging (Alpha Innotech Corporation). Relative levels refer to ratios of *Mbra-EST* versus *rpL8* intensities that were converted to percentages of maximal level for each experiment.

In situ hybridization

The recombinant pCR®II-Topo plasmid, containing the 447 bp *Mbra-EST* insert, was purified and linearized. RNA sense and antisense probes were *in vitro* transcribed from linearized plasmids using T7 and SP6 RNA polymerase (Promega) following recommended protocol. Probes quality was confirmed under denaturing conditions by formaldehyde agarose gel electrophoresis. Hybridization protocol was performed on whole-mount pieces of antennae as described in Jacquin-Joly *et al.* (2000), with a hybridization temperature of 65°C. Longitudinal sections were performed at 5 µm and were photographed, then pictures were digitized and processed using Adobe Photoshop® 5.5.

Native PAGE separation of antennal extracts and esterase staining

Male and female antennae were homogenized on ice in 10 mM Tris-HCl buffer (pH 7.0). Homogenates were centrifuged at 5000 r.p.m. for 15 min and the supernatants were immediately used 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 by Vogt and Riddiford (1981).

Results

Cloning and cDNA sequencing of Mbra-EST, an antennal esterase cDNA

A 1218 bp cDNA product was amplified by RT–PCR on male antennal RNAs by 3′ RACE using a degenerate oligonucleotide primer deduced from a conserved region of various insect esterases. After cloning and sequencing, this product was translated and the deduced amino-acid sequence appeared to belong to the carboxylesterase family. It was then extended to the 5′ region by 5′ RACE using a specific primer. This allowed us to obtain the sequence of a full-length cDNA of 1758 bp (Figure 1). This sequence has been deposited in the GenBank database with accession no. AY390258. Nucleotide sequence analysis revealed that this cDNA contains a putative coding region (ORF) of 1638 bp,

```
                                                                          gtgtcattgatcaaaacagccaacaaa     -1

ATG AAG AAG TAT TTG AAG TCC TTG TCG AGT TTG AAG CTG TTG GTG CTG ATC TCG CTG TGG GTG GGT CCG CTG    72
 M   K   K   Y   L   K   S   L   S   S   L   K   L   L   V   L   I   S   L   W   V   G   P   L     24

GTG TCA CAG CCC ACA GCC CCG GTG CAG CTG AGT GGA CAG GCC CGG GGC GAG GTG GCC TCT GAT GGA TCA CAC   144
 V   S   Q   P   T   A   P   V   Q   L   S   G   Q   A   R   G   E   V   A   S   D   G   S   H     48

CTG CAG TAC TTC GGC ATA CCG TAC GCC ACC GTC ACA AAC AGG TTC CAA GAA GCA ACA CCG AAT CCG AAA TGG   216
 L   Q   Y   F   G   I   P   Y   A   T   V   T   N   R   F   Q   E   A   T   P   N   P   K   W     72

GAA GGA GTT TAC GAC GCA AAC AAT GAA CAC ATC AGA TGC AAG CAA CGC TTC CAC CCC ACC CCG GAT ATG GGC   288
 E   G   V   Y   D   A   N   N   E   H   I   R   C   K   Q   R   F   H   P   T   P   D   M   G     96

GAC GAG GAC TGC CTC ACC GTC AAC GTG TAC ACC CCC GTG GAG CCC AGC GAC AGC CTC CGA CCC GTT ATG GTC   360
 D   E   D   C   L   T   V   N   V   Y   T   P   V   E   P   S   D   S   L   R   P   V   M   V    120

TTC ATA CAT GGA GGA GCA TTC AGG GAC GGC TCC GGA TCC CCC TTC CTC TAT GGC CCC AAG TAC CTA GTA AAA   432
 F   I   H   G   G   A   F   R   D   G   S   G   S   P   F   L   Y   G   P   K   Y   L   V   K    144

CAT GGA GTC ATC CTA GTC ACC TTC AAC TAC AGA CTC GAA ATT CTT GGC TTC CTA TGT CTA GGC ATC AAG GAA   504
 H   G   V   I   L   V   T   F   N   Y   R   L   E   I   L   G   F   L   C   L   G   I   K   E    168
                        3'EstUp
GCA CCC GGC AAT ATT GGC CTG AAA GAT CAA GTG CAA GCA CTC AAG TGG GTG AAG AGA AAC ATA AGA GTT TTC   576
 A   P   G   N   I   G   L   K   D   Q   V   Q   A   L   K   W   V   K   R   N   I   R   V   F    192

GGA GGT GAT CCC GAT AAT ATA ACC ATT TTT GGA GAA AGT GCG GGG TCT GCT TCA GTT TCT TAC CAT TTA CTC   648
 G   G   D   P   D   N   I   T   I   F [ G   E   S   A   G ] S   A   S   V   S   Y   H   L   L    216

TCT CCA ATG TCT AAA GGA CTC TTT AAC AGA GCT ATC CTG CAA AGT GGC TCA GCT TTA AGC TTT TGG GCC TTA   720
 S   P   M   S   K   G   L   F   N   R   A   I   L   Q   S   G   S   A   L   S   F   W   A   L    240

CAG TTT GAA CCT TTG AAG ATA GCT AGT CTA CAC GCT CAA CAA ATG GGG TAC ACA ACC ACT GAT CCT AAG GAA   792
 Q   F   E   P   L   K   I   A   S   L   H   A   Q   Q   M   G   Y   T   T   T   D   P   K   E    264

ATC TAC AAT TTA TTC AAT AAT GTG ACT GCT GAT GAA CTG TTA AGC TAT TAT GTT CCA AGA AAG GAA GGT GAT   864
 I   Y   N   L   F   N   N   V   T   A   D   E   L   L   S   Y   Y   V   P   R   K   E   G   D    288
                                                                             5'Estdo / EstUp
ATA GTG GAA TCA GAC ATT ATT TTC GTG CCA TGC GTT GAA AAG AAA ATT CGT AGA GTA ACC CCA TTC CTG CCT   936
 I   V   E   S   D   I   I   F   V   P   C   V   E   K   K   I   R   R   V   T   P   F   L   P    312

GAC AGT CCC TAC AAT CTG ATC ACA CAA GGA AAA TAT AAC AAA GTC CCT ATC ATC ATT GGT TTC AAT AGC GCC  1008
 D   S   P   Y   N   L   I   T   Q   G   K   Y   N   K   V   P   I   I   I   G   F   N   S   A    336

GAA GGT TAC TAT TTT GCT GGT AAA GAG AAC GAT ACT ACA TTG TCT AAG ATA GAT TTC TAT AAG GCC ATG CCG  1080
 E   G   Y   Y   F   A   G   K   E   N   D   T   T   L   S   K   I   D   F   Y   K   A   M   P    360

AGA GAT TTA ACT TTC CCT TTT GAT GAG GAA AAG GTG AAA ACA GCT AAT AGA TTG AAT GAG CTG TAC TTG GCT  1152
 R   D   L   T   F   P   F   D   E   E   K   V   K   T   A   N   R   L   N   E   L   Y   L   A    384

GGA GAT AAA ATT ACT AAA GAA AAG TCT TCG TTG GAA AAG TTG GCA CGG TTC AAA GGA GAT GCA GAT ATT ATT  1224
 G   D   K   I   T   K   E   K   S   S   L   E   K   L   A   R   F   K   G   D   A   D   I   I    408

TAC CCT GTA TTA GCA ACC ATA GAT CTA TTT CTA CAG ACG ACA AGG AAA CCT GTG TAC GCT TAC AAA TTC AAT  1296
 Y   P   V   L   A   T   I   D   L   F   L   Q   T   T   R   K   P   V   Y   A   Y   K   F   N    432
                                                                               EstDo
TAC GAT GGG TTA CTA AAC CTC GCT AAG ATG ACG CAT GGG TTC CGC AAG TCC CCC GGA GCG ACG CAC GGC GAT  1368
 Y   D   G   L   L   N   L   A   K   M   T   H   G   F   R   K   S   P   G   A   T   H   G   D    456

GAA TTA TTC TAC CTG TTC AGC ACC TTG ACG TTA CCG GCG TTG TCA GAA GTG AGC TTT ATT GAC AAG TTT ACT  1440
 E   L   F   Y   L   F   S   T   L   T   L   P   A   L   S   E   V   S   F   I   D   K   F   T    480

ACT CTG TGG ACC AAC TTT GCG AAG TAC AGT GAT CCT ACT CCA GCC TCA TCT TCA ATC TCT CCG AAG TGG GAG  1512
 T   L   W   T   N   F   A   K   Y   S   D   P   T   P   A   S   S   S   I   S   P   K   W   E    504

CCT GCA GCA GTG GAG GAC CCT CGC CTT CTG CTC ATA GAC AGG GAG TGC TCT ATG GAA CCT GTT TGG GGC GAC  1584
 P   A   A   V   E   D   P   R   L   L   L   I   D   R   E   C   S   M   E   P   V   W   G   D    528

AAC GAA GCC TGG CGG TTT TGG AAC GAG ACA TAC GCT TTG TAT AGG AGG AAG AAT  taaagtagtttgtcttagagtta 1661
 N   E   A   W   R   F   W   N   E   T   Y   A   L   Y   R   R   K   N                            546

atcttaaactgagattaaaaggtgacacttgaagaaatcggcaaaaaaaaaaaaaaaaaaaaaaaaaa                          1731
```

Figure 1 Nucleotide and deduced amino acid sequence of *M. brassicae* antennal carboxylesterase-like cDNA (*Mbra-EST*; GenBank accession no. AY390258). Primers used for 3′–5′ RACE and RT–PCR are doubly underlined and the putative polyadenylation signal sequence is dotted underlined. The predicted cleavage site is in bold, the Ser catalytic site is boxed and the potential N-glycosylation sites are in bold and underlined.

a 27 bp 5′-untranslated region (5′-UTR) and a 93 bp 3′-UTR, with putative polyadenylation signal upstream of the poly(A).

Analysis of the primary structure of *M. brassicae* antennal esterase

Mbra-EST encodes a 546 amino-acid protein, with a signal sequence cleavage site predicted between Ser-26 and Gln-27 by SignalP V 2.0 (Nielsen *et al.*, 1997). Mbra-EST predicted protein exhibits important characteristics of carboxylesterases, namely the size (~550 amino acid residues) and the putative Ser active site with the conservated sequence Gly^{203}–Glu–**Ser**–Ala–Gly–Ala (Figures 1 and 2), which is found in many esterases. The predicted protein has a theoretical molecular mass of 61.5 kDa and an isoelectric point of 7.96, as determined using MWCALC (Infobiogen). This size is close to the size of the ODE cloned from *A. polyphemus* (59.9 kDa). For this latter, the size of the predicted protein is somewhat larger than the size of the native protein determined by electrophoresis, i.e. 55 kDa, suggesting possible post-translational modifications (Ishida and Leal, 2002). Indeed, the Apol-ODE exhibits five putative *N*-glycosylation sites, whereas *M. brassicae* predicted protein presents two potential *N*-glycosylation sites (NetNGlyc 1.0). Search in the GenBank database revealed that the sequence of Mbra-EST predicted protein is most similar to the antennal Apol-ODE deduced protein, with 34.4% of sequence identity. It shares, respectively, 27.7 and 25.5% of identity with the carboxylesterase precursor of the cotton aphid *Aphis gossypii* and with the esterase of the Hymenoptera *Anisopteromalus calandrae*, which are both involved in insecticide resistance (Suzuki, 1998; Zhu *et al.*, 1999). Mbra-EST presents only 24.5% of identity with Apol-IE, which encodes an integumental esterase expressed in various tissues including the antennae of *A. polyphemus* (Ishida and Leal, 2002). Mbra-EST is more distant to the juvenile hormone esterases of the tobacco budworm *Heliothis virescens* and the yellow mealworm *Tenebrio molitor*, with, respectively, 21.2 and 22.1% of amino acid identities (Figure 2).

Characterization of the ribosomal *rpL8* cDNA and expression studies

Ribosomal proteins (r-proteins), which are an important part of the ribosome complex, are highly conserved genes. They are considered as housekeeping genes, as shown in *Drosophila melanogaster* for *rpL8* (Rufh and Meister, 1999), *rpL17* and *rpL27a* genes (Noselli and Vincent, 1992; Garwood and Lepesant, 1994). In order to have a normalized tool for the comparative gene expression studies, we characterized the *rpL8* homolog gene from *M. brassicae*. A full cDNA sequence was obtained consisting of 877 bp, with an ORF of 774 bp. The 258 amino acid encoded protein reveals high similarities with rpL8 proteins, in particular it shares 100% of identity with *S. frugiperda* rpL8 and 78.9% of identity with rpL8 from rat. Because of these high percentages of similarity, we designate this *M. brassicae* cDNA as *Mbra-rpL8* (accession no. AY485337). Ribosomal proteins from eukaryotes share common structural features, i.e. high percentage of basic amino acids arranged in clusters, short 3′-UTR and a typical mRNA 5′-UTR oligopyrimidine stretch followed by a downstream GC-rich sequence (Levy *et al.*, 1991; Wool *et al.*, 1995). In comparison with other *rpL8*s, the *M. brassicae rpL8* cDNA presents a short 3′-UTR of 62 bp with a typical polyadenylation signal, and the 41bp 5′-UTR contains a short oligopyrimidine motif with a 5′-terminal cystidine residue. The predicted protein is a highly basic molecule, with 9.72% arginin and 10.89% lysin residues, some of them arranged in cluster (positions 21–24, for example).

In Northern-blot experiments, performed with total RNA samples, a single *Mbra-rpL8* transcript of around 900 bp was detected (Figure 3A). This abundant transcript is present in all tissues analyzed, namely adult male antennae, brains, gut, fat body, proboscis, thorax, abdomen and legs, but also in female antennae and brains. The ubiquitous and apparently constant expression of the *rpL8* mRNA from *M. brassicae* is similar to the expression pattern of the other ribosomal protein genes, consistent with their role in housekeeping functions. This gene may thus serve as a control in comparative gene expression studies by Northern-blot or RT–PCR, as done previously for the *rpL8* gene from *D. melanogaster* (Rufh and Meister, 1999).

Expression studies of *Mbra-EST*

The tissue-related expression of *Mbra-EST* was determined by Northern-blot hybridizations of total RNA samples from various tissues of adult male and female moths using DIG-labeled cDNA probes (Figure 3B) and by RT–PCR (Figure 3C). In addition to the antennae of both sexes, which carry olfactory sensilla, other chemosensory tissues were used, such as proboscis, legs and ovipositors, where taste sensilla are located. The size observed for the transcript (~2000 kb) is in agreement with the corresponding cDNA length. In Northern blot, the *Mbra-EST* expression is restricted to the male and female antennae and to the male proboscis. This was confirmed in RT–PCR analysis. For similar levels of *rpL8* expression, positive signals were restricted to the same tissues, with no expression detected in thorax or abdomens after 35 rounds of amplification (Figure 3C). After densitometry quantification, the relative level of *Mbra-EST* is stronger after Northern blot in female than in male antennae (86% of female relative level), but this result is inverted after RT–PCR. In the proboscis, the ratio of *Mbra-EST*:*rpL8* is quite the same with the two techniques (70–75% of maximal level). This suggests that there is no real difference between male and female antennal signal intensities and that the corresponding gene is not male specific or male enriched.

A precise localization of *Mbra-EST* within the antennae of males and females was achieved using *in situ* hybridization. The antennae are filiform, ~1 cm long, with a flagellum of 72

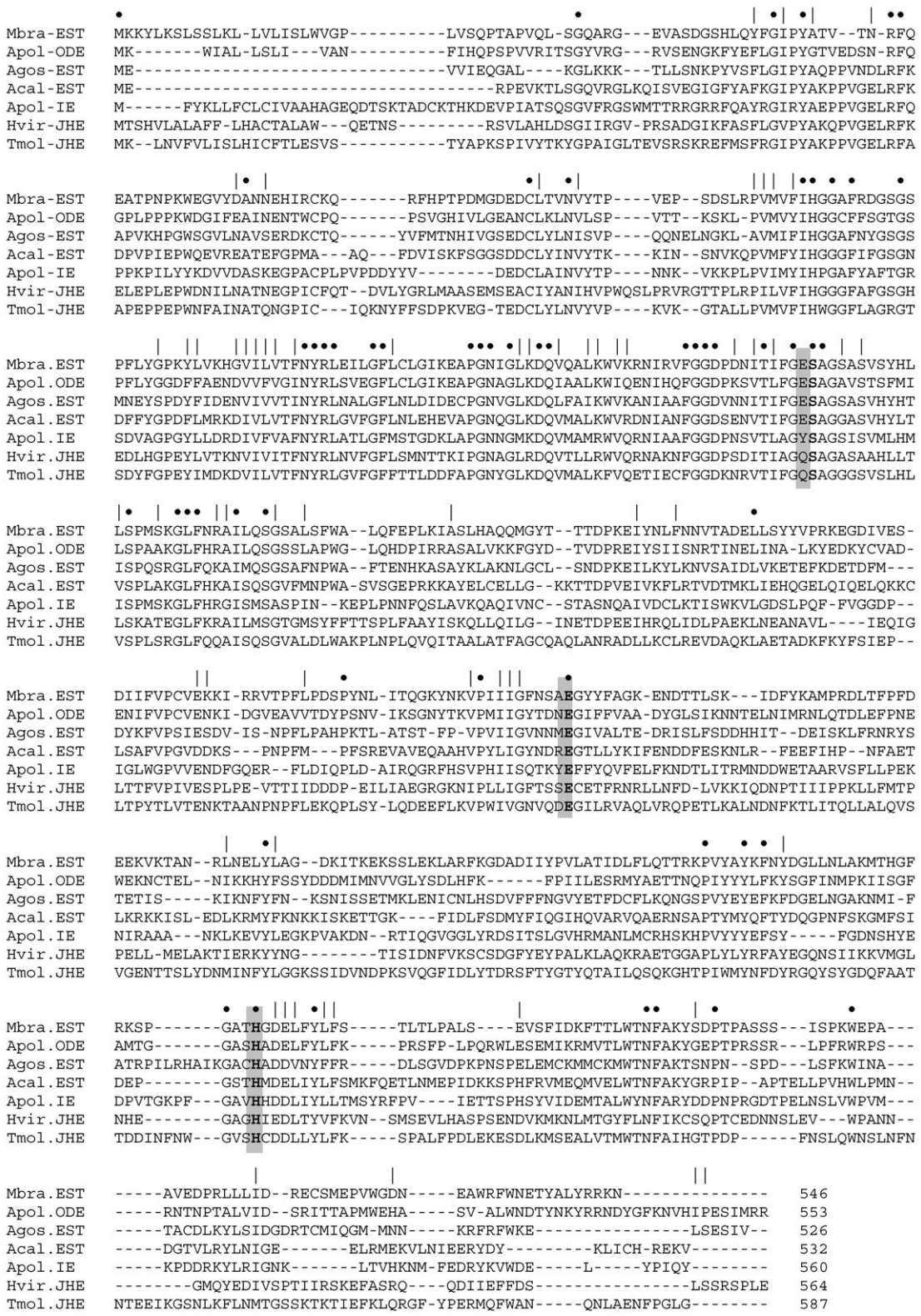

Figure 2 Predicted amino acid sequence of Mbra-EST and alignment with esterases from other six insect species. Apol-ODE, *Antheraea polyphemus* putative odorant-degrading enzyme (GenBank accession no. AAM14415); Agos-EST, *Aphis gossypii* carboxylesterase precursor (GenBank accession no. BAA32385); Acal-EST, *Anisopteromalus calandrae* carboxylesterase (GenBank accession no. AAC36245); Apol-IE, *Antheraea polyphemus* putative integumental esterase (GenBank accession no. AAM14416); Hvir-JHE, *Heliothis virescens* juvenile hormone esterase precursor (GenBank accession no. AAB96654); Tmol-JHE, *Tenebrio molitor* juvenile hormone esterase (GenBank accession no. AAL41023). Dots and vertical lines indicate respectively identical and similar amino acid residues among all seven sequences. Functionally important residues of the catalytic triad are indicated by shaded boxes.

segments (Renou and Lucas, 1994). Each segment exhibits the same general organization: the dorsal side is covered with scales and the olfactory hairs are located on the unscaled ventral side. In males, the olfactory hairs are distributed in two classes according to their length: the long sensilla trichodea (60–190 µm) are located on the lateral part of the ventral area and are settled in parallel rows, whereas short sensilla (35–55 µm) are medio-ventral and randomly distributed (Figure 4A). In females, the long hairs are absent, the short sensilla trichodea are located in the lateral and medio-ventral parts of the ventral area.

Sense strand controls gave no signals (not shown), whereas antisense *Mbra-EST* probe hybridization was clearly restricted to the sensilla side of the antenna, with no signal on the scale side (Figure 4B,E). Sections through the cuticle in the ventro-lateral region of male antennae reveal labeled spots distributed in rows, a pattern which is consistent with a localization of the *Mbra-EST* transcripts in the long sensilla trichodea (Figure 4C). On the same sections, short olfactory sensilla, which are randomly distributed, are also labeled. In female antennae, short sensilla are well labeled (Figure 4E,F). In both sexes, close examination revealed hybridization in cells localized at the base of sensory hairs (Figure 4D,F). This could correspond to the olfactory neurons, or to the accessory cells surrounding the bipolar neurons of the sensilla trichodea. The labeling is clearly observed in male long sensilla trichodea as well as in male and female short sensilla, indicating that the *Mbra-EST* transcripts are thus associated with olfactory sensilla of both sexes.

Esterase activities in male and female antennae

As *Mbra-EST* transcripts were found equally in male and female antennae, esterase activities in antennae homogenates of both sexes were investigated: after separation of proteins by 10% native PAGE, esterase staining revealed the presence of four different bands in male and female extracts (Figure 5). The intensities of the four bands are similar between male and female extracts.

Discussion

In the present work, we describe the cloning and characterization of an esterase cDNA expressed in the antennae of the moth *M. brassicae* and called *Mbra-EST*. Northern-blot and RT–PCR analysis demonstrate that *Mbra-EST* is expressed in both male and female antennae, as well as in male proboscis.

Esterase activities were found in the antennae of various moth species that use acetate compounds as pheromone, including the cabbage looper *T. ni* (Ferkovich *et al.*, 1980), the diamondback moth *Plutella xylostella* (Prestwich *et al.*, 1989) and the most studied wild silk moth *A. polyphemus* (Vogt and Riddiford, 1981; Vogt *et al.*, 1985; Maida *et al.*, 1993). Two different esterases were characterized in *A. polyphemus* antennae: one is only associated with male antennae (ApolSE), whereas the other (integumental esterase) is expressed in male and female antennae and in all cuticular tissues tested, including legs. Both antennal and integumental esterases degraded the pheromone to the corresponding alcohol (Vogt *et al.*, 1985). In the cabbage

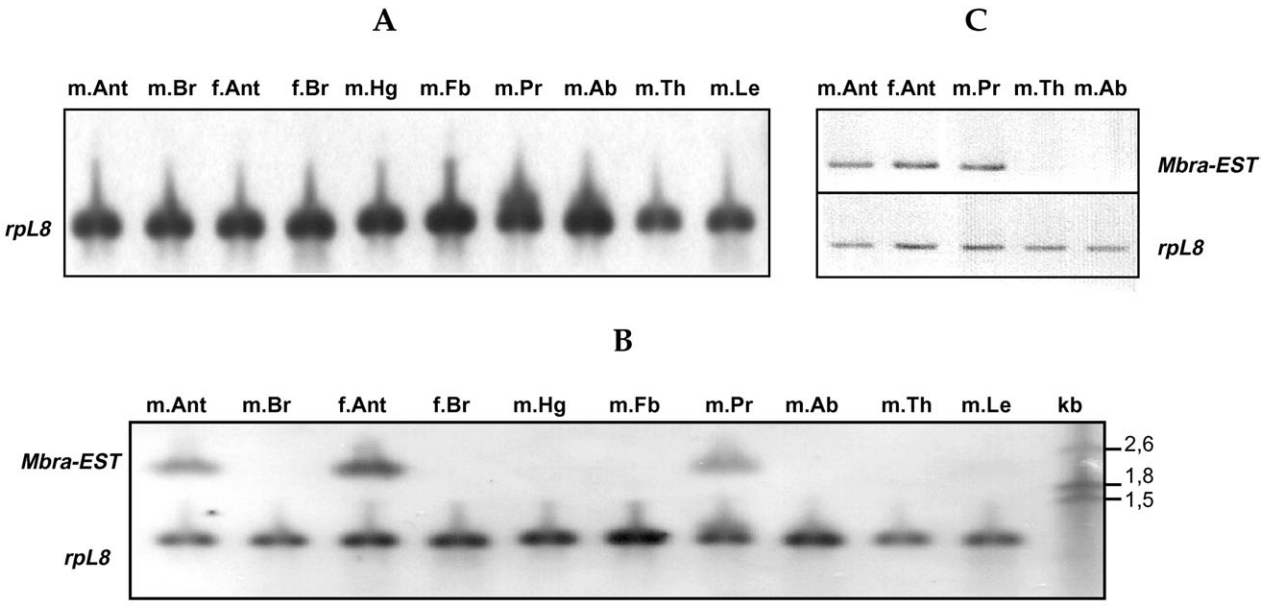

Figure 3 Tissue expression of *Mbra-EST* transcripts in adult *Mamestra brassicae*. **(A)** Northern-blot of total RNAs prepared from various tissues analyzed with the rpL8 bp probe. RNAs templates are from male antennae (m.Ant), brain (m.Br), hindgut (m.Hg), fat body (m.Fb), proboscis (m.Pr), thorax (m.Th), abdomen (m.Ab) and legs (m.Le). Templates from female are antennae (f.Ant) and brain (f.Br). **(B)** Hybridization signals detected after Northern blot with *Mbra-EST* and *rpL8* DIG-labeled probes. RNAs templates as described above. **(C)** RT–PCR analysis of *Mbra-EST* and *rpL8* expression: cDNAs amplified with gene specific primers from female antennae (f.Ant), male antennae (m.Ant), proboscis (m.Pr), thorax (m.Th) and abdomen (m.Ab).

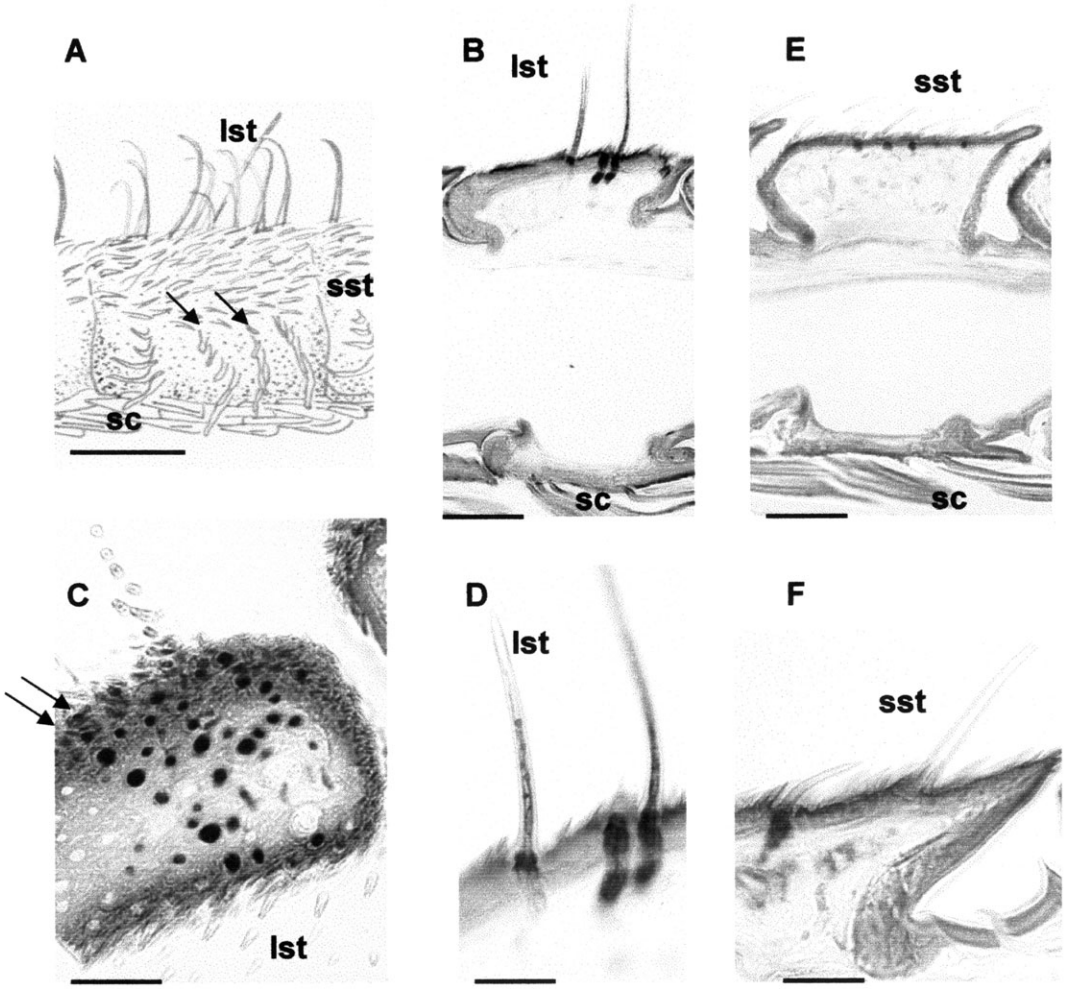

Figure 4 Expression patterns of *Mbra-EST* after *in situ* hybridization on sections of *M. brassicae* male **(B–D)** and female **(E, F)** antennae. **(A)** Schematic representation of a male antennal segment, showing the disposition of the short (sst) and long sensilla trichodea (lst) and the dorsal localization of the scales (sc). Arrows indicate the long sensilla trichodea arranged in parallel rows. (C) Arrows show the labeling of the long sensilla trichodea. (B, E) Complete antennal segments. (D, F) Details of sensilla trichodea, with intense labeling at the base of the sensory hairs. (D) Magnification of (B). Bars: 50 μm in (A), 10 μm in (B, E), 5 μm in (C, D, F).

looper, both antennal and cuticular esterases were also found (Ferkovich *et al.*, 1980). Male integumental esterase could ensure that the pheromone absorbed to the male's body surface, preventing the body from itself becoming a pheromone source (Vogt and Riddiford, 1981). In the female, the cuticular esterase could clear the pheromone from the female body to avoid later pheromone release when the female is no longer receptive to mating (Ferkovich *et al.*, 1980).

After Northern-blot studies, *Mbra-EST* expression was not found in the legs, abdomen or thorax where integument is present and *in situ* hybridization revealed that the labeling is restricted to the olfactory sensilla trichodea. Taken together, these results strongly suggest that *Mbra-EST* encodes a sensillar esterase rather than an integumental enzyme, but its precise localization in the proboscis, where taste sensilla are found (Nagnan-Le Meillour *et al.*, 2000), remained to be established. In *M. brassicae* males, sensilla

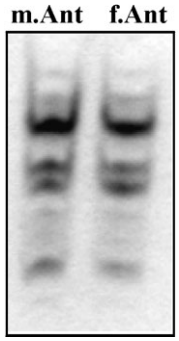

Figure 5 Esterases from *M. brassicae* male and female antennal extracts, after separation on a 10% native polyacrylamide gel and staining with α- and β-naphthyl acetate. m.Ant, six male antenna-equivalents; f.Ant, six female antenna-equivalents.

trichodea respond to specific and heterospecific acetate pheromone components: long sensilla trichodea are tuned to *Z*11–16:Ac (sex attractant) and to the antagonist *Z*9–14:Ac

(9-*cis*-tetradecenyl acetate), whereas short sensilla respond to *Z*9–14:Ac (Renou and Lucas, 1994). In females, plant volatiles such as hexyl acetate, are detected by the short sensilla (M. Renou, personal communication). All these acetate components are thus potential substrates for antennal esterases from both sexes, including Mbra-EST.

Putative odorant-degrading enzyme (Apol-ODE) and integumental esterase (Apol-IE) cDNAs have been cloned recently from *A. polyphemus* (Ishida and Leal, 2002). Apol-ODE could correspond to the sensillar esterase ApolSE previously described in this species (Vogt and Riddiford, 1981; Vogt *et al.*, 1985). By comparison with other esterases from insects, Mbra-EST is closest to Apol-ODE, whereas overall comparison at the amino acid level revealed lower identities than expected: Mbra-EST shares only 34.4% of identity with Apol-ODE and 24.5% with Apol-IE. The main sex pheromone components of *M. brassicae* (*Z*11–16:Ac) and *A. polyphemus* (6-*trans*,11-*cis*-hexadecadienyl acetate) have the same length and differ only by the double bond configuration. Inhibition studies with analogs of *A. polyphemus* pheromome showed that the sensillar esterase has a strong preference for substrates with conformations most similar to the natural pheromone and that the length of the chain is less important that the presence of unsaturations (Prestwich *et al.*, 1986). The structural divergence between the sensilla esterases of these two species may reflect evolutionary divergences between noctuid and saturnid moths as well as different substrate specificity.

Moreover, *Mbra-EST* labeling is present in olfactory sensilla of males but, in contrast to Apol-SE (and Apol-ODE), which is male specific, it is also found in the olfactory sensilla trichodea of females. Other ODEs able to degrade sex pheromones and expressed in both sexes were previously described, but most of them are enriched in male antennae. This is the case for the aldehyde-oxidases from *M. sexta*, *A. polyphemus*, *B. mori* (Rybczynski *et al.*, 1989, 1990) and *Heliothis virescens* (Tasayco and Prestwich, 1990) or for the antennal specific GST from *M. sexta*, MsexGSTolf, which is able to transform aldehyde odorants (Rogers *et al.*, 1999). These enzymes are supposed to modulate the perception of both pheromone and general odorants. Here, the expression level of *Mbra-EST* seems to be identical in both sexes, and this is confirmed by biochemical data: the electrophoresis patterns from crude antennal extracts revealed by α- and β-naphthyl acetate are similar in males and females. The same situation was observed in the cabbage looper *T. ni*, another noctuid moth: the electrophoresis patterns of the antennal esterases from males and females were similar and both of them hydrolyzed the sex attractant, 7-*cis*-dodecen-1-ol acetate (Ferkovich *et al.*, 1980).

The strong expression of *Mbra-EST* in the short sensilla of females, which are tuned to 'general' odors, suggests thus a putative dual function for the corresponding enzyme in the inactivation of pheromone as well as plant-derived compounds with acetate functional groups. However, we could not exclude a possible function in acetate pheromone degradation in female antennae too. Although *M. brassicae* females have not been shown to respond to their own pheromone, autodetection of sex pheromone by female moths has been demonstrated for several species (reviewed in Callahan *et al.*, 2000). For example, females of *S. littoralis* are able to detect their own pheromone and other acetate compounds (Ljüngberg *et al.*, 1993) and recent EAG recordings from females of *M. sexta* showed that some sensilla respond to at least one sex pheromone component (Kalinovà *et al.*, 2001).

In *M. brassicae*, we have previously characterized antennal cytochrome P450 cDNAs expressed in the accessory cells surrounding the olfactory neurons of male sensilla trichodea. The corresponding enzymes may be involved in odorant and pheromone degradation (Maïbèche-Coisne *et al.*, 2002). However, the encoded enzymes present the structural features of endoplasmic reticulum membrane-bound P450, suggesting that they could act on odorant molecules only after their internalization in the cells. In this work, we described the molecular characterization of an antennal cDNA encoding a putative extracellular esterase expressed in both males and females from the same species. This is, together with the esterase of *A. polyphemus*, only the second enzyme of this category cloned to date from insect antennae. In order to further understand pheromone and odorant inactivation in insects, more molecular data on these enzymes are needed. We will now focus our work on the characterization of other antennal esterases from *M. brassicae*, as well as on the development of functional studies to determine their substrate specificity towards pheromones and other plant-derived acetates.

Acknowledgements

This study was supported by INRA and Université Paris VI.

References

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

Bau J., Justus, K.A. and **Carde, R.T.** (2002) *Antennal resolution of pulsed pheromone plumes in three moth species.* J. Insect Physiol., 48, 433–442.

Callahan, F., Vogt, R., 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.

Farine, J.P., Frérot, B. and **Isart, J.** (1981) *Facteurs d'isolement chimique dans la sécrétion phéromonale de deux noctuelles Hadeninae:* Mamestra brassicae *(L.) et* Pseudatellia unipuncta *(Haw.)*. C. R. Acad. Sci. III, 292, 101–104.

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.

Garwood, J. and **Lepesant, J.A.** (1994) *The* Drosophila melanogaster *homolog of ribosomal protein L27a*. Biochem. Biophys. Res. Commun., 198, 748–744.

Györgyi, T., Roby-Shemkovitz, A. and **Lerner, M.** (1988) *Characterization and cDNA cloning of the pheromone-binding protein from the tabacco hornworm* Manduca sexta*: a tissue-specific developmentally regulated protein*. Proc. Natl Acad. Sci. USA, 85, 9851–9855.

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

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

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

Kalinovà, B., Hoskovec, M, Liblikas, I., Unelius, C.R. and **Hansson, B.S.** (2001) *Detection of sex pheromone components in* Manduca sexta *(L.)*. Chem. Senses, 26, 1175–1186.

Levy, S., Avni, D., Hariharan, N., Perry, R. and **Meyuhas, O.** (1991) *Oligopyrimidine tract at the 5′ end of mammalian ribosomal protein mRNAs is required for their translational control*. Proc. Natl Acad. Sci. USA, 88, 3319–3323.

Ljüngberg, 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: Noctuidea)*. J. Insect Physiol., 39, 253–260.

Maïbèche-Coisne, M., Sobrio, F., Delaunay, T., Lettere, M., Dubroca, J., Jacquin-Joly, E. and **Nagnan-Le Meillour, P.** (1997) *Pheromone-binding proteins of the moth* Mamestra brassicae*: specificity of ligand binding.* Insect Biochem. Mol. Biol., 27, 213–221.

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.

Maida, R., Ziegelberger, G. and **Kaissling, K.** (1993) *Esterase activity in the olfactory sensilla of the silkmoth* Antheraea polyphemus. Neuroreport, 6, 822–824.

Nagnan-Le Meillour, P., Huet, J.C., Maïbèche, M., Pernollet, J. and **Descoins, C.** (1996) *Purification and characterization of multiple forms of odorant/pheromone-binding proteins in the antennae of* Mamestra brassicae *(Noctuidae)*. Insect Biochem. Mol. Biol., 26, 59–67.

Nagnan-Le Meillour, P., Cain, A.H., Jacquin-Joly, E., François, M.C, Ramachandran, S., Maida, R. and **Steinbrecht, R.** (2000) *Chemosensory proteins from the proboscis of* Mamestra brassicae. Chem. Senses, 25, 541–553.

Nardi, J., Miller, L., Walden, K., Rovelstad, S., Wang, L., Frye, J., Ramsdell, K., Deem, L. and **Robertson, H.** (2003) *Expression patterns of odorant-binding proteins in antennae of the moth* Manduca sexta. Cell Tissue Res., 313, 321–333.

Nielsen, H., Engelbrecht, J., Brunak, S. and **von Heijne, G.** (1997) *Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites*. Protein Eng.**,** 10, 1–6.

Noselli, S. and **Vincent, A.** (1992) *The* Drosophila melanogaster *ribosomal protein L17A-encoding gene*. Gene, 118, 273–278.

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

Prestwich, G., Vogt, R. and **Riddiford, L.** (1986) *Binding and hydrolysis of radiolabeled pheromone and several analogs by male specific antennal proteins of the moth* Antheraea polyphemus. J. Chem. Ecol., 12, 323–333.

Prestwich, G., Graham, S., Handley M., Latli, B., Streinz, L. and **Tasayco, M.** (1989) *Enzymatic processing of pheromones and pheromone analogs.* Experientia, 45, 263–270.

Renou, M. and **Lucas, P.** (1994) *Sex pheromone reception in* Mamestra brassicae*: responses of olfactory receptor neurons to minor components of the pheromone blend*. J. Insect Physiol., 40, 75–85.

Rogers, M., Jani, M. and **Vogt, R.** (1999) *An olfactory-specific gluthanione* S*-transferase in the sphinx moth* Manduca sexta*.* J. Exp. Biol., 202, 1625–1637.

Rufh, M.L and **Meister, M.** (1999) *The* Drosophila *homologue of ribosomal protein L8.* Insect Biochem. Mol. Biol., 29, 349–353.

Rybczynski, R., Reagan, J. and **Lerner, M.** (1989) *A pheromone-degrading aldehyde-oxidase in the antennae of the moth* Manduca sexta. J. Neurosci., 9, 1341–1353.

Rybczynski, R., Vogt, R. and **Lerner, M.** (1990) *Antennal-specific pheromone-degrading aldehyde-oxidases from the moths* Antheraea polyphemus *and* Bombyx mori. J. Biol. Chem., 265, 19712–19715.

Steinbrecht, R. (1998) *Odorant-binding proteins: expression and function.* Ann. N Y Acad. Sci., 855, 323–332.

Suzuki, K. (1998) *cDNA sequence for a carboxylesterase in an organophosphorus insecticide resistant cotton aphid clone*. Direct submission in GenBank database.

Tasayco, M. and **Prestwich, G.** (1990) *Aldehyde-oxidizing enzymes in an adult moth:* in vitro *study of aldehyde metabolism in* Heliothis virescens. Arch. Biochem. Biophys*.,* 278, 444–451.

Vogt, R. (2002) *Biochemical diversity of odor detection: OBPs, ODEs and SNMPs*. In Blomquist, G. And Vogt, R. (eds), Insect Pheromone Biochemistry and Molecular Biology. Elsevier Academic Press, London, pp. 391–445.

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

Vogt, R., Riddiford, L. 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.

Vogt, R., Prestwich, G. and **Lerner M.** (1991) *Odorant-binding proteins subfamilies associate with distinct classes of olfactory receptor neurons in insects*. J. Neurobiol., 22, 74–84.

Vogt, R., Callahan, F., Rogers, M. and **Dickens, J.** (1999) *Odorant binding protein diversity and distribution among the insect orders, as indicated by LAP, an OBP-related protein of the true bug* Lygus lineolaris *(Hemiptera, Heteroptera)*. Chem. Senses, 24, 481–495.

Wang, Q., Hasan, G. and **Pikielny, C.** (1999) *Preferential expression of biotransformation enzymes in the olfactory organs of* Drosophila melanogaster, *the antennae.* J. Biol. Chem., 274, 10309–10315.

Wojtasek, H. and **Leal, W.** (1999) *Degradation of an alkaloid pheromone from the pale-brown chafer,* Phyllopertha diversa *(Coleoptera: Scarabaeidae), by an insect olfactory cytochrome P450*. FEBS Lett., 458, 333–336.

Wool, I., Chan, Y. and **Gluck, A.** (1995) *Structure and evolution of mammalian ribosomal proteins.* Biochem. Cell Biol., 73, 933–947.

Zhu, Y.-C., Dowdy, A. and **Baker, J.E.** (1999) *Differential mRNA expression levels and gene sequences of a putative carboxylesterase-like enzyme from two strains of the parasitoid* Anisopteromalus calandrae *(Hymenoptera:Pteromalidae)*. Insect Biochem. Mol. Biol., 29, 417–425.

Accepted March 2, 2004