

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

Martine Maibèche-Coisne^{1,2}, Christine Merlin^{1,2}, Marie-Christine François¹,
Isabelle Queguiner², Patrick Porcheron² and Emmanuelle Jacquin-Joly¹

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

Correspondence to be sent to: M. Maibèche-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 diamond-back 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 (Z11-16: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-transcribed 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 Jacquín-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	taaagtagtttgtcttagagttta					1661		
N	E	A	W	R	F	W	N	E	T	Y	A	L	Y	R	R	K	N						546		
atcttaaaactgaagattaaaaggtgacacttgaagaaatcggcaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa																									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 conserved sequence Gly²⁰³-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 MWALC (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 percent-

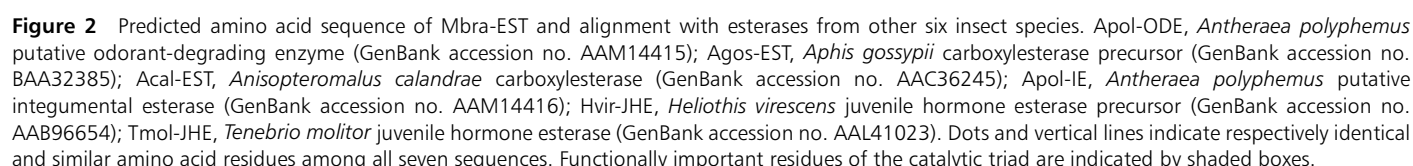
ages of similarity, we designate this *M. brassicae* cDNA as *Mbra-rpL8* (accession no. AY485337). Ribosomal proteins from eukaryotes share common structural features, i.e. high percentage of basic amino acids arranged in clusters, short 3'-UTR and a typical mRNA 5'-UTR oligopyrimidine stretch followed 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



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

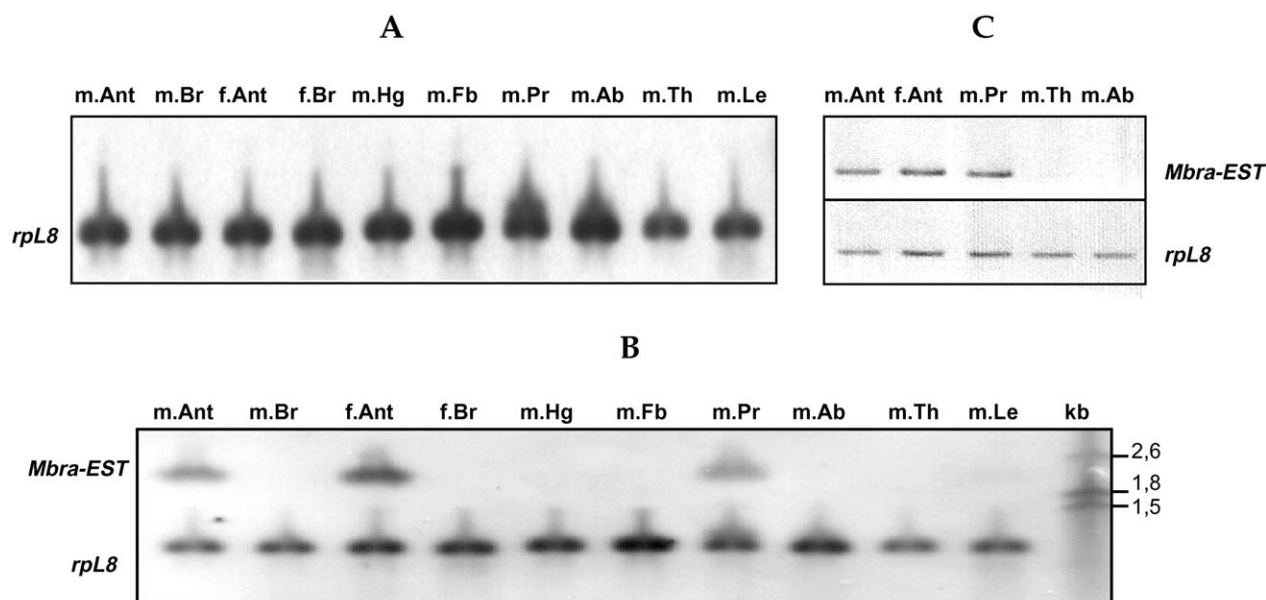


Figure 3 Tissue expression of *Mbra-EST* transcripts in adult *Mamestra brassicae*. **(A)** Northern-blot of total RNAs prepared from various tissues analyzed with the *rpl8* bp probe. RNAs templates are from male antennae (m.Ant), brain (m.Br), hindgut (m.Hg), fat body (m.Fb), proboscis (m.Pr), thorax (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).

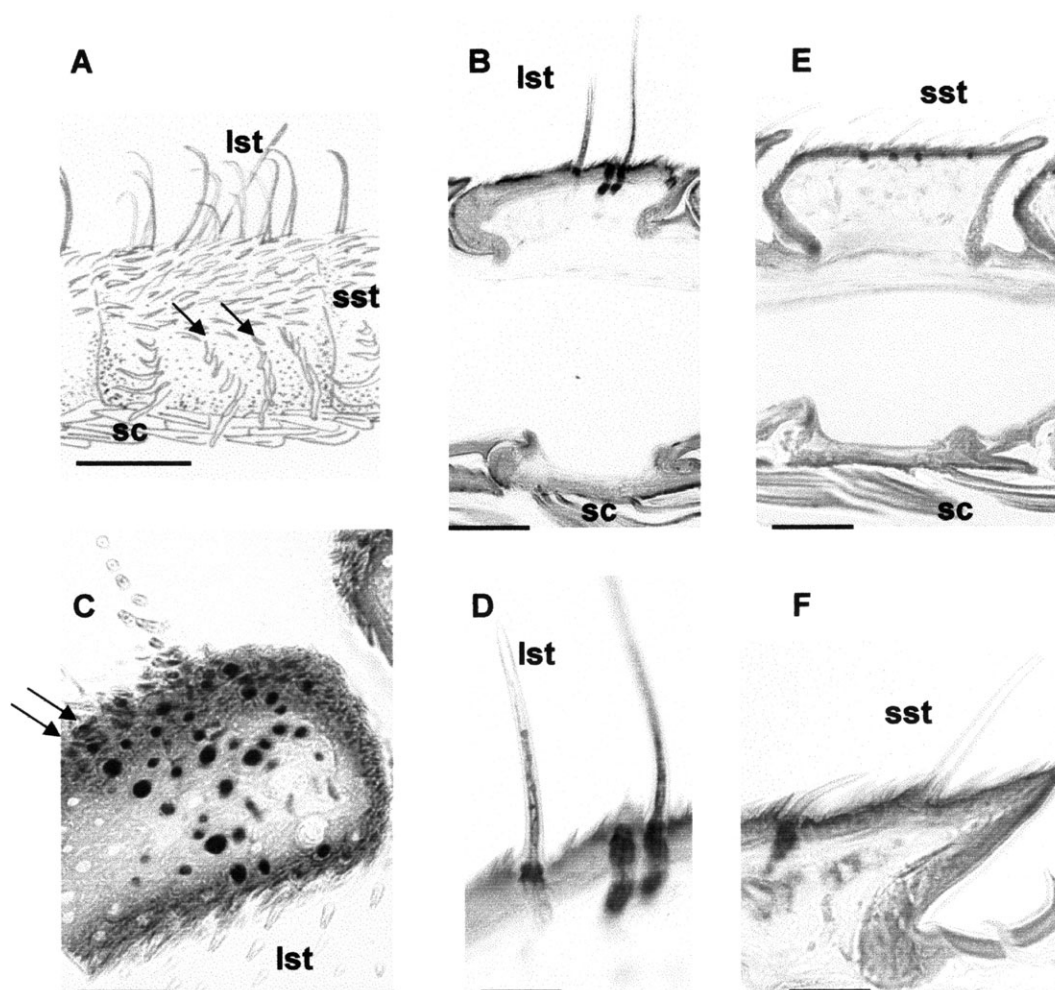


Figure 4 Expression patterns of *Mbra-EST* after *in situ* hybridization on sections of *M. brassicae* male (B–D) and female (E, F) antennae. (A) Schematic representation of a male antennal segment, showing the disposition of the short (sst) and long sensilla trichodea (lst) and the dorsal localization 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 μ m in (A), 10 μ m in (B, E), 5 μ m 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 Z11–16:Ac (sex attractant) and to the antagonist Z9–14:Ac

(9-*cis*-tetradecenyl acetate), whereas short sensilla respond to Z9-14: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* (Z11-16: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* pheromone 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 than 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 (Kalinová *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.

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