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Functional and Expression Pattern Analysis of Chemosensory Proteins Expressed in Antennae and Pheromonal Gland of *Mamestra brassicae*

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Abstract

Sequences coding for chemosensory proteins (CSP) CSPMbraA and CSPMbraB, soluble proteins of low mol. wt, have been amplified using polymerase chain reaction on antennal and pheromonal gland complementary DNAs. On the basis of their sequences, these proteins could be classed in the 'OS-D like' protein family whose first member was described in *Drosophila*, and that includes proteins characterized in chemosensory organs of many insect phylla, including our recent identification in *Mamestra brassicae* proboscis. Binding assays have shown that these proteins bind the pheromonal component (*Z*)-11-hexadecenyl-1-acetate (Z11–16:Ac) as well as (*Z*)-11-octadecenyl-1-acetate (Z11–18:Ac), an other putative component of the *M. brassicae* pheromonal blend. Furthermore, binding with fatty acids, but not with progesterone that is a structurally unrelated compound, leads to the hypothesis that the odorant-binding capability of the MbraCSPs may be restricted to fatty acids and/or to 16–18 carbon backbone skeletons. Thus, these proteins do not show the same highly binding specificity as the pheromone-binding proteins do. The CSP-related proteins appear homologous based on sequence identity, conserved cysteine residues and general patterns of expression. However, phylogenetic analyses suggest the presence of multiple classes of CSP within a given species and possible diversification of CSPs within different orders. This diversity perhaps contributes to the many CSP functions proposed in the literature. In *M. brassicae*, we localized the CSPMbraA expression to the sensilla trichodea, devoted to pheromone reception, suggesting a role in the chemosensory pathway. However, we also localized such proteins in the pheromonal gland, devoid of any chemosensory structure. This suggests that the *M. brassicae* CSP could be involved in transport of hydrophobic molecules through different aqueous media, such as the sensillar lymph, as well as the pheromonal gland cytosol.

Introduction

Biochemical surveys of insect antennae have identified two abundant but unrelated families of small soluble proteins with proposed odorant transport function: odorant-binding proteins (OBPs) and olfactory specific-D (OS-D) related proteins (Pelosi and Maida, 1995; Vogt *et al.*, 1999). OBPs are a multigene family of antennal-specific proteins, comprised of several classes, including the pheromone-binding proteins, the general odorant-binding proteins and the antennal-binding proteins (ABP)X-related proteins (Robertson *et al*., 1999; Vogt *et al*., 1999). OBPs reside in the extracellular fluid surrounding olfactory neurons, they bind odorants entering this fluid and are thought to transport them to membrane-bound receptor proteins in the olfactory neurons in a more or less specific manner (Vogt *et al.*, 1985, 1999; Prestwich *et al.*, 1995; Ziegelberger, 1996; Steinbrecht, 1996, 1999; Kaissling, 1998; Maida *et al*., 2000; Plettner *et al*., 2000; Sandler *et al*., 2000). OBPs are relatively divergent in sequence; at least seven distinct OBPs have been identified through EST sequencing from the moth *Manduca sexta* (Robertson *et al*., 1999); more than 30 are readily identifiable in *Drosophila melanogaster* by analysis of the fly genome (Robertson, personal communication). OBPs have been shown to associate differentially with functionally distinct classes of olfactory sensilla (Vogt *et al*., 1991a; Steinbrecht *et al*., 1992, 1995; Laue *et al*., 1994; Laue and Steinbrecht, 1997).

OS-D related proteins were first identified by subtractive hybridization experiments using antennae of *D. melanogaster* (McKenna *et al*., 1994; Pikielny *et al*., 1994). OS-D homologues were subsequently identified based on sequence similarity: PEBmeIII in the ejaculatory bulb of *D. melanogaster* (Dyanov *et al*., GenBank Accession No. U08281); p10 in the regenerating legs of *Periplaneta americana* (Nomura *et al*., 1992; Kitabayashi *et al*., 1998); CLP-1 of

Cactoblastis cactorum (Maleska and Stange, 1997); several proteins described by N-terminal sequence from *Apis mellifera* (Danty *et al.*, 1998), Phasmatodea (Mameli *et al.*, 1996; Tuccini *et al.*, 1996) and Dictyoptera (Picimbon and Leal, 1999); the CSPs (chemosensory proteins) of *Schistocerca gregaria* (Angeli *et al.*, 1999), *Eurycantha calcarata* (Marchese *et al.*, 2000) and *Locusta migratoria* (Picimbon *et al.*, 2000); and SAPs (sensory appendage proteins) of *M. sexta* (Robertson *et al.*, 1999).

OS-D related proteins differ from OBPs on several points (Vogt *et al.*, 1999). OS-D related proteins share no sequence similarity with OBPs and contain four spatially conserved cysteine residues, while OBPs contain six spatially conserved cysteine residues. Both families are represented by multiple genes within a given species. However, OS-D related proteins are more highly conserved than OBPs, both within a species and between species of different insect orders. OS-D related proteins have been identified in a variety of tissues (sometimes without any obvious olfactory function), while most OBPs appear to be restricted to olfactory tissue. OS-Ds are known in orthopteroid (phasmid and grasshopper) and holometabolous (Lepidoptera and Diptera) insects and thus may be present throughout the Neoptera, while OBPs to date are only known within a subdivision of the Neoptera, namely the holometabolous and hemipteroid lineages (Vogt *et al.*, 1999).

The role of OS-D related proteins in odorant binding was suggested by biochemical studies of OBP-related function in the cabbage armyworm, *Mamestra brassicae*. OS-D related proteins were identified in male antennae (Bohbot *et al.*, 1998) and proboscis (Nagnan-Le Meillour *et al.*, 2000) by N-terminal sequencing, cross-reactivity with anti[ApolGOBP2] antisera and binding of sex-pheromone components. One of these proteins was identified and the coding cDNA cloned in the proboscis (Nagnan-Le Meillour *et al.*, 2000), leading to complete sequences of two distinct OS-D related proteins named CSPMbraA and CSPMbraB, for 'chemosensory proteins', a designation proposed by Angeli *et al.* (Angeli *et al.*, 1999). CSPMbraA and CSPMbraB were shown to bind the tritiated analogues of the *M. brassicae* pheromone components Z11–16:Ac and 16:Ac, as well as the behavioral inhibitor towards male attraction: (*Z*)-11-hexadecenol-1-OH (Z11–16:OH) and a structurally related compound, Z11–18:Ac (Bohbot *et al.*, 1998; Nagnan-Le Meillour *et al.*, 2000). Z11–18:Ac is not known as a Lepidopteran sex pheromone, but was previously described in traces in *M. brassicae* pheromonal blend (Struble *et al.*, 1980). These studies highlight an additional difference between OS-D related proteins and OBPs: OS-Ds interact with odorants with far less specificity than do the OBPs (Vogt *et al.*, 1989; Du and Prestwich, 1995; Feng and Prestwich, 1997; Maibèche-Coisné *et al.*, 1997; Maida *et al.*, 2000; Plettner *et al.*, 2000; Jacquin-Joly *et al.*, 2000). Nevertheless, their association with chemosensory tissues and the demonstrated ability to bind odors suggests that the OS-D related proteins, referred to as CSPs, do indeed have some chemosensory function.

In this current study, we looked for the presence of putative binding proteins of the OS-D class in adult male antennae as well as in the female pheromone gland of *M. brassicae*, starting from their ability to bind pheromone analogues using our binding assay. Also, the expression pattern of one of the newly identified CSPs, CSPMbraA, was examined in the antenna.

Materials and methods

Insects

Animals were reared in Domaine du Magneraud (INRA, France) on a semi-artificial diet (Poitout and Bues, 1974) at 20°C, 60% relative humidity, exposed to a 16 h/8 h light/dark photoperiod and sexed as pupae. Antennae and pheromonal glands from 3-day-old adults were dissected and stored at –80°C until use.

Protein extraction, electrophoresis and binding experiment

Antennae and pheromonal glands were manually homogenized in 1% trifluoroacetic acid and centrifuged 15 min at 15,000 *g*. Supernatants were filtered by centrifugation on Anapore filters (Millipore), then evaporated under vacuum (Savant Speed-Vac) and stored at –70°C until use.

The synthesis of tritiated analogue [^{3}H]Z11–16:Ac (specific activity: 1.7 TBq/mmol) of the main pheromone component Z11–16:Ac was described in Maïbèche-Coisné *et al.* (Maïbèche-Coisné *et al.*, 1997). The [^{3}H]Z11–18:Ac (*cis*-11-tetradecenyl acetate, sp. act. 0.8 TBq.mmol^{-1}) was a gift of Dang Ba-Pho (Université Paris XI). Tritiated palmitic acid (9,10[^{3}H]16:COOH, sp. act. 1.591 TBq/mmol), oleic acid (9,10[^{3}H]Z9–18:COOH, sp. act. 1.221 TBq/mmol) and tritiated progesterone (sp. act. 3.589 TBq/mmol) were from Sigma. The protocol of binding assay was fully described in Bohbot *et al.* (Bohbot *et al.*, 1998). Briefly, protein extract (100 tissue equivalent) and tritiated compound (1 µCi) were incubated for 30 min on ice, then the mixture was loaded on non-denaturing gel, submitted to electrophoresis and electroblotted onto a PVDF membrane that was then processed by fluorography. Protein bands giving a positive binding were sequenced from the *N*-terminus by J. d'Alayer (Institut Pasteur, France), using a gas-phase microsequencer (Applied Biosystems) and the reagents and methods of the manufacturer.

RNA extraction and cDNA synthesis

Total RNAs were extracted from 200 male antennae and 100 female pheromonal glands with the Tri-Reagent (Euromedex). Single-stranded cDNA was synthesized from 1 µg of total RNA with M-MLV (USB), using the buffer and protocol supplied with the enzyme. The reaction mixture contained dNTP mix (Pharmacia), Rnasin

(Promega), oligodT_{18} with an anchor: CATGCATGCGG-CCGCAAGCT$_{18}$VN (synthesized by Isoprim, Toulouse, France), sterile water and template RNA to a final volume of 50 µl. The mix was heated at 68°C for 5 min and chilled on ice before adding the M-MLV (600 U), then incubated for 1 h at 37°C and finally the reverse transcriptase was inactivated at 95°C for 5 min. For the 5′ RACE, cDNA was synthesized from 1 µg of male antennal total RNA at 42°C for 1.5 h using the SMARTT RACE cDNA Amplification Kit (Clontech) with 200 U of Superscript II (Gibco, BRL), 5′ CDS-primer and SMART II Oligonucleotide, according to the kit instructions.

3′ and 5′ RACE-polymerase chain reactions

Approximately 1 ng of cDNA was used for polymerase chain reaction (PCR). The sense primers were designed based on protein N-terminal sequences: GAGGACAAG-TACACIGAYAAGTACGA for the N-terminal EDKY, i.e. CSPMbraA (Bohbot *et al*., 1998) and GAGGAGGCICAY-TAYACIGAYCG for the N-terminal EEAH, i.e. CSPMbraB (Nagnan-Le Meillour *et al.*, 2000). Both primers were synthesized by Isoprim. These primers were used in a pair with the primer used for cDNA synthesis (anchor-oligodT_{18}) in order to perform the 3′ RACE. PCR were carried out with Taq polymerase (1 U; Promega) in 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$ and 0.2 mM of each dNTP. Forty amplification cycles were performed with annealing temperature of 60°C for EDKY and 55°C for EEAH.

5′RACE amplifications were performed on antennal cDNA. We used 2.5 µl of 5′ RACE-ready cDNA with Universal Primer Mix (UPM, Clontech) as a sense anchor primer versus an antisense gene specific primer designed according to one of the cDNA sequences obtained from the 3′ cloning: 5′-GGCGGCCTTTGCGCGGTCTTCATA-3′ for EDKY and 5′-TGTTCCTTGAGCTCCTTGGCGT-CAG-3′ for EEAH (Isoprim). The 50 µl amplification mix was prepared according to the SMART™ RACE cDNA Amplification Kit instructions using the Advantage 2 Polymerase mix (Clontech). Touchdown PCR was performed using hot-start as follows: after 1 min at 94°C, five cycles of 30 s at 94°C and 3 min at 72°C, then five cycles of 30 s at 94°C, 30 s at 70°C and 3 min at 72°C, then 25 cycles of 30 s at 94°C, 30 s at 68°C and 3 min at 72°C, then 5 min at 72°C.

Cloning and sequencing

The amplified cDNAs were purified after agarose electrophoresis using GenElute (Supelco) and ligated into the plasmid pCR™-II using the TOPO cloning kit from Invitrogen (The Netherlands). After transformation, positive clones were digested with *Eco*RI (Biolabs) to screen for the presence of inserts. Recombinant plasmids were then isolated using the Plasmid Midi Kit from Qiagen and were subjected to automated sequencing with vector promotors by ESGS (Evry, France). 5′RACE amplifications were directly sequenced from the gene specific primer after elution from the agarose gel using the QIAquick Gel Extraction Kit (Qiagen). Database searches were performed with the BLAST program (NCBI) and sequence alignment with ClustalW (NPS@ IBCP).

In situ hybridization

CSPMbraA6 RNA sense and antisense digoxigenin-labeled probes (600 bp long) were *in vitro* transcribed from linearized pCRII-CSPMbraA6-3′ cDNA plasmid using T7 and SP6 RNA polymerase (Promega) following recommended protocol and in the presence of 1.5 U of Rnasin (Promega). Probe quality was confirmed under denaturing conditions by formaldehyde agarose gel electrophoresis and stored at –80°C until use.

For hybridization, antennae were removed from the adult head, cut into pieces and treated overnight at 4°C in 4% paraformaldehyde (PFA) in PBS. Fixed tissues were dehydrated to 100% methanol and stored at –20°C. Hybridization protocol was performed on whole-mount pieces of antennae as described in Jacquin-Joly *et al*. (Jacquin-Joly *et al*., 2000). Hybridization was revealed using alkaline-phosphatase-conjugated anti-digoxygenin antibody (1:4000) and stained with NBT-BCIP (Boehringer Mannheim). After sufficient staining, specimens were washed in PBS and fixed in 4% PFA for 20 min, then dehydrated through a graded series of ethanol and included in Epon (Agar 100 Resin kit). Longitudinal sections were performed at 4 µm and counter-stained with orange acridin. Sections were photographed, then pictures were digitized and processed using Adobe Photoshop 5.0.

Phylogenetic analysis

CSP-related sequences were initially identified using the NCBI BLAST network server and retrieved using NCBI Entrez from GenBank, Swiss-Prot or EMBL databases. Sequences were aligned in Clustal X (Thompson *et al.*, 1994). An unrooted neighbor joining tree (Saitou and Nei, 1987) was constructed using PAUP (Version 4.0b1 for Macintosh), based on mean character difference (distance). The data matrix was minimally trimmed at the C and N terminal ends. All other characters were included; the program calculated pairwise differences, ignoring missing characters resulting from alignment gapping. Bootstrap support values were determined based on 1000 neighbor joining replicates, again using the PAUP program. The tree presented only includes nodes with 50% or higher bootstrap support; branch lengths are proportional and indicate mean distance (percentage difference) between the sequences.

Results

Functional characterization of proteins in antennae and pheromonal gland

In binding assays, the [^{3}H]Z11–18:Ac was bound by a single band in antennae and pheromonal glands (Figure 1). This band contained the single N-terminal sequences EDKY in antennae and a mixed sequence of EDKY (50%) and EEAH (50%) in female glands. The [^{3}H]Z11–16:Ac was bound in antennae by two bands—an upper band containing the sequence EDKY and a lower band containing the sequence SKELIT (MbraPBP1), as previously reported (Bohbot *et al.*, 1998). The [^{3}H]Z11–16:Ac was bound by one band in pheromonal gland containing the mixed EDKY/EEAH sequence. Binding experiments with non-pheromonal tritiated fatty acids 16:COOH and Z9–18:COOH, as well as progesterone (a non-fatty-acid compound used as control) were performed to study the binding specificity. In antennae as well as in gland extracts, proteins at the migrating position of CSP were able to bind the two fatty acids but not the progesterone (Figure 2).

cDNA cloning and sequencing in male antennae and pheromonal gland

3′ RACE-PCR amplifications were performed on cDNAs from antennae using degenerate primers encoding the determined N-terminal sequences EDKY or EEAH versus the anchor-oligodT. Products of 650 bp and 480 bp were obtained using the EDKY primer and the EEAH primer, respectively. Analysis of cloned PCR products yielded three different sequences, one encoding 112 amino acids with an N-terminal sequence EDKY and two encoding 108 amino acids with an N-terminal sequence EEAH. We have named these proteins CSPMbraA6 (EDKY N-terminal sequence) and CSPMbraB3 and CSPMbraB4 (EEAH N-terminal sequence), according to the nomenclature of chemosensory proteins proposed by Angeli *et al.* (Angeli *et al.*, 1999) and in reference to homologous proteins previously cloned from *M. brassicae* proboscis (Nagnan-Le Meillour *et al.*, 2000). The derived N-terminus of CSPMbraA6 exactly matched the amino acid sequence previously determined by direct sequencing—EDKYTDKYDNINLDE (Bohbot *et al.*, 1998)—as did that of CSPMbraB4—EEAHYTDRYDNVDLDEILGN (Nagnan-Le Meillour *et al.*, 2000). CSPMbraB3 and CSPMbraB4 differed in only one residue within this 20 amino acid N-terminal region, but differed by 19 residues overall (82.5% identity), suggesting they are separate gene products.

To obtain the 5′ sequences of a representative of each protein class (CSPMbraA6 and CSPMbraB3), antennal cDNAs were amplified using specific antisense internal primers based on the obtained 3′ sequences and a sense anchor primer. Amplified fragments were gel purified and sequenced, producing 100% base identity, except in the degenerated sense primer regions, which were thus corrected, yielding the full-length cDNA coding sequences of CSPMbraA6 and CSPMbraB3. The nucleotides and the translated amino acid sequences are presented in Figure 3. The first in-frame ATG codons are located 48 and 60 bases upstream of the amino acid sequences EDKY and EEAH, respectively, which represent the N-terminal sequences of the proteins as determined by Edman degradation (Bohbot *et al.*, 1998) and are proposed to be the start of the protein coding domains. The 16 and 20 amino acids from these ATG to the initial amino acid of the N-terminal sequence of the mature protein are presumed to be signal peptides with

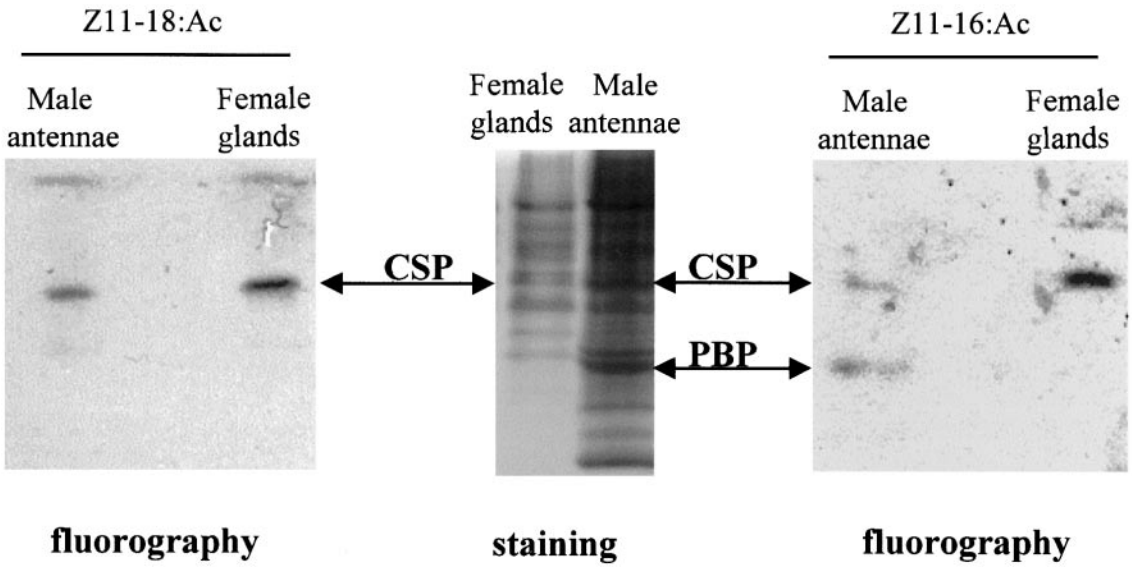

Figure 1 Binding experiment. Extracts of 100 male antennae and 100 female glands were incubated with 1 μCi of each of the tritiated pheromone analogues [^{3}H]Z11–16:Ac and [^{3}H]Z11–18:Ac. Middle: Coomassie blue staining of the gel.

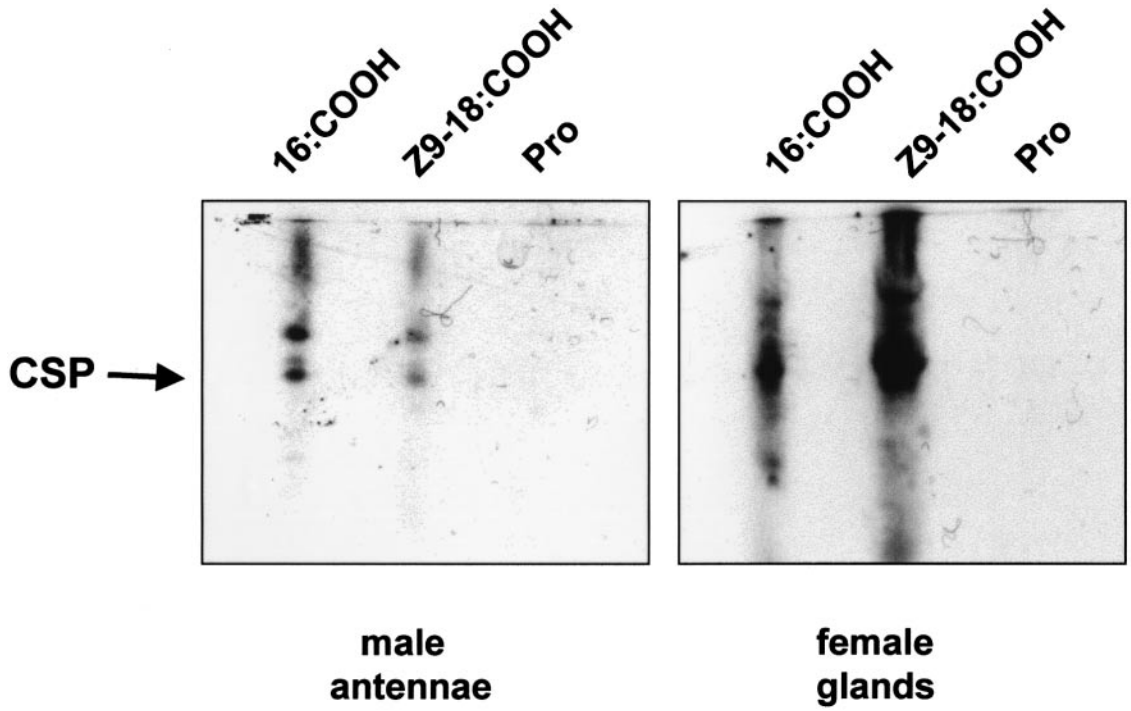

Figure 2 Binding experiment. Extracts of 100 male antennae and 100 female glands were incubated with 1 μCi of each of the following analogues: [^{3}H]16:COOH (palmitic acid), [^{3}H]Z9–18:COOH (oleic acid) and [^{3}H]Pro (progesterone).

cleavage sites at VVA/EDK and ARP/EEA; those presumptive signal peptides are consistent in length with signal peptides of other secreted proteins, including insect OBPs (Vogt *et al*., 1991b).

Pheromone gland derived cDNA was processed identically to the above antennal cDNA, yielding two different nucleotide sequences. One sequence was obtained from amplification using the EDKY primer and was 100% identical to the antennal CSPMbraA6 cDNA. The second sequence was obtained after amplification using EEAH primer and was 100% identical to the proboscis CSP-MbraB1 previously described (Nagnan-Le Meillour *et al.*, 2000).

Figure 4 shows the deduced amino acid sequences of the cloned cDNAs compared with those of proteins from the same class reported in other insect species, including the proboscis CSP recently identified in *M. brassicae* (Nagnan-Le Meillour *et al.*, 2000). Among the CSPMbraA proteins, identity is very high, differing by only one to three amino acids (97–99% identities). Among the CSPMbraB proteins, sequences are more variable, pairwise comparisons ranging from 82 to 99% identity. Identities between CSPMbraA and CSPMbraB proteins are consistently around 50%, similar to percentage identity values between all the CSP-related proteins from different species. Percentage identity values between all the proteins used in the analysis are quite consistent, from 40 to 50%, with two exceptions: 16% between CSPMbraA6 and SAP1, and 77% between CSPMbraB1 and SAP3. Throughout all of these proteins, the four cysteines are highly conserved at positions 29, 36, 55 and 58 from the N-terminal of the mature protein (Figure 4). Nucleotide sequences have been deposited in the GenBank Database with the accession numbers AF255918, AF255919 and AF255920 for CSPMbraA6, B3 and B4, respectively.

Phylogenetic analysis of CSP-related proteins

All currently available CSP-related sequences were identified and collected using NCBI Entrez and BLAST network servers (Table 1) and aligned in Clustal W (Figure 4) and Clustal X. Figure 5 shows an unrooted neighbor joining tree derived from this character matrix, and represents the sequence similarities of all these CSP-related proteins. This analysis is phylogenetically consistent in that no branches contain proteins from multiple insect orders. The CSPMbra proteins, along with proteins from other Lepidoptera, appear to define two distinct structural classes, characterized by the CSPMbraA and CSPMbraB proteins respectively. The CSP proteins show consistent patterns of sequence difference regardless of the phylogenetic distance of the organisms: 50–60% identity between proteins of the two orthopteran species (*S. gregaria* and *L. migratoria*); 38–54% identity between proteins of the two lepidopteran groups (CSPMbraA and CSPMbraB); and 37–50% identity between proteins of Orthoptera versus Lepidoptera.

In-situ hybridization studies of CSPMbraA expression

In-situ hybridization analysis was performed using dig-

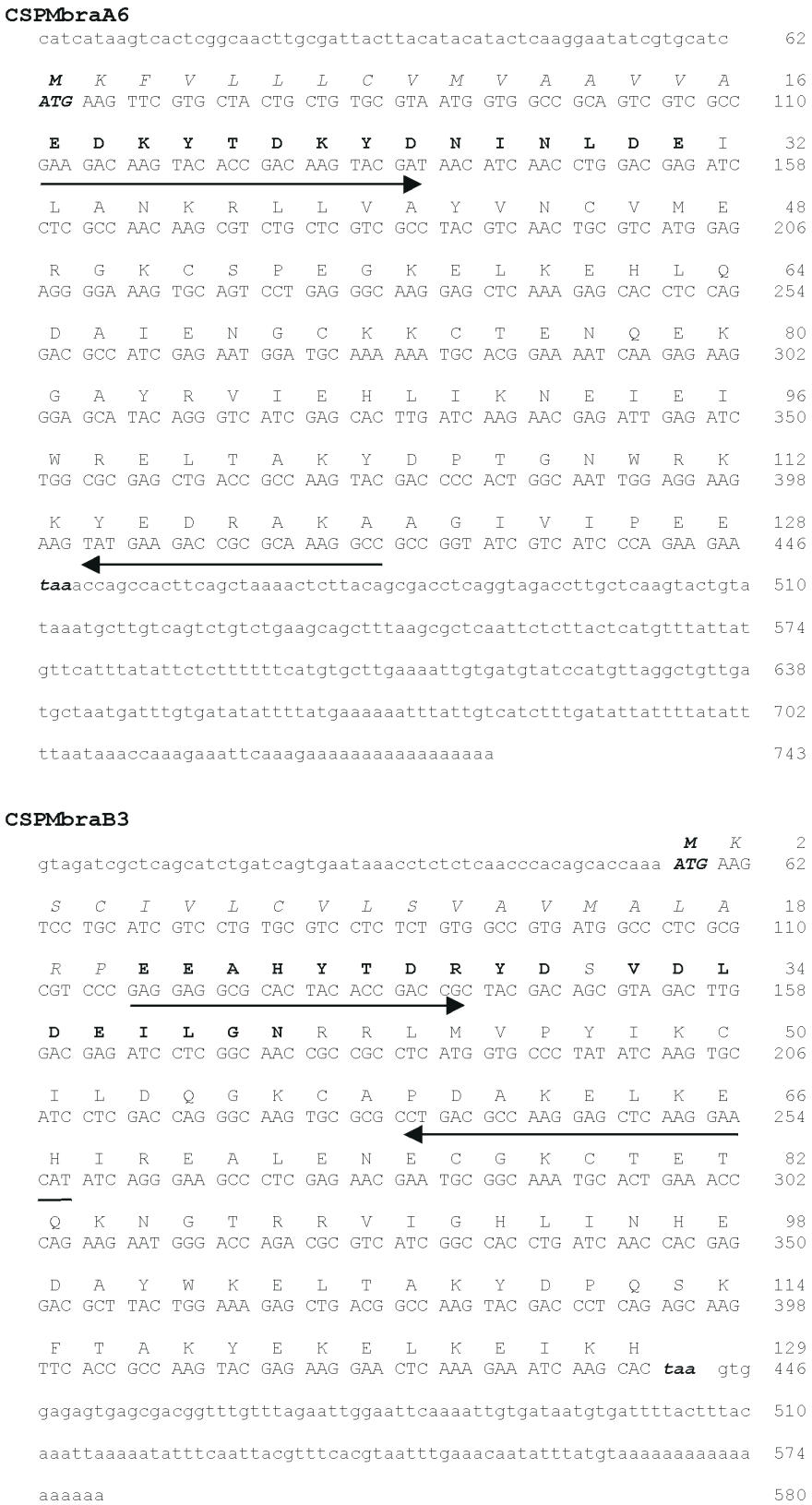

Figure 3 CSPMbraA6 and CSPMbraB3 full length cDNA sequences. The cDNA and deduced amino acid sequences of one of the CSPMbraA (No. 6) and one of the CSPMbraB (No. 3) are shown. The previously determined N-terminal sequences are in bold type and the suggested leader sequences are in italics. The suggested start ATG and stop TAA codons are in bold italics. The positions of sense primers (3′ RACE) and antisense primers (5′ RACE) used for PCR are underlined.

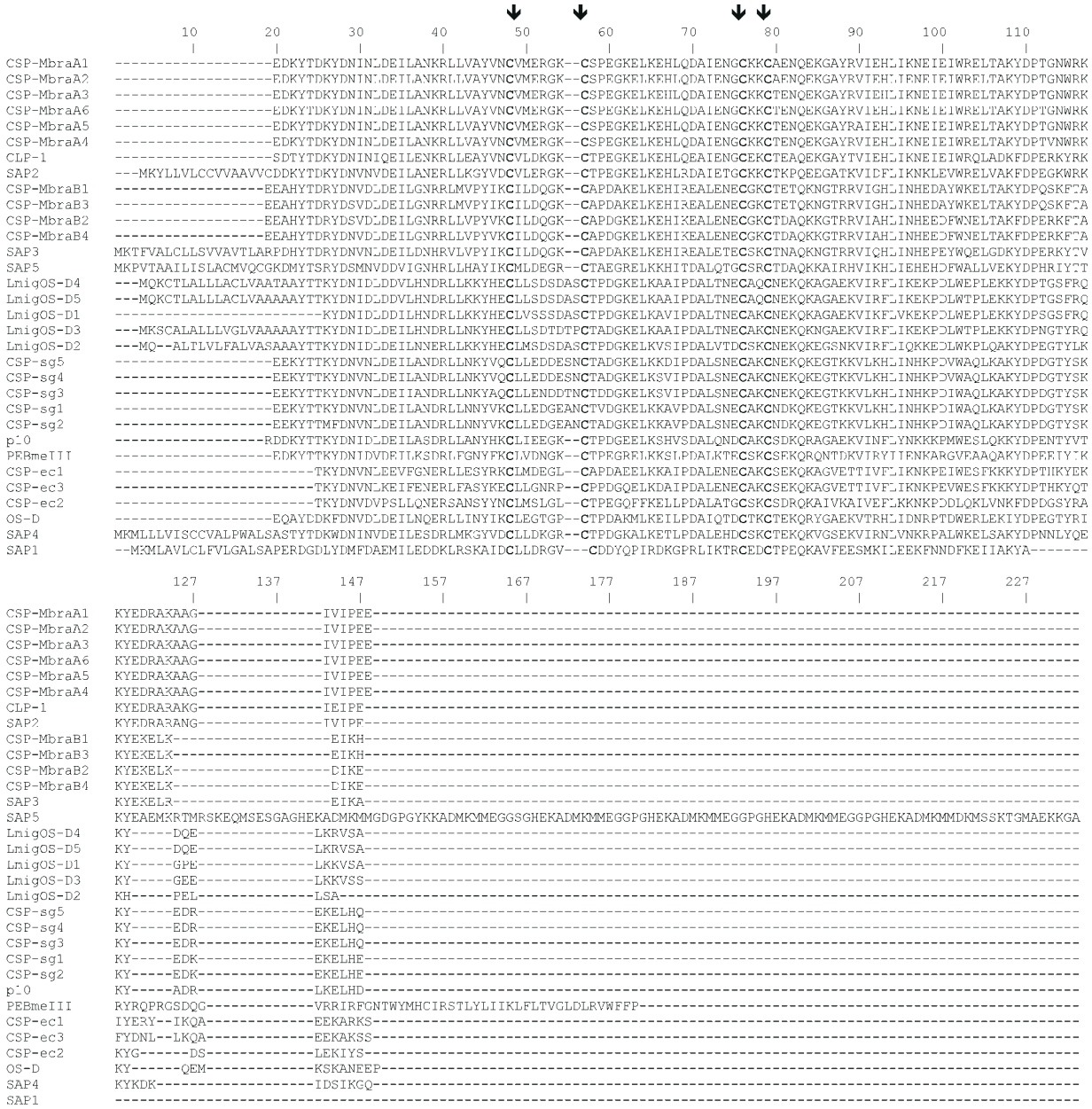

Figure 4 CSP alignment. Alignment was done in Clustal W. Sequence names, GenBank accession numbers and sources are indicated in Table 1. The four conserved cysteines are in bold and indicated by arrows.

oxigenin-incorporated CSPMbraA6 antisense and sense RNA probes against adult male antennae. The *M. brassicae* antenna is filiform, ~1 cm long and comprises ~72 segments (Renou and Lucas, 1994). Each segment exhibits the same general organization: the dorsal side is covered with two rows of scales and the olfactory hairs, the sensilla, are located on the ventral side (Figure 6A). In the male, the olfactory hairs are distributed in two classes according to their length. The long sensilla trichodea are located on the lateral part of the ventral area and are settled in four or five parallel rows (Renou and Lucas, 1994) (Figure 6A, white arrows). Short sensilla are located medio-ventrally and are not arranged in rows. Sense strand controls gave no signals (not shown), whereas antisense probe hybridization is restricted to the sensilla side of the antenna, with no labeling on the scale side (Figure 6B). Close examination revealed hybridization in cells localized at the base of sensory hairs (Figure 6D). The CSPMbraA expressing cells are presumably the sensilla supporting cells, by comparison with what has been observed with OBP probes or antibodies in other insects (Steinbrecht *et al.*, 1992, 1995; Hekmat-Scafe *et al.*, 1997; Vogt *et al.*, 1999) and in *M. brassicae* (Jacquin-Joly *et al.*, 2000). On longitudinal sections through the antennae, it is difficult to distinguish between long and

Table 1 CSP protein sequences used in the phylogenetic analysis

Order	Species	Protein name	GB accession no.	Reference
Lepidoptera	*Manduca sexta*	SAP1	AF117574	(Robertson *et al*., 1999)
		SAP2	AF117592	(Robertson *et al*., 1999)
		SAP3	AF117585	(Robertson *et al*., 1999)
		SAP4	AF117599	(Robertson *et al*., 1999)
		SAP5	AF117594	(Robertson *et al*., 1999)
	Mamestra brassicae	CSP-MbraA1	AF211177	(Nagnan-Le Meillour *et al*., 2000)
		CSP-MbraA2	AF211178	(Nagnan-Le Meillour *et al*., 2000)
		CSP-MbraA3	AF211179	(Nagnan-Le Meillour *et al*., 2000)
		CSP-MbraA4	AF211180	(Nagnan-Le Meillour *et al*., 2000)
		CSP-MbraA5	AF211181	(Nagnan-Le Meillour *et al*., 2000)
		CSP-MbraA6	AF255918	this paper
		CSP-MbraB1	AF211182	(Nagnan-Le Meillour *et al*., 2000)
		CSP-MbraB2	AF211183	(Nagnan-Le Meillour *et al*., 2000)
		CSP-MbraB3	AF255919	this paper
		CSP-MbraB4	AF255920	this paper
	Cactoblastis cactorum	CLP-1	U95046	(Maleska and Stange, 1997)
Diptera	*Drosophila melanogaster*	OS-D	U02546	(McKenna *et al*., 1994)
		PEBmelII	U08281	(Dyanov *et al*., unpublished data)
Orthoptera	*Schistocerca gregaria*	CSP-sg1	AF070961	(Angeli *et al*., 1999)
		CSP-sg2	AF070962	(Angeli *et al*., 1999)
		CSP-sg3	AF070963	(Angeli *et al*., 1999)
		CSP-sg4	AF070964	(Angeli *et al*., 1999)
		CSP-sg5	AF070965	(Angeli *et al*., 1999)
	Locusta migratoria	LmigOS-D 1	AJ251075	(Picimbon *et al*., 2000)
		LmigOS-D 2	AJ251076	(Picimbon *et al*., 2000)
		LmigOS-D 3	AJ251077	(Picimbon *et al*., 2000)
		LmigOS-D 4	AJ251078	(Picimbon *et al*., 2000)
		LmigOS-D 5	AJ251079	(Picimbon *et al*., 2000)
Phasmatodea	*Eurycantha calcarata*	CSP-ec1	AF139196	(Marchese *et al*., 2000)
		CSP-ec2	AF139197	(Marchese *et al*., 2000)
		CSP-ec3	AF139198	(Marchese *et al*., 2000)
Dictyoptera	*Periplaneta americana*	p10	AF030340	(Kitabayashi *et al*., 1998)

short sensilla as only parts of the sensilla are visible (Figure 6B, D). However, sections through the cuticle allowed us to reveal labeled spots distributed in the ventro-lateral region with a row pattern consistent with the distribution of the long sensilla trichodea (Figure 6C, black arrows, and 6F). Typical structures of sensilla coeloconica, that resemble flowers in scanning microscopy (Figure 6E), can be observed on sections through the cuticle, without any associated labeling (Figure 6F), whereas on the same section other sensilla are labeled, some in rows and others without any particular distribution.

Discussion

CSPMbra proteins

CSPMbraA and CSPMbraB proteins were previously characterized in *M. brassicae,* in male adult antennae (Bohbot *et al*., 1998) and proboscis (Nagnan-Le Meillour *et al*., 2000), using a binding assay with pheromone components. Although binding assays revealed that CSPMbra proteins bind all the tritiated pheromone analogues tested without specificity, their role in *M. brassicae* is not yet clearly understood. To further characterize these CSP proteins, degenerate PCR primers were designed against previously obtained N-terminal amino acid sequences and used to clone partial cDNAs of CSP proteins from male antennae and female pheromone glands. Full-length CSPMbraA and CSPMbraB cDNA sequences were obtained from antennal-derived mRNA by 5′RACE. The deduced amino acid sequences revealed the occurrence of a putative signal peptide at their N-terminus, confirming that these proteins are secreted into the sensillar lymph of the antennae, as other OBPs.

Comparison of these proteins with the OS-D like protein family revealed the conservation of four cysteines, a consistent hallmark of all the proteins identified so far as OS-D like or CSP proteins. In *S. gregaria*, the four cysteines are involved in two disulfide bonds (Angeli *et al*., 1999), linking

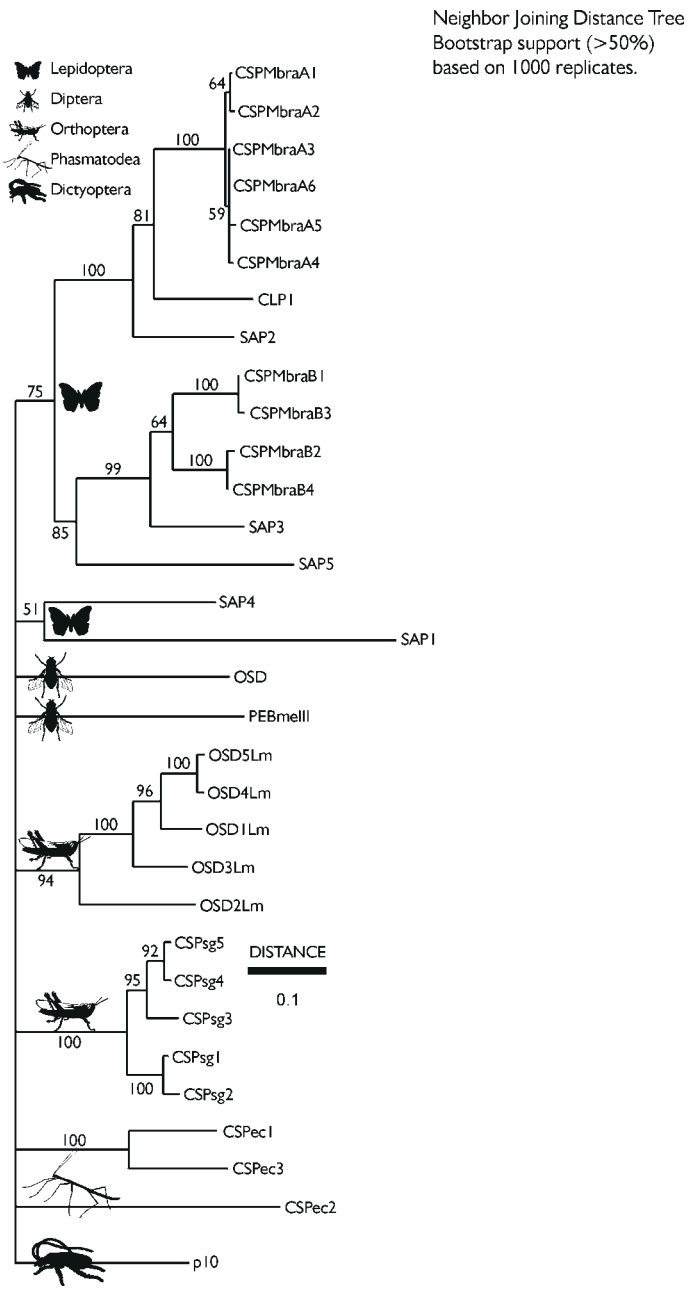

Figure 5 Neighbor joining distance tree of CSP sequences. Branch lengths are proportional and the scale of distance is indicated. Bootstrap support values (%) based on 1000 replicates are indicated.

Cys57–Cys60 and Cys29–Cys38, which create two small protruding loops. The topology of the CSP proteins thus appears very different from that of OBPs, where six conserved cysteines form three disulfide bridges (Leal *et al.*, 1999; Scaloni *et al.*, 1999). Amino acid identity among the CSPs from different species is high (40–50%), and higher within a single species, e.g. CSPMbraA, CSPsg or LmigOS-D (80–99%). In contrast, OBPs and OBP-related proteins of different species are highly divergent, as are

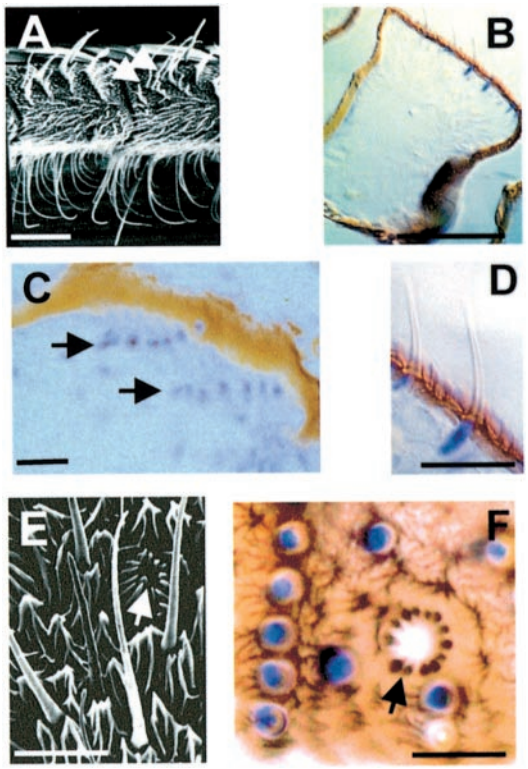

Figure 6 Expression patterns of CSPMbraA6 gene revealed by *in-situ* hybridization to mRNA in longitudinal sections of *M. brassicae* male antennae. **(A, E)** Scanning electron microscopy of a male antennae. **(A)** Ventral surface showing disposition of short and long hairs, the last being arranged in parallel rows (white arrows). **(E)** Detail of the ventral side showing morphology of a typical sensillum coeloconicum (white arrow) surrounded by sensilla trichodea (hairs). **(B)** Expression of CSPMbraA6 restricted to the sensilla side of the antennae, with no labeling on the scale side. **(D)** Sensilla trichodea at higher magnification. **(C, F)** Sections through the cuticle in the ventro-lateral region of the antennae showing CSPMbraA6 expression in row patterns (black arrows) consistent with the distribution of the long sensilla trichodea. **(F)** Detail of a sensillum coeloconicum without any labeling (black arrows) surrounded by labeled sensilla trichodea bases. Bar: 50 μm in A, B and 10 μm in C– F.

different classes of OBP within a single species (Vogt *et al.*, 1999).

Multiple classes of insect CSP

In our study, the sequencing of multiple clones encoding CSPMbraA and CSPMbraB revealed variations suggesting that these proteins are expressed in multiple isoforms. Isoforms for each class of proteins, CSPMbraA and B, have already been observed in the *M. brassicae* proboscis (Nagnan-Le Meillour *et al.*, 2000), as well as in other species such as *S. gregaria* (Angeli *et al.*, 1999), *E. calcarata* (Marchese *et al*, 2000), or *L. migratoria* (Picimbon *et al.*, 2000). In particular, CSPMbraA deduced proteins are 97–99% identical. When PCR amplification is performed on cDNA from a pool of organs, this identity could be the reflection of individual differences. However, many CSP isoforms were found in individual *S. gregaria* legs, reflecting

a real microdiversity in the same animal, although we do not know if this is the case in *M. brassicae*.

In the present tree analyses, we were able to differentiate CSP-related proteins into multiple classes. First, there is strong support (99 or 100% bootstraps) for distinct Lepidoptera classes (CSPMbraA, CLP1, SAP2/CSPMbraB, SAP3) and subclasses (e.g. CSPMbraB1, B3/CSPMbraB2, B4). In *M. brassicae,* CSPMbraA1/A6 may be different alleles of the same gene, which is also likely the case for CSPMbra B1/B3 and for CSPMbraB2/B4; however, these groupings represent at least three distinct CSPs in *M. brassicae.* Second, the tree revealed that no branch contains proteins from multiple orders. The different CSPs identified within different insect orders are presumably homologous proteins, but their orthogologous/paralogous relationships are as yet unclear, and the diversification of CSP proteins within an order may have derived from duplications within that order.

Role of the CSP proteins

Several different roles have been proposed for the CSP proteins based on their tissue localization. For example, p10 is expressed during leg regeneration in the cockroach *P. americana* and thus has a proposed function related to limb regeneration (Nomura *et al*., 1992). CLP-1 is found in the labial palps of the moth *C. cactorum* and has been proposed to function in CO_2 detection (Maleszka and Stange, 1997). However, no binding of radioactively labeled CO_2 could be observed with the CSP proteins of *S. gregaria* and immunocytochemical analysis led to the suggestion that these proteins have a role in contact chemoreception in Orthoptera (Angeli *et al.*, 1999).

In *M. brassicae*, CSPs have been characterized with respect to their patterns of tissue expression and their ability to bind small ligands. First, binding assays performed using crude protein extracts from the different organs (antennae, pheromonal glands) and tritiated major pheromone component analogue Z11–16:Ac, as well as the 18-carbon homologue Z11–18:Ac, showed that these proteins could bind these odorants. Similar binding was recently observed with CSPMbra proteins from the proboscis (Nagnan-Le Meillour *et al*., 2000); partial sequences of these proboscis proteins were identical with sequences of the proteins described here. Moreover, in antennae and gland extracts, proteins migrating at the CSP position were able to bind the two fatty acids 16:COOH and Z9–18:COOH, that are not pheromone components of the species studied, but no binding with progesterone was observed (Figure 2). It appeared that CSP can bind all the fatty acids and fatty-acid derivatives tested in this study, but not progesterone that is a structurally unrelated compound. So, the odorant binding capability of the MbraCSPs seemed to be restricted to fatty acids and/or to 16–18 carbon backbone skeleton. It is quite different from that of the OBPs that bind selectively the fatty-acid derivatives tested (Maïbèche-Coisné *et al*., 1997; Maida *et al*., 2000; Plettner *et al*., 2000), suggesting that the diversity of OBPs may play a role in the prefiltration of the odorant message.

In addition to the three more prominent components described by Farine *et al.* (Farine *et al.*, 1981) in the *M. brassicae* female pheromone blend, Z11–18:Ac was reported in trace amounts in a *M. brassicae* strain by Struble *et al.* (Struble *et al.*, 1980), but was never further mentioned. However, no positive signal in response to Z11–18:Ac was recorded in EAG screenings (Bohbot *et al*., 1998), but its binding with MbraCSPs in the proboscis (Nagnan-Le Meillour *et al.*, 2000), antennae (Bohbot *et al*., 1998) and female gland (this study) suggests that this compound might play a role in *M. brassicae* chemical communication.

Additional information regarding CSP function comes from the CSPMbraA *in-situ* hybridization studies in adult male antennae. CSPMbraA was expressed in the supporting cells of both long sensilla trichodea and short sensilla; the identity of the short sensilla, as short trichoid or basiconic, could not be determined. No expression occurred in the sensilla coeloconica, a structure easily recognized on histological sections. These results are different from those observed in other species. In *S. gregaria*, immunocytochemical localization of CSP proteins revealed that they are abundant in the outer lymph of contact sensilla in all chemosensory organs investigated (antenna, maxillary palps and tarsi) (Angeli *et al*., 1999). Labeling was observed in antennal sensilla chaetica, but not in olfactory sensilla or sensilla coeloconica, leading to the suggestion that in Orthoptera, CSPs are involved in contact chemoreception. In *Drosophila* antennae, OS-D expression appeared to be associated with sensilla coeloconica, although it was not definitely established (McKenna *et al*., 1994). A strong signal was observed surrounding the sacculus, a sensory cavity containing sensilla coeloconica, but also other sensilla types, and the resolution did not permit the authors to establish clearly that labeling is associated with one sensilla type. Thus, it appears that the site of CSP expression is different depending on the species studied, suggesting that the role of CSPs may differ from one insect order to another. In our study, expression of secreted proteins in olfactory sensilla trichodea supporting cells leads us to hypothesize that, in *M. brassicae*, at least CSPMbraA is implicated as having some function in pheromone reception.

The discovery of CSP proteins in the pheromonal gland of *M. brassicae* females is provocative. Unlike other organs in which CSPs have been identified, the pheromone glands are devoid of any sensory structures; in our preparations the glandular epithelium was meticulously separated from the ovipositor that is known to carry such sensilla. The pheromones of the Lepidoptera are generally a blend of long-chain unsaturated aldehydes, alcohols and acetate esters derived from fatty acids. If desaturation and reduction steps occur on acyl moities, they produce free hydrophobic fatty acid derivatives in the cytosol that need to be dissolved

in the otherwise aqueous medium of the cells and brought to the cell surface for diffusion into the air, in a process that is similar (and reverse) to pheromone reception in the olfactory sensilla of the antennae. CSPs expressed in pheromone glands may function to non-specifically bind and transport pheromone components through the cytosol to the outward facing cell membranes. Such a function may explain the localization of the CSP protein PEBmeIII in the ejaculatory bulb of *Drosophila.* The ejaculatory bulb synthesizes vaccenyl acetate, Z11–18:Ac, which is transferred from the male to the female during copulation and may serve as a repellent for other courting males (Jallon *et al*., 1981). One can then imagine that the Z11–18:Ac, a hydrophobic molecule, would be carried by a soluble protein, such as PEBmeIII. Other proteins with four conserved cysteines, but also with poor sequence similarities with OBPs, were identified in the beetle *Tenebrio molitor*: the B proteins in accessory sex glands (Paesen and Happ, 1995) and THP12 in hemolymph (Rothemund *et al*., 1997). Their three-dimensional structure (Rothemund *et al*., 1999) indicates that they could have a similar function as small lipid carriers in body fluids. More generally, transporters of small hydrophobic ligands are common throughout animal species, from invertebrates (OBPs, blood carriers such as insecticyanin, etc.) to vertebrates where they bind pheromones in the nasal mucus (Pelosi, 1994; Flower, 1996), but also in physiological fluids such as urine, saliva or vaginal secretion, known to carry pheromones (Pelosi, 1994).

The presence of CSPMbra in both antennae and pheromonal gland, sites where pheromone molecules are localized within the animal, extend the model of CSP function, suggesting that the proteins may have a general interaction with small, hydrophobic molecules in a non-specific manner, analogous to the interaction of serum albumens with steroids in mammalian blood, maintaining their solubility until interactions with more specific proteins (e.g. OBPs) can take place. These proteins might then represent a novel class of binding proteins that could be referred to as OBP type 2 (Vogt *et al*., 1999), although they share only a few features, such as acidity and low mol. wt, with known insect OBPs.

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