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P450 and P450 reductase cDNAs from the moth *Mamestra brassicae*: cloning and expression patterns in male antennae

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Abstract

The involvement of cytochrome *P*450 (CYP) enzymes in olfaction has been demonstrated in vertebrates over the past decade. In insects, these enzymes are well known for their role in biosynthesis of endogenous compounds as well as xenobiotic metabolism, but the presence of olfactory cytochrome *P*450s was poorly investigated. Using a PCR-based strategy, we have isolated cDNAs of two new microsomal P450s from the antennae of the cabbage armyworm *Mamestra brassicae*, *CYP9A13* and *CYP4G20* of two new microsomal P450s, as well as their red-ox partner, the cytochrome *P*450 reductase (CPR). Their distribution through the body and their cellular localization within the antennae were studied by RT-PCR and in situ hybridization. The three genes are strongly expressed in some sensory units of the antennae, the sensilla trichodea, which are tuned to odorants detection. The putative functions of the corresponding enzymes are discussed in regard to their respective expression patterns and to our knowledge on olfactory P450 metabolism in mammals.

Keywords: Cytochrome *P*450; NADPH-P450-reductase; Olfaction; Odorant/xenobiotic degradation; *Mamestra brassicae*

1. Introduction

All organisms live in environments that contain potentially harmful chemicals, natural or anthropic. In vertebrates, extensive studies of detoxification mechanisms, especially in liver, have revealed the great variety of chemical transformation involved, including red-ox reactions catalyzed by cytochrome *P*450 (CYP) enzymes. Cytochrome *P*450 monooxygenases (P450s or CYPs) consist in a large superfamily of enzymes that catalyzes the transfer of one atom from O2 into various substrates (monooxygenase reactions), leading to a great diversity of catalytic reactions, such as hydroxylations (for a review, see Mansuy, 1985). P450s play a fundamental role as phase I transformation enzymes during detoxification of exogenous compounds, such as drugs, toxic pollutants or pesticides. The products of phase I reactions are then metabolized by phase II enzymes, such as UDP-glucoronosyl transferases and glutathione *S*-transferases, leading to the production of hydrophilic compounds that can no longer cross the membranes and are eliminated by secretion. P450-mediated reactions play also a key role in the metabolism of endogenous compounds, such as steroid hormones of vertebrates (Meyer, 1996) or insects (review in Feyereisen, 1999).

In mammals, the CYP1, 2 and 3 families are particularly involved in xenobiotic metabolism, and most of them are expressed in the liver, which plays a dominant role in the first-pass clearance of ingested compounds and controls the systemic levels of drugs and other chemicals substrates (Ding and Kaminsky, 2003). In insects, a great number of P450 genes have been isolated to date in more than 39 species

Abbreviations: CYP, cytochrome *P*450; CPR, cytochrome *P*450 reductase; 20E, 20-hydroxyecdysone; JH, juvenile hormone.

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(http://www.drnelson.utmen.edu/cytochromeP450.html). Some of them are involved in fundamental physiological functions, such as growth, development or reproduction through the biosynthesis and the catabolism of key hormones, such as juvenile hormone (JH) or 20-hydroxy-ecdysone (20E; Feyereisen, 1999). Recent molecular genetic and biochemical approaches in *Drosophila* and *Bombyx mori* have lead to the identification of four P450 enzymes (CYP302A1, CYP315A1, CYP314A1 and CYP306A1) involved in hydroxylation steps during 20E biosynthesis (Niwa et al., 2004). Another P450-mediated reaction (26-hydroxylation) is involved in 20E inactivation and the corresponding enzyme could correspond to CYP18A1 in *Drosophila* (Feyereisen, 1999). In the cockroach *Diploptera punctata*, CYP15A1, a newly identified microsomal P450, was showed to catalyze the last step of JH biosynthesis via a highly selective epoxidation reaction (Helvig et al., 2004). However, most of the P450s studied to date, especially in agronomical pest insects, were found to be involved in insecticide metabolism and inactivation of plant toxins (Stevens et al., 2000; Li et al., 2002). Among them, the P450s belonging to the CYP4, CYP6, CYP9 and CYP12 families are generally expressed in the digestive tract and fat body (Feyereisen, 1999; Petersen et al., 2001).

The olfactory system of terrestrial animals, which is specialized in the detection of volatile hydrophobic molecules, has a similar organization in mammals and insects. The dendrites of the olfactory neurons, which carry the olfactory receptors, are bathed in an aqueous medium, the mucus layer in vertebrates or the sensillum lymph in insect antennae, medium that odorants must cross before interaction with receptors. In both cases, the sensory neurons are thus indirectly in contact with the external environment and therefore exposed to odorants but also to volatile xenobiotics. In mammals, olfactory and respiratory tissues, which are exposed to both inhaled and blood-borne compounds, were found to express xenobiotic metabolism P450s (Lazard et al., 1990; Ding and Coon, 1990; Peng et al., 1993). The nasal mucosa is considered as a "metabolic hot spot" (Ding and Kaminsky, 2003) and many P450s found in liver, as well as other biotransformation enzymes, such as UDP-glucoronosyl-transferases, were also found in the respiratory tract (Lazard et al., 1990), leading to a tissue-selective response to chemical exposure. These biotransformation enzymes have been proposed to participate in the termination of odorant signals, as well as the protection of the sensory neurons against potentially harmful compounds (Lazard et al., 1990).

In insects, the presence of P450s in olfactory tissues was demonstrated only recently. One cytochrome P450 cDNA belonging to the CYP6 family was found to be expressed in the antennae and chemosensory organs of fruit fly *Drosophila melanogaster* (Wang et al., 1999) and ESTs encoding P450s were reported from the antennae of the tobacco hornworm *Manduca sexta* (Robertson et al., 1999). In addition, a biochemical study of the degradation of the pheromone from the scarab beetle *Phyllopertha diversa* demonstrated the involvement of antennal P450 metabolism (Wojtasek and Leal, 1999). More recently, two P450 cDNAs belonging to the CYP4 family were characterized in the antennae of a Lepidoptera, the cabbage armyworm *Mamestra brassicae*, and were shown to be expressed in the olfactory sensilla trichodea, devoted to odorant detection (Maïbèche-Coisne et al., 2002). Microsomal P450-mediated monooxygenase activity is supported by NADPH and thus requires an interaction with NADPH-cytochrome *P*450 reductase (CPR). As CPR plays an essential role in the transfer of reducing equivalents from NADPH to cytochrome *P*450 enzymes, its presence in olfactory tissues is a necessity. In mammals, CPR is widely expressed in all tissues examined, including the olfactory mucosa, but its expression in insect antennae has been reported only once in *D. melanogaster* (Hovemann et al., 1997).

In this study, we characterized two new P450 cDNAs strongly expressed in the antennae of *M. brassicae*, in association with a CPR cDNA, leading to a total of four antennal P450s in the same species. The expression of all these genes in olfactory sensilla is strongly suggestive of a role in odorant and/or xenobiotic clearance.

2. Materials and methods

2.1. Animals and tissue collection

Insects were purchased as pupae from Domaine du Magneraud (INRA, France) and were maintained at 20 °C and 60% relative humidity until emergence. Dissected tissues from sexual mature 3-day-old males (antennae, proboscis, brains, legs, thorax and abdomens) and females (antennae, ovipositors) were stored at −80 °C until use for total RNA isolation. For in situ hybridization experiments, male antennae were immediately fixed in 4% paraformaldehyde.

2.2. RNA isolation and cDNA synthesis

Total RNAs were extracted with TRI-Reagent (Euromedex) and were quantified by spectrophotometry at 260 nm. Single-stranded cDNAs for RT-PCR were synthesized from total RNAs (1 μg) from the various tissues with 200 units of M-MLV reverse transcriptase using the RT-for-PCR-Advantage™ kit (Clontech). For 5′and 3′RACE-PCR, single-stranded cDNAs were synthesized from 1 μg of male antennal total RNA using the SMART™ RACE cDNA Amplification kit (Clontech), according to the manufacturer's instructions.

2.3. Molecular cloning of two putative P450 antennal cDNAs by 3′ and 5′ RACE

3′ RACE-PCR were carried out on antennal cDNA with *Taq* polymerase (Promega) using the Universal Primer Mix (Clontech) and a degenerated primer (5′-GGICCI(A/C)GIAA(C/T)TG(C/T)ATIGG-3′), corresponding to the consensus sequence GPRSCVG of the heme-binding domain of P450 enzymes. Touchdown PCR was done as follows: 1 min at 94 °C, 3× (30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C), 3× (30 s at 94 °C, 30 s at 50 °C, 1 min at 72 °C), 3× (30 s at 94 °C, 30 s at 45 °C, 1 min at 72 °C), then 30× (30 s at 94 °C, 30 s at 40 °C, 1 min at 72 °C), then 5 min at 72 °C.

Two fragments of around 300 and 500 bp were amplified, gel purified (GenElute™, Sigma) and ligated into pCR®II using the TOPO cloning kit (Invitrogen). Recombinant plasmids were purified (QIAprep® Spin Miniprep kit, Qiagen) and subjected to automated sequencing (Genomexpress). Gene sequence analyses and database comparisons were carried out using the BLAST program or GeneJockey software (Biosoft, Cambridge, UK). Two different cDNA products of 312 and 523 bp were identified as putative new P450 fragments and named *MbraCYP4* and *MbraCYP9*, because of their sequence similarities with these two CYP families.

The 5′ regions of the corresponding cDNAs were obtained using the SMART™ RACE cDNA Amplification kit. Two specific antisense primers were designated according to the cDNA sequences obtained after 3′RACE, 5′-AGTCGGACTCCTTAAGATCTGAGTGGACGCGG-3′ for *MbraCYP4* (encoding amino acids RVHSDLKESD) and 5′-CTTATCAAGCTTGGCCGGGATTGTAGTCTTCTC-3′ for *MbraCYP9* (encoding amino acids EKTTIPAKLDK). Touchdown PCR was performed as described in Maïbèche-Coisne et al. (2002). PCR products were cloned as described above.

By merging the overlapping sequences obtained by 3′and 5′ RACE, two putative full-length cDNAs were generated. After analysis by the P450 Gene Family Nomenclature Committee (Dr. Nelson D. R., University of Tennessee, Memphis), the two cDNAs were qualified as *CYP4G20* for *MbraCYP4* and *CYP9A13* for *MbraCYP9* because of their greatest similarity to members of subfamilies 4G and 9A, respectively. Indeed, all members of a subfamily have to share at least 55% identity (Feyereisen, 1999).

2.4. Molecular cloning of M. brassicae NADPH-cytochrome P450 reductase from antennae

Two degenerated primers deduced from conserved amino-acid sequences YGEDPTDN (5′-TAYGGYGARGGMGATCCCAC-3′) and HPFPCPT (5′-GTGGGRCARGGGAATGGRTG-3′), found in house fly *Musca domestica*, fruit fly, silk moth *B. mori* and rat CPR sequences, were used to carry 35 cycles of PCR on male antennal cDNA with annealing temperature of 45 °C. A 680 bp product was amplified, then cloned and after analysis, the sequence suggests that this cDNA fragment was derived from P450-reductase gene. The 550 bp 5′ and 3130 bp 3′ parts of the corresponding cDNA were obtained as described above, using two specific primers for 5′ RACE (5′-GCCAAGGCCAAACACAGCATAATTCAAACCAGT-3′) and for 3′ RACE (5′-GTGGAACGTCTGGGCCAGTTAACTGGAGCT-3′).

2.5. RT-PCR detection of CYP4G20, CYP9A13 and M. brassicae CPR in different tissues

Equal amounts of RNA (1 μg) extracted from male antennae, proboscis, brains, legs, thorax, abdomens and from female antennae and ovipositors were reverse-transcribed and simultaneously amplified with *Cyp4G20*, *CYP9A13*, and CPR specific primers: (5′-GAGTTCACCGGTGGCTCTCACGACATCTTC-3′/5′-AGCGGCAGTTGTGTCGTGACCCTC-3′ for *CYP4G20*; 5′-GTCACGATGGAGGAGGTGGAAAA-3′/5′-CCTTCAGCCCGAAGGCGCAGGAGGCT-3′ for *CYP9A13*; 5′-AATTATGCTGTGTTTGGCCTTGGC-3′/5′-AGTTAACTGGCCCAGACGTTCCAC-3′ for CPR). Fragments of, respectively, 906, 340 and 522 bp were obtained. The *M. brassicae* ubiquitous ribosomal protein gene *rpL8* (accession number AAR36138), that presents a constant expression in all tissues checked, was used as a control of gene expression (Maïbèche-Coisne et al., 2004). The sense primer 5′-GAGTCATCCGAGCTCARMGNAARGG-3′ and the antisense primer 5′-CCAGCAGTTTCGCTTNACYTTRTA-3′ allowed amplification of a 580 bp fragment. PCR products were loaded on 1.8% agarose gel and visualized by ethidium-bromide.

2.6. In situ hybridization

The recombinant pCR®II-TOPO plasmids, containing the 312, 523 and 680 bp insert fragments of *CYP4G20*, *CYP9A13* and CPR cDNAs, were linearized for in vitro transcription of RNA sense and antisense probes using T7 and SP6 RNA polymerase (Promega), following recommended protocol. Hybridizations on male antennae were performed as described in Maïbèche-Coisne et al. (2002). Five-micron longitudinal sections were performed and photographed. Pictures were digitized and processed using Adobe Photoshop® 5.5.

3. Results

3.1. Cloning of two new antennal cytochrome P450 genes, CYP4G20 and CYP9A13

Two cDNA products of 312 and 523 bp were amplified after RT-PCR on male antennal RNA by 3′ RACE, using a degenerate primer deduced from the conserved heme-

binding region of cytochrome *P*450s. After cloning and sequencing, the deduced amino-acid sequences appeared to belong to the cytochrome *P*450 superfamily. They were then extended to the 5′ region by 5′ RACE using specific primers. Two full-length cDNAs of 2155 and 1779 bp were then obtained and designated by the P450 Nomenclature Committee as *CYP4G20* and *CYP9A13*, respectively. These sequences have been deposited in the GenBank database with accession numbers AY390259 and AY3902260. *CYP4G20* nucleotide sequence analysis revealed that this cDNA contains a putative ORF of 1671 bp, a 153 bp 5′-untranslated region (5′UTR) and a 328 bp 3′ UTR, with two putative polyadenylation signals upstream of the poly(A). *CYP9A13* cDNA is constituted of a 1593 bp ORF, with a 153 bp 5′UTR and a 331 bp 3′UTR with only one polyadenylation signal.

CYP4G20 encodes a 557 amino-acid protein, with a theorical molecular mass of 63272 Da as determined using MWCALC (Infobiogen). The *CYP9A13* encoded protein is relatively smaller, with 531 amino acids and a putative molecular weight of 61168 Da. These protein-deduced sizes are in good agreement with the size of other already known P450s, in particular the CYPs belonging to the CYP4G subfamily are somewhat larger that the other invertebrate P450s. The two CYP-deduced amino-acid sequences are aligned in Fig. 1 with the two other antennal P450s from *M. brassicae*, CYP4L4 and CYP4S4 (Maïbèche-Coisne et al., 2002). Both CYP4G20 and CYP9A13 exhibit important characteristics of members of the P450 superfamily and their deduced amino-acid sequences share a number of common structural features with other P450s: the typical P450 signature corresponding to the heme-binding domain

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CYP9A13      LIRLTIGQAVVTTQSTRGDAFRSCSEQHIMIILLIWVAVLIAVLVLYLRQVYSRSSRYGVKHFKPVPLLGDMTG
CYP4G20      ----------MSYAAAESVVPTSTWAATSLFYVLLVPALILWYAYWRMSRRHMYELAAKLHGPPGLPLLGNALE
CYP4L4       ------------------------MIVLLVFVVVVLALLVSWISLVRVSRR-----FN-VPGPSPLPLVGNAHL
CYP4S4       -------------------------MLLTLALLGFITFVIWRLLADEENT-----LDQLPGPPRRPIIGCALE
                                                                        *  *

CYP9A13      TGILFRVNHFTDDIVNLYDSFPEEKFVGRFEFLNEMVMIRDIELVKKITVKDFEHFLDHRSIFSTSDSFFSRNLF
CYP4G20      LEFTGGSHDIFRNVIEKSIPYDGESVVKIW-IGPRFWCSCTILVTWSLILSSHTH-IDKADEYRFFKPWLGDGLL
CYP4L4       HLFVVKSSEFLNLVQRLSEK-YGHAFRVHF-FSTPYVVICHSKYA-EPLVSSTEH-ITKGRSYSFLTCWLGQGLL
CYP4S4       LEFLKISPRELFLKMRQYKKEYGDRYVIKM-MKRRILHLSNEKDV-EVVLSHSRN-IKKSKPYTFLEAWLGTGLL
                                                                              *

CYP9A13      SLKGQEWKDMRSTLSPAFTSSKMRLMVPFMVEVGDQMMSSLQKKIKESESGYIDIDCKDLTTRYANDVIASCAFG
CYP4G20      ISTGQKWRSHRKLIAPTFHLNVLKSFIDLFNANSRAVVSKLKK--EAGE-----FDCHDYMSECTVEILLETAMG
CYP4L4       TATGQRWKSHRKFLTPAFHFNILQNFLPVFCKNQRILTEKIRGMADGRP-----IDMFPIIALAALDNVNESIMG
CYP4S4       LSSGAKWHRRRKILTPTFHFNILKSFTVVMKEKSRSLVETLNNL-EGTE-----VNLLPVISDYTLYTICETAMG
                *  *   *    * *                                                      *

CYP9A13      LKVDSHNDVDNEFYAMG--KVSSTFNFRQMLMFFLIVNAPKLAKV-LKLDFLSEASKIFFRKLVLETMANREMKN
CYP4G20      VSKSTQDQSGF-EYAMAVMKMCDILHLRHTKIWLRPNLLFKLTDYAKKQTKLLDVIHGLTKKVIRRKKEEFNSGK
CYP4L4       VCMDAQKHSQS-EYVKSIEELSAIVTMRMQIPFFGEDAIFNLLPYKTKQDKALKVLHGQTNKVINARRAELKKAN
CYP4S4       TQLDADKSSKTLEYKTAILEIGNLLLGRLTRVWLHNEYLFKKFPIGRRFENCLEKVHSFADDVIMERKKNWKPGQ
                          *               *

CYP9A13      VIRP----------------DMIHLLMEAKKGKLTHEDIKSSDEAAGFATVEESSVGQKEINRVWTDDDLIAQAV
CYP4G20      RPTILQDCTTTTTEEANKTTSVEGLSFGQSAGLKDDLDVDDADVGQKKRLAFLDLLLESSQSGVVISDEEIKEQV
CYP4L4       ITTL--------------------------------NDSSDIGTKNKHTFLDLLLLAEIDGKKIDDESVREEV
CYP4S4       SEFT--------------------------------EDDVSIGGKKRYAMLDLLLEAENKGE-IDLEGIREEV
                                                                              *

CYP9A13      -LFFIAGFETVSAGMSFLLYELALNPDVQERLAQEIKETDAKN-GGKFDFNSIQNMPYMDMVVSELLRLWPPGIA
CYP4G20      DTIMFEGHDTTAAGSSFFLSMMGIHQDIQDKVIDELDKIFGDS-DRPATFQDTLEMKYLERCLMETLRMFPPVPI
CYP4L4       DTFMFEGHDTTTSGIVYTLHCLSKRRDVQEKIYEELKTIFGSEIHRDPTYHELQQMKYLELVIKESMRLFPPVPL
CYP4S4       NTLMFEGHDTTAMAIVLGLMLIADHEEVQDRIFEECQKILGDS-DRTPTMSDLADMKYLEAVIKETLRLYPSVPF
               * *                    *    *                        *     *  *  *
               i-helix

CYP9A13      LDRICTKDYNLGKPNEKAEKDFIVRKGTGISIPAYAFHRDPQFFPNPDKFDPERFSEENKHNIQMFAYMPFGVGP
CYP4G20      IARHLKQDITLPSCGKQ------VPAGTTVVVATYKLHRRPDVYPNPTEFDPDNFLPEKSANRHYYAFVPFSAGP
CYP4L4       IERRIMKDCEVG--GLK------LVKGTSVVMNIFQIQRQPDLFDDPLEFRPERF---EAPLKNPFSWLAFSAGP
CYP4S4       IAREITEDFMLD--DLP------VKKGSEVSIHIYDLHRRPDLFPEPDAFIPDRFL--SGAPMHPYAFVPFSAGP
               *                    *          * *     *  * *  *                  *  **
                                                                        heme-binding

CYP9A13      RNCIGSRFALCEMKVMAYQILQHLEVSPCEKTTIPAKLDKQTFNIRLEGGHWLRFRARK-----
CYP4G20      RSCVGRKYAMLKLKIILSTILRSFRVHSDLKESDFKLQ--ADIILKRAEGFKVRLEPRKTTKAY
CYP4L4       RNCIGQKFAMMELKITISEIVKNFFILPAAE--EPELS--ADLVLRSKNGVNVKFLPRK-----
CYP4S4       RNCIGQRFAMLEMKCVFSAICRNFRLAPKVQGYRPALL--ADMLIRPEGPVYVKFSRR------
             * * *   *    *     *                                  *
             domain
```

Fig. 1. Comparison of CYP9A13 and CYP4G20 amino-acid sequences with two other *M. brassicae* antennal cytochrome *P*450s, CYP4L4 (GenBank n° AY063501) and CYP4S4 (GenBank n° AY063500). From the N-terminal to the C-terminal ends, the clusters of prolines are simply underlined, the I-helix and the heme-binding regions are boxed. Identical residues among the four sequences are marked by asterisks.

(most commonly FXXGXRXCXG), which is found in the carboxyl-terminal parts, and the consensus sequence (XGX(D/E)T) within the I-helix region (EGHDT for CYP4G20, AGFET for CYP9A13). In addition, CYP4G20 and CYP9A13 have characteristics that are unique to, respectively, CYP4 and CYP9 families: the SR(F/I/L)(A/G)XX(Q/E) sequence immediately following the heme-binding domain, specific to family 9, is found in CYP9A13 (SRFALCE, position 478–484) and the invariant I-helix region with amino-acid sequence DTF(I/M)FEGHDT, that is typical of the CYP4 family, is found in CYP4G20 sequence (DTIMFEGHDT, position 352–362). The presence of a hydrophobic N-terminal segment, followed by a short cluster of prolines that form a hinge, strongly supports an endoplasmic reticulum location for both CYP4G20 and CYP9A13, as also previously suggested for the two other antennal P450s from *M. brassicae*, CYP4L4 and CYP4S4.

CYP4G20 and CYP9A13 have significant amino-acid similarities with CYPs from other insects: CYP9A13 shares 82.2% and 62.6% identity with CYP9A12 and CYP9A14

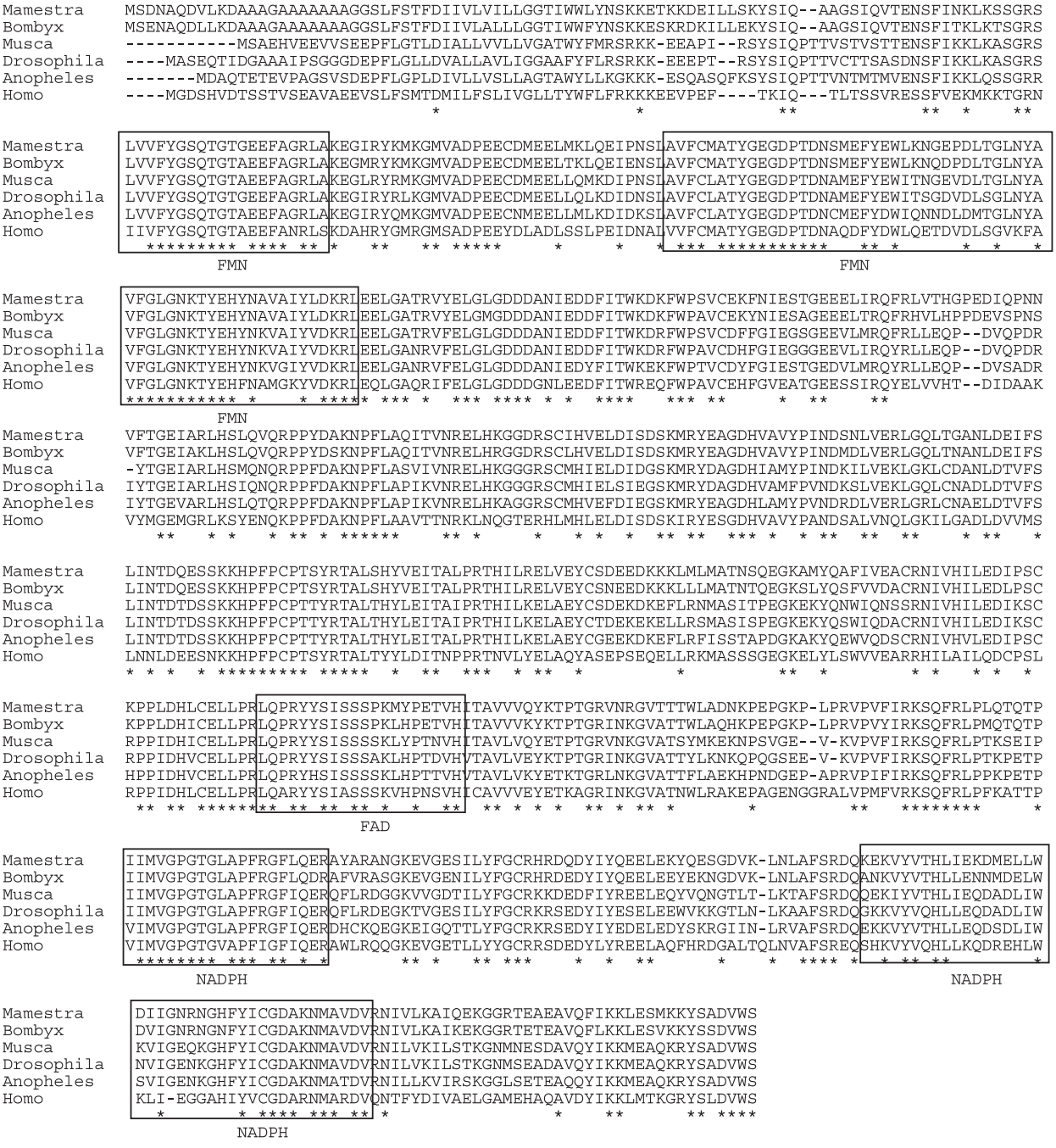

Fig. 2. Comparison of the amino-acid sequence of *M. brassicae* P450 reductase with those of silk worm (GenBank n°AB042615), house fly (n° AAA29295), fruit fly (n° NP723173), African malaria mosquito (n° AA024765) and human (n° NP000932). Identical residues among the six sequences are marked by asterisks. Several regions reported to be the binding sites of the cofactors are boxed.

from the cotton bollworm *Helicoverpa armigera*, 57.2% with CYP9A4 from the tobacco hornworm *M. sexta* and 41.6% with CYP9E2 from the cockroach *Blattella germanica*. CYP4G20 is closest to CYP4G15 from the fruit fly (69.2%), CYP4G19 from *B. germanica* (62%) and CYP4G13 from the house fly (41%). It presents 69.8% of identity with one antennal EST sequence from *M. sexta* (pMsma65, accession number AI187496), which may encode a P450 belonging to the CYP4G subfamily too. However, CYP9A13, CYP4G20, CYP4L4 and CYP4S4 from *M. brassicae* antennae share less than 25% of identity with each other.

3.2. Cloning of *M. brassicae* P450 reductase

A cDNA fragment of 680 bp encoding a P450 reductase ortholog was amplified by RT-PCR on male antennal cDNA and subjected to 5′ and 3′ RACE. This leads us to obtain a full-length cDNA of 4154 bp (Genbank accession number AY390257), with an ORF of 2061 bp and a 3′UTR of 2093 bp containing 7 putative polyadenylation signals. The deduced protein is constituted of 687 amino acids and has a calculated molecular mass of 77538 Da. This amino-acid sequence was aligned with those of P450 reductases found in other insect species and human (Fig. 2). The proposed FMN, FAD and NADPH-binding sites described in the house fly CPR sequence (Koener et al., 1993) are found. The *M. brassicae* sequence is closest to silk worm CPR with 88.2% identity. It shares 67.8%, 66.8%, 65.3% and 55% identity with house fly, fruit fly, African malaria mosquito *Anopheles gambiae* and human CPR sequences, respectively. Because of these high degrees of sequence conservation, we conclude that this sequence correspond to a *M. brassicae* CPR ortholog.

3.3. Tissue distribution of *CYP4G20, CYP9A13* and CPR

The tissue-related expressions of the *M. brassicae* P450 and CPR cDNAs were determined by RT-PCR, using the ribosomal gene rpL8 as positive control. For similar levels of rpL8 expression, the three genes are amplified in male and female antennae, as in other chemosensory tissues tested, such as male proboscis and legs, or female ovipositors (Fig. 3). The three genes are also detected in thorax or abdomens and thus present an overall distribution through the body. However, if *CYP9A13* and CPR are clearly detected in brains, *CYP4G20* was never amplified in this tissue (three independent repetitions on different cDNAs from male brains, 35 cycles of PCR).

3.4. Localization of *CYP4G20, CYP9A13* and CPR expression within the antennae

Precise localizations of *CYP4G20*, *CYP9A13* and *M. brassicae* CPR ortholog within the antennae were achieved using in situ hybridization. The cabbage armyworm antennae are filiform and segmented, approximately 1 cm long and each segment exhibits the same general organization: the dorsal side is covered with two rows of scales, and the olfactory hairs (among them the sensilla trichodea) are located on the ventral side, as shown in Fig. 4A. In males, the sensilla trichodea are distributed in two classes according to their length: long sensilla trichodea (60–190 μm) are located on the lateral part of the ventral area and are settled in four to five parallel rows, whereas the short ones (35–55 μm) are medio-ventral and not arranged in rows.

Sense strand controls gave no signals (not shown), whereas antisense CPR probe hybridization is clearly restricted to the sensilla side of the antenna, with no signal on the scale side (Fig. 4A). The same patterns are observed for *CYP9A13* and *CYP4G20* probes (Fig. 4D and F). For the three genes, labeling is observed in sensilla trichodea and close examination revealed hybridization in cells localized at the base of sensory hairs (Fig. 4C, E, and G). Sometimes, two labeled somata can be seen at the base of one sensillum (Fig. 4C and F: arrows). On longitudinal sections through the antennae, it is difficult to distinguish between long and short sensilla, as only fragment parts of the sensilla are visible. However, on some sections through the cuticle of the antennae (Fig. 4B), labeled spots distributed in the ventro-lateral region with a row pattern correspond to long olfactory sensilla, whereas labeled spots randomly distributed in the median part of the segment correspond to short sensilla trichodea. Sensilla coeloconica and chaetica are not labeled (Fig. 4B and D).

4. Discussion

In the present work, we described in the noctuid moth *M. brassicae* the molecular cloning, the tissue distribution and the cellular expression in the antennae of two new members of the cytochrome *P*450 family, assigned to the CYP4 and

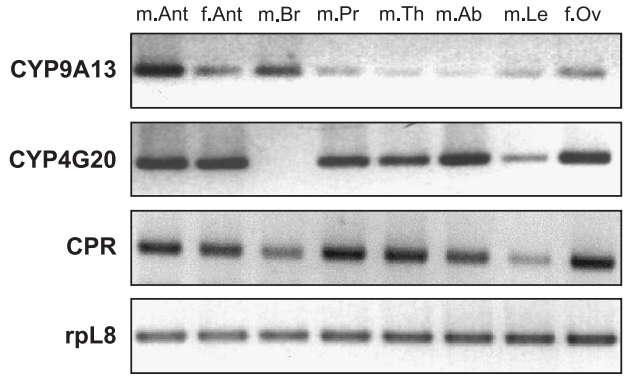

Fig. 3. Tissular distribution of *M. brassicae CYP9A13*, *CYP4G20* and CPR after RT-PCR analysis. The ubiquitous *rpL8* gene serves as positive control. m./f.: male or female; Ant: antennae; Br: brains; Pr: proboscis; Th: thorax without wings; Ab: abdomens; Le: legs; Ov: ovipositors.

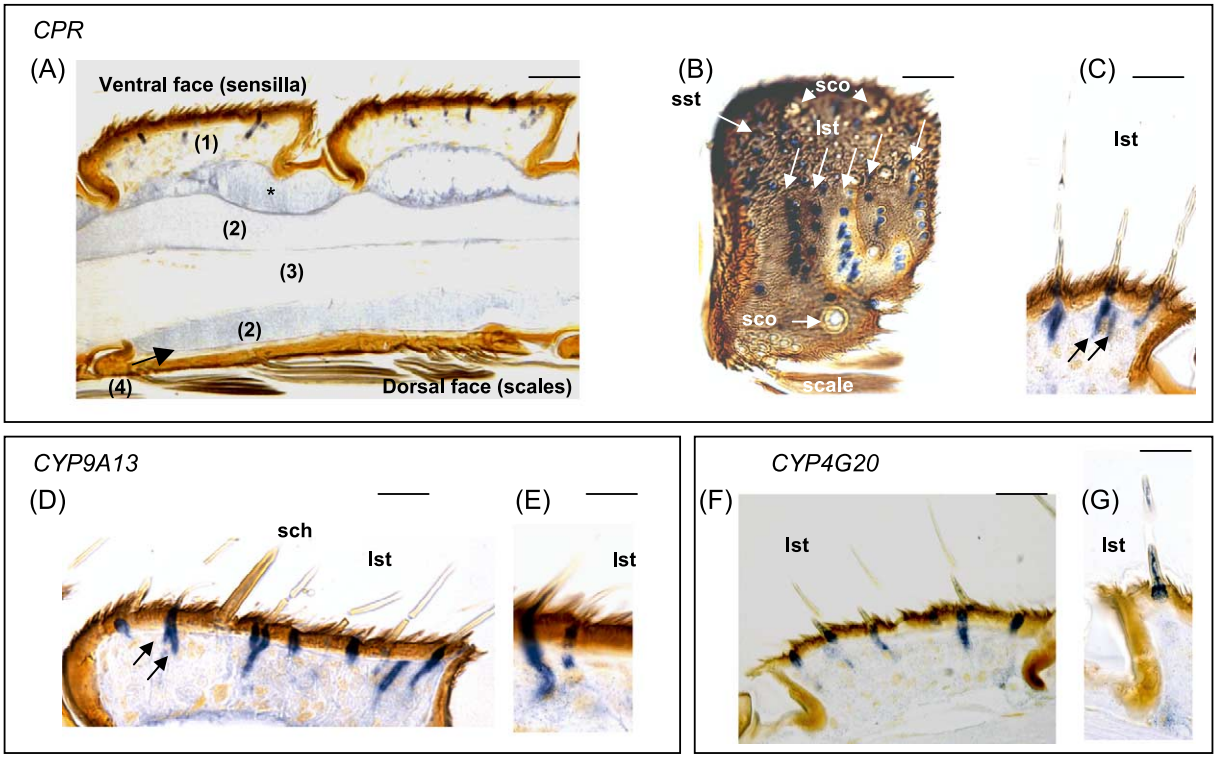

Fig. 4. Expression patterns of *Mamestra* CPR (A, B, C), *CYP9A13* (D, E) and *CYP4G20* (F, G) after in situ hybridization on sections of *M. brassicae* male antennae. (A) Longitudinal section of two male antennal segments, showing the ventral disposition of the olfactory sensilla and sensory neurons (1), the dorsal localization of the scales, the antennal lumen filled with haemolymph (2), the antennal nerve (3) and the epidermis (4). Asterisk marks an artifact. (B) Section through the cuticle. Arrows show the labeling of the long sensilla trichodea (lst), arranged in parallel rows. Medio-ventral short sensilla trichodea (sst) are randomly distributed. (C, E, G) Details of sensilla trichodea, with intense labeling at the base of the sensory hairs. (B, D) Sensilla coeloconica (sco) and sensilla chaetica (sch) are not labeled. Scale: 50 μm in A, B and F; 25 μm in D; 10 μm in C, E and G.

CYP9 families, as well as their red-ox partner, the cytochrome *P*450 reductase. The CYP4 is one of the most ancient and diversified group of P450s; they are found in vertebrates and insects and are related to endogenous as well as xenobiotic metabolism (Feyereisen, 1999). Few data are available on the CYP9 family: some of them, characterized in lepidopteran species, such as the tobacco budworm *Heliothis virescens* and the tobacco hornworm *M. sexta*, were shown to be involved in xenobiotic metabolism (Stevens et al., 2000) but most of them are of unknown function.

Contrary to the previously identified P450s from *M. brassicae* antennae, CYP4S4 and CYP4L4, whose expressions were restricted, respectively, to the antennae and to the chemosensory tissues, *CYP9A13* and *CYP4G20* transcripts are strongly expressed in the antennae of males, but are also found expressed in all other chemosensory tissues tested, including the male proboscis, legs and female ovipositors, organs where taste sensilla are located. This suggests that they may probably not only be involved in the metabolism of odorant compounds, but that they may also play a role in taste. For a similar level of *rpL8* expression, *CYP4G20* is strongly expressed in all tissues tested, except a faint expression in the legs, and no expression was found in the brains, whereas *CYP9A13* expression is abundant in male antennae as well as in brains. In mammals, most of the P450s expressed in the olfactory tract are also present in the liver, but some of them are preferentially expressed in this extra-hepatic location. In the rabbit, for example, CYP2G1 is olfactory specific (Ding and Coon, 1990) whereas CYP2A10 and A11 are found in olfactory mucosa as well as in liver (Peng et al., 1993). These three isoforms have distinct substrate specificities against endogenous compounds or nasal toxicants (Ding and Coon, 1994).

The detection of *M. brassicae CYP9A13* in brains is of particular interest. Indeed, few insect P450s were found to be expressed in the central nervous system, among them *CYP4G15* in the fruit fly (Maïbèche-Coisne et al., 2000) and *CYP6D1* in the house fly (Korytko and Scott, 1998), which is associated with insecticide resistance. The presence of various hepatic P450s in brain has been demonstrated over the past decade in rat, mouse and human, more precisely in neuronal cells, where they could play an important role in xenobiotic (review in Ravindranath, 1998) and endogenous steroid metabolism (Hiroi et al., 2001).

In situ hybridization analyses in the antennae demonstrated that, although RT-PCR revealed that the three genes have a broad expression through the body, they are also expressed in olfactory structures, the long and short sensilla trichodea, devoted to sex pheromones and general odorant

detection in male moths. They are also found in female antennae that also carry olfactory sensilla, devoted to food and host plant odor detection. Moreover, no signal was found at the base of the mechanoreceptor sensilla chaetica, nor in the sensilla coeloconica, whose function is unknown in *M. brassicae*, suggesting that expression is restricted to olfactory sensilla trichodea.

The *M. brassicae* CPR sequence is almost identical to the *B. mori* one, both being remarkably conservated with vertebrate CPR sequences. This protein, which supports P450 activity, was shown to be strongly expressed in the antennae of fruit fly, and it may be related to odorant clearance via P450 metabolism (Hovemann et al., 1997). Here, for the first time, we clearly demonstrated that its expression is associated with insect olfactory sensilla, reinforcing the potential importance of cytochrome metabolism in insect antennae.

More information about the possible intervention of CYPs in insect olfaction comes from in situ hybridization. The three genes are expressed at the base of olfactory sensilla, in cells that could be either accessory cells embedding sensory neurons or the neurons themselves. On Fig. 4G, for instance, protrusions emanating from the soma and entering the base of the olfactory hair are labeled with the *CYP4G20* probe that could correspond to the dendrite of the olfactory neurons. Furthermore, with the CPR and *CYP9A13* probes, two labeled somata can be seen at the base of the same sensillum (Fig. 4C and D), possibly corresponding to the two receptor neurons usually observed in olfactory sensilla. Although some P450s were found in brain neurons of mammals (Hiroi et al., 2001), their occurrence in olfactory neurons of vertebrates has not been reported yet, and their expression seems restricted to nonneuronal cells of the olfactory epithelium and in Bowman's gland (Piras et al., 2003; Ding and Kaminsky, 2003).

CYP4G20- and CYP9A13-deduced protein sequences present the structural characteristics of microsomal P450s. This potential location in the membrane of endoplasmic reticulum implies that they could presumably act on odorants only after their internalization in the cell. Indeed, odorant internalization in the accessory cells has already been suggested, that could involve Odorant-Binding Proteins (OBP), which bind and thus solubilize hydrophobic odorant molecules in the sensillum lymph and are degraded by the accessory cells, or, alternatively, odorant molecules may directly enter the supporting cells (review in Vogt, 2005) where they could be degraded by intracellular enzymes. Internalization of odorants in the olfactory neurons may also occur during the endocytosis of the membrane-bound olfactory receptor, as it is known for other G-protein-coupled receptors (GPCR; Fergusson, 2001). After interaction with the receptor, odorant molecules could be internalized together with the olfactory receptor into the sensory neuron during the desensitization step and become accessible for intracellular-degrading enzymes. It has also been hypothesized that another kind of proteins, the Sensory Neuron Membrane Proteins (SNMP), may act as scavengers, allowing internalization of odorants, OBPs or odorant/OBP complexes into the sensory neurons, leading to metabolism of odorants, OBPs or the complexes by intracellular enzymes (Vogt, 2005).

Moreover, in addition to their "classic" localization in the endoplasmic reticulum and in the mitochondria, numerous studies have also provided evidences for the presence of microsomal P450s in the plasma membrane of mammalian cells, including rabbit pulmonary cells (Serabjit-Singh et al., 1988; Loeper et al., 1998). More recently, a microsomal CYP2D human isoform has been detected in synaptic terminals of neurons (Chinta et al., 2002), with a possible localization in the plasma membrane, where interactions between neurotransmitter receptors/transporters take place. With such potential localization in the plasma membrane of the dendrite, where olfactory receptors are located, it could be hypothesized that antennal P450s could act directly on the odorant molecules, as do other extracellular biotransformation enzymes present in the sensillum lymph. In several moth species, secreted enzymes, such as esterases or aldehyde-oxidases, are indeed localized within the extracellular sensilla lumen where they are able to rapidly inactivate pheromone compounds (Vogt, 2005). However, after transformation into biological inactive compounds by these extracellular enzymes, we can suppose that the odorant molecules still have to be cleared from the sensillum lymph and thus to be internalized into the cells for further enzymatic degradation by various enzymes, among which are P450s. Further studies using double-labeling in situ *hybridization* are now required to precisely identify in which kind of cells, neurons or accessory cells (or both) are CYP4G20 and CYP9A13 expressed.

Together with our previous results, this study clearly shows for the first time the diversity of cytochrome *P*450s in the olfactory organ of a single insect species that may be correlated with the diversity of odorants or xenobiotics entering the olfactory sensilla from external environment. Although 83 P450 genes have been identified in fruit fly, belonging to 25 different families, with more than half from the CYP4, CYP6 and CYP9 families, only one, CYP6W1, has been found in the antennae to date (Wang et al., 1999). Furthermore, we cannot exclude the presence of other P450 antennal members in *M. brassicae*, since only two families are here represented, the CYP4 and the CYP9. As a comparison, in human, around 60 P450 genes have been sequenced (http://www.drnelson.utmen.edu/cytochromeP450.html), and more than 10 isoforms are found in nasal mucosa, trachea and lungs (Ding and Kaminsky, 2003). Predominant expression of CYP forms in the olfactory mucosa of mammals would increase clearance of airborne compounds in the neuroepithelium but might also lead to a decreased transfer of potential

neurotoxicants to the brain via the olfactory pathways: the axons of olfactory neurons project directly into the olfactory bulb, establishing a connection that may be a portal of entry of xenobiotics into the brain (Piras et al., 2003). The finding of four different cytochrome *P*450s expressed in the olfactory sensilla of a single insect species, together with CPR expression, suggests that odorant and xenobiotic P450-mediated metabolism in insect's olfactory system is certainly as complex and diversified as in mammals.

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