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A new aldehyde oxidase selectively expressed in chemosensory organs of insects

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Abstract

Signal termination is a crucial step in the dynamic of the olfactory process. It involves different classes of odorant-degrading enzymes. Whereas aldehyde oxidase enzymatic activities have been demonstrated in insect antennae by previous biochemical studies, the corresponding enzymes have never been characterized at the molecular level. In the cabbage armyworm *Mamestra brassicae*, we isolated for the first time an aldehyde oxidase partial cDNA specifically expressed in chemosensory organs, with the strongest expression in antennae of both sexes. In these organs, expression was restricted to the olfactory sensilla. Our results suggest that the corresponding enzyme could degrade aldehyde odorant compounds, such as pheromones or plant's volatiles.

Keywords: Odorant-degrading enzyme; Aldehyde oxidase; Molybdo-flavoenzyme; Olfaction; *Mamestra brassicae*

Insect's olfaction takes place in the antennae, composed of morphofunctional units, the sensilla. These sensilla contain olfactory receptor neurons (ORNs), whose dendrites are bathed in an aqueous sensillar lymph. Olfaction results from spatial and temporal dynamic interactions between incoming odorant molecules and various components of the antennae. These interactions consist of sequential steps: the odorant binding, reception, termination, and the signal transduction that lead to the transformation of the chemical signal into an electrical message [1,2]. Signal termination is a critical step as it prevents the olfactory organs to a continuous chemical stimulation and minimizes signal saturation. Enzymatic degradation of odorant molecules has been proposed to participate in signal termination. It involves extracellular as well as intracellular odorant-degrading enzymes (ODEs).

Only a few insect ODEs of different classes have been identified at the molecular level. Some biotransformation enzymes, such as glutathione-*S*-transferases or cytochromes P450, were characterized in the antennae of some insect species [3–8]. They could be implicated in degradation of odorant molecules and/or in protection against harmful volatile xenobiotics, since the ORNs are among the few nervous cells directly exposed to external environment. Other types of ODEs specifically involved in odorant degradation have been identified by biochemical approaches, in particular esterases, alcohol dehydrogenases, and aldehyde oxidases [1,9–12]. These enzymes could degrade components of Lepidopteran pheromone blends composed of volatile esters, alcohols or aldehydes, whose presence and relative abundance ensure the specificity of communication and contribute to reproductive isolation between species. Antennal esterases have been cloned only recently in two moth species, the wild silk moth *Antheraea polyphemus* [13] and our model, the cabbage armyworm

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Mamestra brassicae [14], both of them using an acetate as major female sex pheromone component. To date, no alcohol dehydrogenases or aldehyde oxidases have been structurally characterized.

In our model *M. brassicae*, the female pheromone blend possesses not only a major acetate component 11-*cis*-hexadecenyl acetate (Z11-16: Ac), but also minor components, in particular 11-*cis*-hexadecenyl aldehyde (Z11-16: Ald) [15,16]. This aldehyde is detected by specialized ORNs housed in short olfactory sensilla of males [15] and is a synergist for their attraction behavior [17,18]. In addition, males emit different pheromone components during the sexual courtship, among them benzaldehyde [19], a common substrate for aldehyde oxidases.

We focused our work on enzymes able to degrade aldehyde pheromones in male and female *M. brassicae* antennae. We hypothesized that this enzyme should belong to the molybdo-flavoenzyme (MFE) family. Indeed, the single AOX characterized to date at the molecular level in insects is a MFE associated with insecticide resistance in the mosquito *Culex quinquefasciatus* [20]. MFEs are present throughout evolution, from bacteria to human, and include two classes of enzymes, xanthine dehydrogenases (XDHs), and aldehyde oxidases.

Using a PCR-based strategy with primers deduced from *C. quinquefasciatus* AOX sequence, a partial cDNA coding for a putative antennal aldehyde oxidase (*Mbra-AOX*) was isolated from *M. brassicae* male antennae. Its expression pattern was studied by RT-PCR, Northern-blot, and in situ hybridization. The Mbra-AOX deduced polypeptide shared the common structural features of known aldehyde oxidases and the corresponding transcript was selectively expressed in chemosensory organs, with a strong expression in male and female antennae, and a faint expression in taste organs such as proboscis and legs. In antennae of both sexes, expression was restricted to the olfactory sensilla trichodea, which strongly suggests a potential role in aldehyde odorant clearance for this new member of insect AOX family.

Materials and methods

Animals and tissue collection. M. brassicae males and females were reared in our laboratory on a semi-synthetic diet [21]. Pupae were maintained at 20 °C and 60% humidity until emergence. Tissues from sexually mature 3-day-old males (antennae, heads, proboscis, legs, thorax, and abdomens) and females (antennae) were dissected and used for RNA isolation. For in situ hybridization, antennae from both sexes were fixed in 4% paraformaldehyde.

RNA isolation and cDNA synthesis. Single-stranded cDNAs from various tissues were synthesized from total RNAs (1 μg) extracted with TRIzol reagent (Gibco-BRL) using the Advantage RT-for-PCR kit (Clontech). For 5′ RACE PCR, antennal cDNA was synthesized from male antennal total RNA (1 μg) using the SMART RACE cDNA Amplification kit (Clontech) with 200 U of Superscript II (Gibco-BRL), according to the manufacturer's instructions.

PCR amplification and partial cloning of the putative aldehyde oxidase. Two degenerate primers (up 5′-GARGGIGGITGYGGIGYITG YRT-3′ and down 5′-AAISCISCRTTIACRTAIGCRTG-3′), corresponding to the amino acid sequences EGGCG(A/V)C(I/V) and HAYVN(A/G)(A/G)F, respectively, were deduced from consensus motifs after alignment of various AOX sequences, including *C. quinquefasciatus* AOX (GenBank Accession No. AAF87601) and *Drosophila melanogaster* AOX1 (Accession No. AAF55207). They were used to generate a cDNA fragment of 1182 bp by PCR. After 35 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a 72 °C final elongation of 10 min, the fragment was gel purified (GenElute, Sigma) and cloned into pCR II-TOPO plasmid (Invitrogen). Recombinant plasmids were purified (QIAprep Spin Miniprep Kit, Qiagen) and sequenced (Genomexpress). The 5′ region of the cDNA was obtained using a specific primer (5′-CAACTGTGGTAACCTCCCA GCCATGGCAGG-3′) and touchdown PCR was performed as described in [5]. The product of 343 bp was cloned as described above. By merging the two overlapping sequences, a 1394 bp partial cDNA sequence called *Mbra-AOX* was generated and identified as a putative new aldehyde oxidase after database comparisons using BLAST [22].

In situ hybridization. The recombinant pCR II-TOPO plasmid containing the 343 bp AOX insert was purified and linearized for RNA sense and antisense probes' transcription using T7 and SP6 RNA polymerases (Promega). Hybridization was performed at 65 °C as described in [23].

Northern blot and RT-PCR studies. For Northern blot analysis, total RNAs (20 μg) from male antennae were separated on a 1% formaldehyde gel and transferred onto a positively charged nylon membrane. Two specific digoxigenin labeled probes were produced by PCR on antennal cDNAs using the Dig-DNA labeling mixture (Roche) and two specific primers: (1) a 450 bp fragment of the *Mbra-AOX* cDNA (5′-GCGAAAATTGTTTTCAAAATTAATGG-3′ and 5′-GATTACTTGCAAAAGAATTTTCTACTTCTCTC-3′) and (2) a 508 bp fragment of the *rpL8* cDNA encoding the rpL8 ribosomal protein of *M. brassicae* (GenBank Accession No. AY485337) that was used as control probe. They were sequentially hybridized to the membrane at 40 °C as described in [5]. For RT-PCR, equal amounts of RNAs (1 μg) extracted from various tissues were treated with DNase, reverse-transcribed, and simultaneously amplified with *Mbra-AOX* and *rpL8* primers (to obtain fragments of 450 and 508 bp, respectively). Products were visualized on 2% agarose gel.

Phylogenetic analysis. AOX-related amino acid sequences were aligned using Multalin program [24]. Maximum parsimony was used to build a strict consensus tree in Paup 4.0b10 software (Sinauer associates, Sunderland, Massachussets, USA). Branch support was assessed by bootstrap analysis based on 1000 replications and the bacterial XDH from *Rhodobacter capsulatus* (Rho-XDH, GenBank Accession No. CAA04470) was used as outgroup.

Results and discussion

This work contributes to the understanding of the dynamic of the insect olfactory mechanism by the identification and characterization of *M. brassicae* aldehyde oxidase (*Mbra-AOX*), a novel insect member of the AOX family specifically expressed in the chemosensory organs.

Characterization of Mbra-AOX

The PCR-based strategy allowed us to obtain a partial nucleotide sequence of 1394 bp called *Mbra-AOX*. This sequence has been deposited in GenBank under

Accession No. AY947538. *Mbra-AOX* contained a partial ORF of 1311 bp encoding a 437 amino acid deduced polypeptide, Mbra-AOX, found to be the most similar to proteins belonging to the MFE family. Mbra-AOX was aligned in Fig. 1 with *C. quinquefasciatus* AOX (AAF87601), *D. melanogaster* AOX1 (AAF55207), mouse AOX3 (NP001008419), and with various xanthine dehydrogenases, including the Mediterranean fruit fly *Ceratitis capitata* XDH (AAG47345), *D. melanogaster* XDH (Y00308), and mouse XDH (NP035853). MFE proteins are characterized by a similar general structure: these enzymes are homodimers consisting of identical subunits of approx. 150 kDa [25]. Each subunit has a typical structure with a N-terminal domain containing two iron–sulfur (2Fe–2S) redox centers followed by a flavin-containing region (FAD-binding domain) and a C-terminal domain comprising the molybdenum cofactor (MoCo)-binding site within the substrate binding pocket domain. As shown in Fig. 1, the N-terminal region of Mbra-AOX contained the conserved domains corresponding to the two (2Fe–2S) centers. In particular, Mbra-AOX presented the eight cysteine residues necessary for the coordination of the iron ions [25]. These structural domains were followed by a relatively

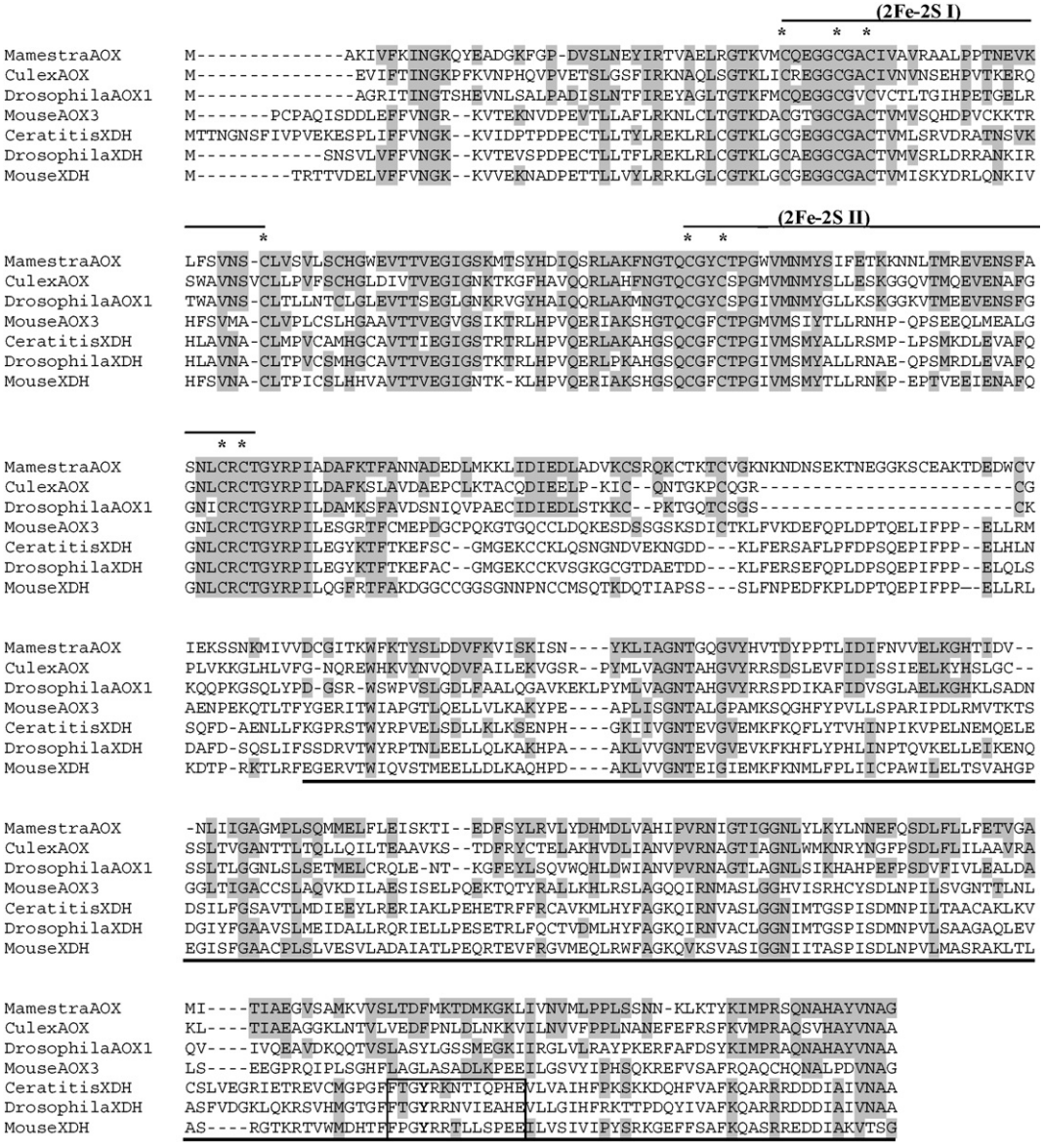

Fig. 1. Amino acid sequence comparison of *M. brassicae* AOX polypeptide fragment with *C. quinquefasciatus* AOX, *D. melanogaster* AOX1, mouse AOX3, and xanthine dehydrogenases (XDHs), including *C. capitata*, *D. melanogaster*, and mouse XDHs. The residues highlighted in gray represent identical amino acids relative to *Mamestra* AOX. The domains corresponding to the two (2Fe–2S) redox centers are overlined. In these domains, the asterisks above the sequences mark the positions of the cysteine residues reported to be involved in the formation of the iron–sulfur centers. The underlined domain indicates the FAD-binding domain. The boxed sequences with the tyrosine in bold correspond to the putative NAD^+-binding site characteristic of XDHs.

non-conserved hinge region that connected them to the segment containing the FAD-binding site. This hinge was slightly longer in Mbra-AOX than in the other known insect AOX sequences. More importantly, the FAD-binding domain of Mbra-AOX was lacking the short conserved sequence corresponding to the NAD^+-binding site typical of XDHs [25,26]. In particular, the tyrosine residue necessary for the binding of NAD^+ in XDHs was replaced by different amino acids in AOXs, such as a phenylalanine in Mbra-AOX and in *Culex* AOX. This lack of NAD^+-binding site was consistent with the fact that Mbra-AOX, as other AOXs and contrary to XDHs, was a pure oxidase that did not bind NAD^+. Taken together, these structural characteristics of Mbra-AOX deduced from its primary sequence indicated that the corresponding enzyme was a member of the MFE family and more precisely of the AOX subfamily. This assignment to the AOX subfamily was supported by the phylogenetic analysis. Mbra-AOX was compared to various AOXs and XDHs from mammals and insects. As shown in Fig. 2, Mbra-AOX clustered closer to AOXs than to XDHs from other insect species. As previously observed, mammalian AOXs are structurally more similar to mammalian XDHs than to insect AOXs, supporting the view that AOXs have evolved two independent times from XDHs during the process of duplication of MFE genes [25,27]. Interestingly, within the insect grouping, Mbra-AOX clustered with an expressed sequence tag (EST) from *Bombyx mori* (Accession No. BP184157), and as revealed by high branch support values, this lepidopteran group was separated from the dipteran's one. This short *B. mori* EST obtained from a pupae pheromone gland cDNA library could correspond to a putative AOX with still unknown function.

We were not able to amplify the entire sequence of the antennal AOX from *M. brassicae* by 3′RACE-PCR and we still lack the C-terminal domain. However, to determine the size of the corresponding transcript, we performed Northern-blot on male antennal RNA using a dig-labeled probe (Fig. 3). A single transcript was detected in the antennae, with a size of approx. 5500 bp that is in agreement with the already known AOX sizes. The cDNA encoding the *C. quinquefasciatus* AOX consists of 3798 bp with very short 5′ and 3′ untranslated regions (UTR) [28], whereas the human AOX1 is 5125 bp [29] and the mouse AOX3 with an incomplete 3′UTR is 4776 bp long [30]. The corresponding proteins have

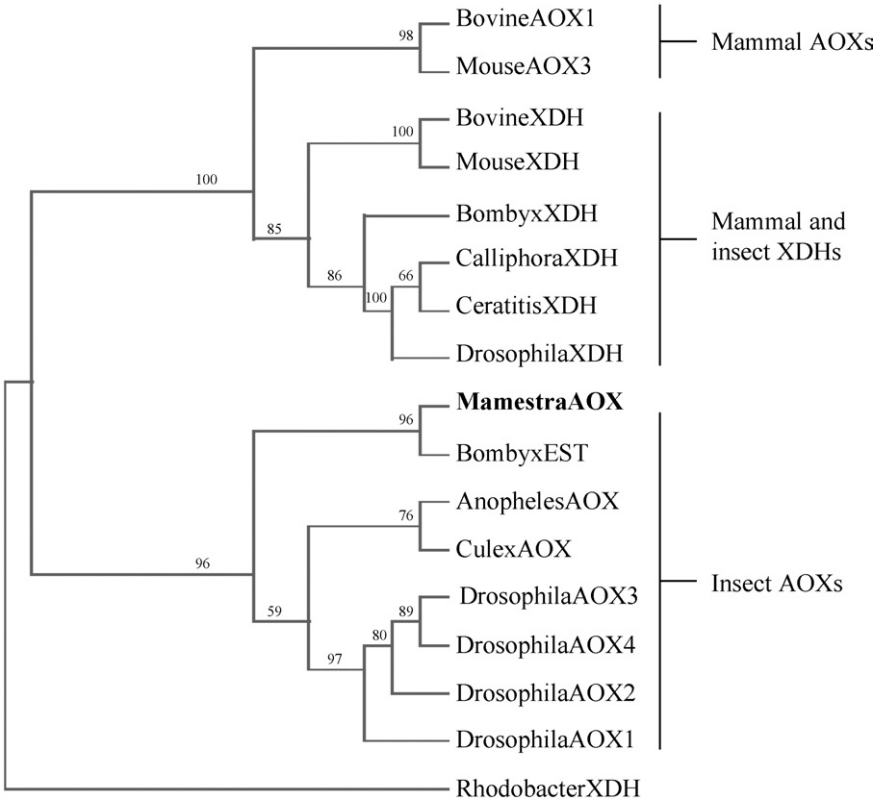

Fig. 2. Phylogenetic analysis of Mbra-AOX and various MFEs. GenBank accession numbers are as follows: *Anopheles gambiae* AOX, XP316290; *B. mori* EST, BP184157; *C. quinquefasciatus* AOX, AAF87601; *D. melanogaster* AOX1, AAF55207, AOX2, AAF55208, AOX3, AAF55209.1, AOX4, AAF55210.1; *Calliphora vicina* XDH, X07323; *C. capitata* XDH, AAG47354; *D. melanogaster* XDH, Y00308; *B. mori* XDH, D38159; mouse AOX3, NP001008419; mouse XDH, NP035853; bovine AOX, X87251.1; and bovine XDH, X83508. Bootstrap support values (in percent) based on 1000 replicates are indicated.

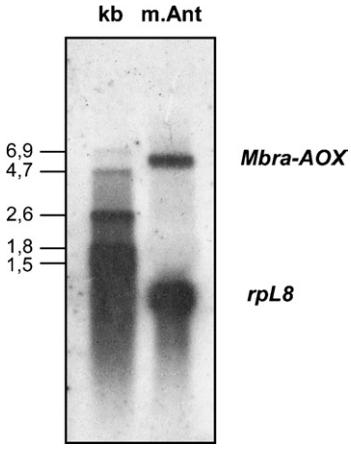

Fig. 3. Determination of the *Mbra-AOX* transcript size by Northern-blot in adult male antennae (m.Ant) using *Mbra-AOX* and *rpL8* Dig-labeled probes.

predicted molecular masses of around 150 kDa that is also in agreement with the data obtained by biochemical studies on AOXs of the moths *Manduca sexta* [10], *A. polyphemus*, and *B. mori* [11]. Indeed, after denaturing-PAGE, the molecular weights of the antennal aldehyde degrading enzymes in these three species were estimated at 150, 175, and 130 kDa for each monomer, respectively.

Selective expression of Mbra-AOX in the chemosensory organs

The tissue-related expression of *Mbra-AOX* was determined by RT-PCR (Fig. 4). In addition to the antennae, which carry olfactory sensilla, other chemosensory tissues were used, such as proboscis and legs where taste sensilla are located. For similar levels of *rpL8* expression, the strongest signals were observed in the antennae of both sexes, whereas a faint expression was found in proboscis and legs, and no expression was detected in other tissues. These results are consistent with previous biochemical data. Indeed, *M. sexta*, *A. polyphemus*, and *B. mori* AOX activities have been shown to be restricted to the antennae [10,11]. However, in the moths *Heliothis virescens* and *Helicoverpa zea*, which are more closely related to *M. brassicae*, enzymatic activities were also found faintly in legs [31,32]. In these two species, proboscis was unfortunately never tested.

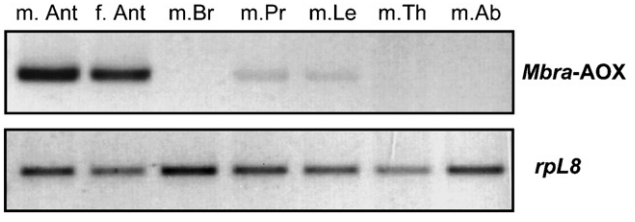

Fig. 4. RT-PCR analysis of *Mbra-AOX* and *rpL8* expression: cDNAs amplified with gene specific primers from antennae (Ant), brains (Br), proboscis (Pr), legs (Le), thorax (Th), and abdomens (Ab). m, male; f, female.

Selective expression of Mbra-AOX in the olfactory sensilla of the antennae

A precise localization of *Mbra-AOX* within male and female antennae was achieved using in situ hybridization. In *M. brassicae*, the antennae are filiform and their dorsal side is covered with scales whereas the olfactory hairs are located on the ventral side. Two types of olfactory hairs are present in males: the long sensilla trichodea are located on the lateral part of the ventral area and the short sensilla are medio-ventral. In females, the long hairs are absent and the short sensilla trichodea are located in the lateral and medio-ventral parts of the ventral area [15]. Sense strand control gave no signals (not shown), whereas antisense *Mbra-AOX* probe hybridizations were clearly restricted to the sensilla side of the antenna, with no signal on the scale side (Figs. 5A and D). The labeling was clearly observed in male long sensilla trichodea as well as in male and female short sensilla (Figs. 5B and D). Moreover, the sensilla chaetica, which are mechanoreceptors, were never labeled. In the antennae, *Mbra-AOX* transcripts were thus restricted to olfactory sensilla of both sexes. This is consistent with AOX activities found in the antennae of both sexes in various lepidopteran species and with their known catalytic activities [10,11,31,32]. In *M. sexta*, male antennal AOX exhibits a wide substrate specificity which includes the pheromone component E10, E12-hexadecadienal, and various aldehydes such as propanal, benzaldehyde, and vanillin [10]. As female moths are generally supposed to be anosmic to their own pheromone, a dual function in the degradation of pheromones (detected by males) and plant's volatiles (detected by males and females) has been proposed for these enzymes [10,11]. *M. brassicae* males detect the minor sex pheromone component, Z11-16: Ald, by specific ORNs located in the short olfactory sensilla, in which *Mbra-AOX* is expressed. However, the *Mbra-AOX* labeling was also abundant in the long olfactory sensilla, specialized in the detection of the major pheromone component, Z11-16: Ac, and sensitive to some heterospecific compounds, such as 9-*cis*-tetradecenyl acetate (Z9-14: Ac) and 11-*cis*-hexadecenyl alcohol (Z11-16: OH). These sensilla are not known to detect any aldehyde compounds, but volatile aldehydes (including Z11-16: Ald) entering the sensillar lumen could be degraded by Mbra-AOX in order to avoid background noise. *M. brassicae* females detect benzaldehyde, a courtship pheromone emitted by males [19], and are able to detect plant's volatiles [33]. Mbra-AOX could thus be involved in the degradation of pheromone com-

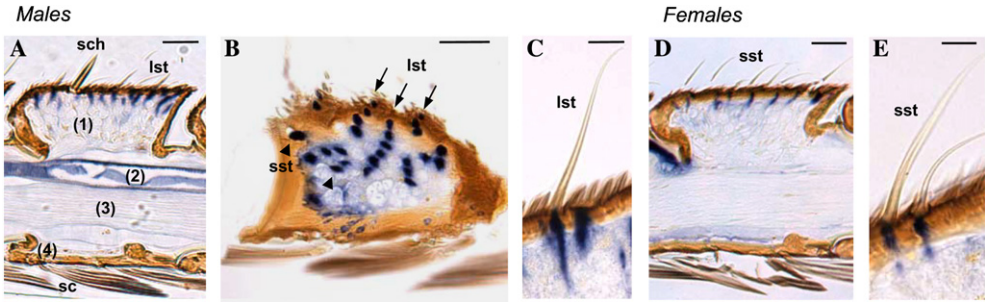

Fig. 5. Expression patterns of *Mbra-AOX* after in situ hybridization on longitudinal sections of male (A–C) and female antennae (D,E). (A) Male antennal segment, showing the ventral disposition of the long sensilla trichodea (lst) and sensory neurons (1), the dorsal localization of the scales (sc), the trachea (2), the antennal nerve (3), and the epidermis (4). Sensilla chaetica (sch) are not labeled. (B) Section through the cuticle. Arrows show the labeling of the lst arranged in parallel rows. Arrowheads show the short sensilla trichodea (sst) randomly distributed in the medio-ventral area. (D) Female antennal segment showing the labeling of the sst. (C,E) Magnification of male and female sensilla trichodea, respectively, with intense labeling at the base of the sensory hairs. Bars: 50 μm in (A, B, and D), 5 μm in (C,E).

ponents in both sexes, as well as in the degradation of aldehydes from host plants.

Closer examination of in situ hybridization experiments revealed labeling in cells localized at the base of the sensory hairs that could correspond to the olfactory neurons or to the accessory cells surrounding the bipolar neurons of the sensilla trichodea (Figs. 5C and E). In *M. sexta*, the antennal AOX activity is found in the sensillar lymph in the absence of any cofactor, and the corresponding enzyme is considered to be extracellular [10]. Expression in the sensillar lymph should allow the rapid degradation of the pheromone entering the sensilla. In this species, the half-life of the aldehyde pheromone is indeed estimated at 0.6 ms. However, no cleavage site was found in the N-terminal part of Mbra-AOX nor in all other mammalian and insect MFEs. These enzymes are thus considered to be primarily cytosolic. No information are available to date on the possible secretion of AOX outside the cell. Although AOXs and XDHs lack a typical secretory sequence, active protein secretion could result from an atypical secretory pathway [25]. This is illustrated with the mouse XDH, which is secreted in association with other major milk proteins in the mammary gland [34], but this phenomenon remains to be elucidated.

Coming from the assumption that insect genes strongly expressed in antennae have an olfactory role, numerous components of olfactory mechanisms have been identified. Among them, no antennal aldehyde oxidase has been characterized until now at the molecular level. The strong expression of *Mbra-AOX* observed in the antennae suggested a role of the corresponding enzyme in the oxidation of volatile aldehyde odorants. Recently, another novel member of the AOX family, AOX3, was identified in mammals [30]. Since its expression was restricted to the olfactory mucosa, this enzyme has been proposed to be involved in the degradation of aliphatic aldehydes, which are strong odorants [30], and thus to serve in controlling the duration and/or strength of olfactory stimuli that activate the ORs. Functional analogies of olfactory reception have already been described between vertebrates and invertebrates [35]. Our results, taken together with the characterization of mouse AOX3, suggest that similar pathway of aldehyde degradation could occur in vertebrates as well as in invertebrates, underlying evolutionary conservation of molecular mechanisms of signal termination.

Acknowledgments

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