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# Molecular Characterization of a Phospholipase C $\beta$ Potentially Involved in Moth Olfactory Transduction

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## Abstract

To clarify the role of phospholipase C (PLC) in insect olfactory transduction, we have undertaken its molecular identification in the moth *Spodoptera littoralis*. From the analysis of a male antennal expressed sequence tag library, we succeeded in cloning a full-length cDNA encoding a PLC that belongs to the cluster of *PLC- $\beta$*  subtypes. In adult males, the *PLC- $\beta$*  transcript was located predominantly in brain and antennae where its presence was detected in the olfactory *sensilla trichodea*. Moreover, *PLC- $\beta$*  was expressed in antennae at the beginning of the pupal stage, then reached a maximum at the end of this stage and was maintained at this level during the adult period. Taken together, these results provided molecular evidence for the putative participation of a *PLC- $\beta$*  in signaling pathways responsible for the establishment and the functioning of insect olfactory system.

**Key words:** expression patterns, olfactory system, Phospholipase C, *Spodoptera littoralis*, signal transduction

## Introduction

In most organisms, olfaction allows to recognize and to discriminate environmental chemical signals that profoundly influence the expression of vital behaviors such as recognition of a mating partner and identification of food sources (Shiple and Ennis 1996; Benton et al. 2006). The quality, intensity, and duration of chemical stimuli are encoded by olfactory receptor neurons (ORNs) into trains of action potentials which propagate along axons toward brain olfactory centers where olfactory information is processed, leading to physiological and/or behavioral responses (Hansson and Anton 2000). In moths, ORNs innervate sensory organs, the sensilla, distributed on the antennae (Kaissling 2004).

From genetic, molecular, and electrophysiological experiments carried out on a variety of model organisms, a common set of chemoelectrical transduction mechanisms begins to emerge (Ache 1994; Hildebrand and Shepherd 1997; Prasad and Reed 1999). The binding of odor molecules to olfactory receptors (ORs) that belong to the G protein-coupled receptors (GPCRs) family, triggers the induction

of G protein-mediated signaling cascades (Buck and Axel 1991; Clyne et al. 1999; Gao and Chess 1999; Vosshall et al. 1999; Fox et al. 2002; Sakurai et al. 2004). The activation of G proteins in turn stimulates key enzymes of the second messenger cascades: adenylate cyclase catalyses the formation of cyclic 3',5'-adenosine monophosphate (cAMP) from adenosine 5'-triphosphate and/or phospholipase C (PLC), the enzyme that hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) into inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). The rapid increase in the concentration of second messengers activates ion channels in the plasma membrane, thereby generating a receptor potential.

The participation of G proteins, second messengers, and second messenger-gated channels in the olfactory transduction process is firmly established in vertebrates as well as in nematodes and lobsters, where the cAMP and the IP<sub>3</sub> cascades appear to operate as 2 alternative pathways (Breer et al. 1994; Roayaie et al. 1998; Schild and Restrepo 1998; Zhainazarov et al. 2004). In insects, recent works revealed

a new mode of olfactory transduction by the functional characterization of a *Drosophila* OR heterodimeric complex that possesses ligand-gated channel properties in vertebrate expression systems (Sato et al. 2008; Wicher et al. 2008). It seems reasonable to assume that insect chemical transduction may be distinct from that of other olfactory model systems, at least for allelochemical olfaction. Nevertheless, numerous biochemical and electrophysiological data strongly support a role of G protein-mediated cascades in insect olfactory signaling. First, addition of odorous compounds to cells expressing ORs resulted in G protein-dependent currents in addition to direct ligand-gated currents (Wicher et al. 2008). Second, stimulation of antennal preparations with pheromone elicited an increase in the IP<sub>3</sub> intracellular level for a variety of insect species (Breer et al. 1990; Boekhoff et al. 1993; Kaissling and Boekhoff 1993; Kaissling 1994). Third, application of a DAG-analogue activated ORN firing activity in *Bombyx mori* and *Antheraea polyphemus* pheromone-sensitive sensilla and generated a nonspecific cationic current in *Spodoptera littoralis* ORNs in vitro (Maida et al. 2000; Pophof and van der Goes van Naters 2002; Lucas and Pézier 2006). These data underline the importance of PLC activity in insect olfactory processes.

PLC is a well-conserved family of proteins found in animals at all stages in evolution. PLC enzymes have been grouped into 6 major families ( $\beta$ ,  $\epsilon$ ,  $\eta$ ,  $\zeta$ ,  $\gamma$ , and  $\delta$ ) that differ in structural organization, regulation, activation, and tissue distribution. Signaling pathways that utilize PLC are activated by a diverse spectrum of stimuli and modulate a wide variety of cellular processes such as phototransduction, secretion, contraction, cell growth, and differentiation (Nebigil 1997; Rebecchi and Pentylala 2000; Oude Weernink et al. 2007). A role for PLC in insect olfactory transduction was supported by reduced odor responses of maxillary palps and antennal neurons in *Drosophila melanogaster* PLC- $\beta$  mutants (Riesgo-Escovar et al. 1995; Kain et al. 2008). In addition, a PLC- $\beta$  was immunolocalized in homogenates of pheromone-sensitive sensilla of the moth *A. polyphemus* (Maida et al. 2000). Nevertheless, there are no molecular data available on this protein that is considered to function in the insect olfactory transduction pathway. In this context, we report here, the cloning, phylogenetic analysis, and spatiotemporal expression patterns of a PLC- $\beta$  that might play a role in odor detection in the moth *S. littoralis*.

## Materials and methods

### Animals and tissue collection

*Spodoptera littoralis* were reared in the laboratory at 24 °C, 60–70% relative humidity and exposed to a 16:8 light:dark cycle. Tissues from adults (antennae, brains, wings, legs, thoracic muscles, eyes, and abdomens) were dissected and used directly for total RNA isolation. For the developmental study, the whole cuticular chambers containing antennae

were collected from early stages (before day 8). In older pupae, a small window was cut into the cuticle at the base of the antennae, which was collected and used for RNA isolation. For in situ hybridization, male antennae were removed from adult head, cut into pieces and fixed overnight in 4% paraformaldehyde (PFA) at 4 °C, then dehydrated in methanol, and stored at –20 °C until use.

### RNA isolation and cDNA synthesis

Total RNAs were extracted with TRIzol reagent (Gibco BRL), according to the manufacturer's instructions and were quantified by spectrophotometry at 260 nm. Single-stranded cDNAs for polymerase chain reaction (PCR) were synthesized from total RNAs (1  $\mu$ g) from various tissues with 200 U of M-MLV reverse transcriptase, using the Advantage RT-for-PCR Kit (Clontech). The reaction contained a deoxy-Nucleotid TriPhosphate mix, Rnasin, Oligo(dT) primer, and sterile water to a final volume of 25  $\mu$ L. The mix was heated at 70 °C for 2 min before adding the enzyme and then incubated for 1 h at 42 °C. For 5'- and 3'-rapid amplification of cDNA ends (RACE) PCR, antennal cDNAs were synthesized from 1  $\mu$ g of male antennal total RNA at 42 °C for 1.5 h using the SMART RACE cDNA Amplification Kit (Clontech) with 200 U of superscript II (Gibco BRL), 5'- or 3'-CDS-primer and SMART II oligonucleotide.

### Cloning of *S. littoralis* PLC $\beta$ isoform

A partial cDNA encoding a putative PLC  $\beta$  isoform was identified from an *S. littoralis* male antennal expressed sequence tag (EST) library (Jacquin-Joly E, unpublished data) by basic alignment search tool (BLAST) analysis compared with public databases (Genbank, trEMBL). The 5' and 3' regions of the corresponding cDNA were obtained by 5'- and 3'-RACE (SMART RACE cDNA Amplification Kit) following the manufacturer's instructions. For 5'-RACE, we used 2  $\mu$ L of 5'-RACE-ready cDNA with a specific reverse primer 5'-RACE PLCX (5'-CCG ACG ACT CCG ACG ACC CTT GCC G-3') and universal primer mix (UPM, Clontech) as the forward anchor primer. The 3'-RACE amplification was carried out with UPM as the reverse primer and a specific forward primer 3'-RACE PLCY (5'-CGC GTC TAC CCC GCA GGG-3'). Touchdown PCR was performed using hot start as follows: after 1 min at 94 °C, 5 cycles of 30 s at 94 °C and 7 min at 72 °C, then 5 cycles of 30 s at 94 °C, 1 min at 66 °C, and 5 min at 72 °C, then 28 cycles of 30 s at 94 °C, 1 min at 66 °C, and 5 min at 72 °C, then 7 min at 72 °C. The PCR products were purified by agarose gel electrophoresis (Gene Clean II kit; Bio101, Inc.) and cloned into pCRII-Topo plasmid (Invitrogen). After colony isolation, DNA minipreps were prepared (QIAprep Spin Miniprep kit; Qiagen), and correct insertion was determined by restriction enzyme analysis. The DNA clone containing the proper insert was sequenced by the dideoxy chain termination method

(Genome Express). By merging the overlapping sequences obtained from the 5'- and 3'-RACE, a putative full-length cDNA of 6546 bp was generated and named *S. littoralis* *PLC-β* (*SLPLC-β*).

### Northern blot

Northern blot hybridization analysis was performed according to the manufacturer's instructions. RNA samples (20 μg) were denatured with formamide (50%) and formaldehyde (1.14 M) separated on a 1% denaturing agarose gel and transferred to a positively charged nylon membrane (Roche). *SLPLC-β* cDNA was digoxigenin (DIG)-labeled by PCR using the PCR DIG probe synthesis Kit (Roche) with a pair of specific primers designed from the carboxyl-terminal extension (CTE) of *SLPLC-β*: SIF 5'-forward primer (5'-CAG CAA GCC AAG ATT GCC AAC-3'), SIR 5'-reverse primer (5'-CCA CTG GTA CTC TCT GCG AAC GGG-3'). A DIG-labeled fragment of the cDNA encoding the *RpL8* ribosomal protein of *S. littoralis* was generated with the primers, RpF 5'-forward primer (5'-TTC CAT GGA AAT TGG CCC-3'), RpR 5'-reverse primer (5'-CCT TAA TTC GGG CTG GCT A-3') and used as control probe. The probes were used at the concentration of 25 ng/mL in hybridization solution. Blotted RNA was hybridized overnight at 58 °C with *SLPLC-β* and *RpL8* probes. An immunological signal detection by chemoluminescence was performed, using the alkaline phosphatase bound anti-body/CSPD system, as described in Roche's DIG system User's Guide for filter hybridization. A molecular RNA marker ladder DIG-labeled (Roche) was run in parallel to determine the molecular mass of hybridizing RNAs.

### PCR and qPCR

PCR were performed on 200 ng cDNA preparations from various tissues with 1.25 units of high expand fidelity DNA polymerase (Roche). *SLPLC-β* and *RpL8* cDNAs were amplified using the couple of primers SIF, SIR and RpF, RpR at 0.4 μM with each dNTP at 0.8 mM. Following an initial 5 min denaturation at 94 °C, the thermal amplification procedure included 30 cycles of denaturation for 30 s at 94 °C, annealing at 65 °C for 30 s, elongation at 72 °C for 30 s and then final elongation at 72 °C for 10 min. *SLPLC-β* (763 bp) and *RpL8* (570 bp) amplification products were loaded on 1.5% agarose gels and visualized with ethidium bromide.

For quantitative PCR (qPCR) experiments, total RNAs were extracted from nymphal and adult antennae of males with TRIzol reagent (Gibco BRL) then reverse-transcribed as previously described. The reactions were performed on the iCycler iQ multicolor real-time PCR detection system using the SYBR green detection system according to manufacturer's instructions (Bio-Rad). Three independent RNA preparations were made for each sample. PCR conditions were 35 cycles of (95 °C 30 s, 65 °C 30 s, 72 °C 30 s) with

the primers as follows: SIF 5'-forward primer (5'-CAG CAA GCC AAG ATT GCC AAC-3'), SIR 5'-reverse primer (5'-CCA CTG GTA CTC TCT GCG AAC GGG-3') for *SLPLC-β*, and RpFq 5'-forward primer (5'-ATG CCT GTG GGT GCT AT GC-3'), RpRq 5'-reverse primer (5'-TGC CTC TGT TGC TTG ATG GTA-3') for *RpL8* reference gene. qPCR reactions were carried out in triplicate. The standard curve was analyzed for all primers and gave amplification efficiencies of 90–100%. Data were analyzed with the iCycler software (Bio-Rad) and results with the geNORM Visual Basic application for Microsoft Excel as described by Vandesompele et al. (2002). Using these algorithms, the expression level of *SLPLC-β* was normalized to that of the reference gene and then calculated using the Q-Gen software (Simon 2003).

### In situ hybridization

The *SLPLC-β* DIG-labeled RNA sense and antisense probes were in vitro transcribed from PCR fragment amplified from the recombinant plasmid *SLPLC-β*/pCRII-Topo with M13 forward and M13 reverse primers. The transcription was performed using T7 and SP6 RNA polymerases following recommended protocol (Riboprobe kit; Promega) to generate *SLPLC-β* sense and antisense probes. The 795 bp probes were purified with RNA G50 sephadex columns (Quick Spin columns; Roche). The hybridization protocol was performed on whole-mount pieces of antennae, as described (Jacquin-Joly et al. 2000), with a hybridization temperature of 65 °C. Pieces of antennae were then embedded on OCT freezing medium (Tissue-tek®) and cryosectioned at –20 °C. Longitudinal and transversal sections at 6 μm thickness were performed and photographed. Pictures were digitized and processed using Adobe Photoshop 7.0 (Adobe).

### Phylogenetic analysis

PLC-related amino acid sequences were retrieved from GenBank and aligned using the Multalin program (Corpet 1988). Maximum parsimony was used to build a strict consensus tree in MEGA version 4 (Tamura et al. 2007). Branch support was assessed by bootstrap analysis based on 1000 replications and the *Arabidopsis thaliana* PLC sequence was used as an out-group.

## Results

### Isolation and characterization of *SLPLC-β* isoform

#### Cloning of an *SLPLC-β* cDNA fragment

In order to characterize the moth *PLC-β* isoform at the molecular level, we conducted a search for the mRNA sequence coding for such a protein. The screening of an *S. littoralis* male antennal EST library with sequences from a variety

of vertebrates and invertebrates PLC isoforms allowed us to identify an *S. littoralis* homologous cDNA sequence. A BLAST search revealed that this partial sequence was most similar to *D. melanogaster PLC21C*.

Utilizing the partial sequence to generate oligonucleotide primers, 5'- and 3'-RACE reactions were performed on single-strand cDNA templates obtained via reverse transcriptase PCR reaction of total RNA isolated from *S. littoralis* male antennae. The nucleic acid sequences for the 5'- and 3'-RACE reaction products were then assembled with the original fragment to generate the full-length cDNA of *SLPLC* which has been deposited in the GenBank database under accession number EU649777. Nucleotide sequence analysis revealed that the *SLPLC* cDNA of 6546 bp contains a putative coding region (open reading frame [ORF]) of 3489 bp, a 234 bp 5'-untranslated region (5'-UTR), and a 2823 bp 3'-UTR, with a putative polyadenylation signal upstream of the poly(A) (Figure 1). The full-length sequence was translated into a predicted amino acid sequence with BioEdit (Ibis Biosciences). The ORF, which starts from ATG, encodes 1163 amino acids (Figure 1) with a theoretical molecular mass of 133 kDa and an isoelectric point of 6.76 as determined using MWCALC (Infobiogen).

#### Phylogenetic analysis and comparison of the primary structure of *SLPLC-β*

There are 6 major known families of PLCs referred to as PLC-β, ε, η, ζ, γ, and δ. In order to determine to which family the *SLPLC* is most similar, a phylogenetic tree of the PLC family was inferred by comparing the amino acid sequence of *SLPLC* with those of known PLCs from 7 different species. Figure 2 shows that *SLPLC* belongs to the cluster of PLC-β subtype and consequently was referred to as *SLPLC-β*. Indeed, the polypeptide *SLPLC-β* contains the characteristic domains that are found in other known PLC-β sequences (Figure 1). The bipartite catalytic domain (X and Y) is flanked by a pleckstrin homology (PH) domain and elongation factor (EF)-hand motifs upstream of the X domain at the amino-terminal end and by a C2 domain followed by a long CTE downstream of the Y domain (Figure 1). In addition, the amino acid sequence of *SLPLC-β* showed 66%, 32%, 36%, 37%, 35%, 33%, 33%, 27%, 31%, and 33% identity with *D. melanogaster PLC21C*, *norpA*, *Xenopus laevis PLC1*, *Homo sapiens PLC-β1*, *PLC-β3*, *PLC-β2*, *PLC-β4*, *Caenorhabditis elegans PLC-β* (*egl-8*), *Homarus americanus PLC-β*, and *Aedes aegypti PLC-β*, respectively (Figure 2). From the obtained values, *SLPLC-β* was clearly assigned as a homologue of *D. melanogaster PLC21C*.

#### Expression studies of *SLPLC-β*

##### Tissue distribution

The tissue-related expression of *SLPLC-β* was then determined by RT-PCR of total RNA samples from various adult

male tissues using a pair of DNA primers (SIF, SIR) designed from the CTE of *SLPLC-β*. RT-PCR analysis revealed the presence of only one PCR product of expected size (763 bp) whose amount was higher in the antennae and brain than in the thoracic muscles, eyes, legs, and abdomen (Figure 3A). No amplification was recorded in the wings (Figure 3A). In addition, using an *SLPLC-β* specific probe, we succeeded in detecting by northern hybridization the *SLPLC-β* transcript in antennae where its level was higher in male than in female (Figure 3B). The estimated size of antennal *SLPLC-β* transcript (6.5 Kb) was in agreement with the corresponding cDNA length.

##### Expression of *SLPLC-β* within the antennae

In *S. littoralis*, the antennae are filiform and consist of segments which exhibit the same general organization: their dorsal side is covered with scales, whereas olfactory hairs (the sensilla) are situated on the ventral side as can be seen using scanning electron microscopy (Figure 4A). The cellular localization of *SLPLC-β* in male antennae was examined from in situ hybridization experiments. Sense strand controls gave no signals (Figure 4G), whereas antisense *SLPLC-β* probe hybridizations were clearly restricted to the sensilla (ventral) side of the antennae (Figure 4B,C). Close examination revealed that signals were located at the base of *sensilla trichodea* and inside the sensilla hair itself (Figure 4D–F). In males, the *sensilla trichodea* are distributed in 2 classes according to their length. The long *sensilla trichodea* are located on the lateral parts of the ventral area, and the short *sensilla trichodea* are medioventrally situated. On longitudinal sections, it is difficult to discriminate between long and short *sensilla trichodea*, as only parts of the sensilla are visible. However, in transversal sections as well as in ventral sections performed through the cuticle, a high number of spots were distributed all over the lateral and medial surface, probably corresponding to the labeling of long and short *sensilla trichodea* (Figure 4B–D). On the other hand, *sensilla coeloconica* and *sensilla chaetica* were also visualized on antennal sections but without any associated labeling (Figure 4D,F).

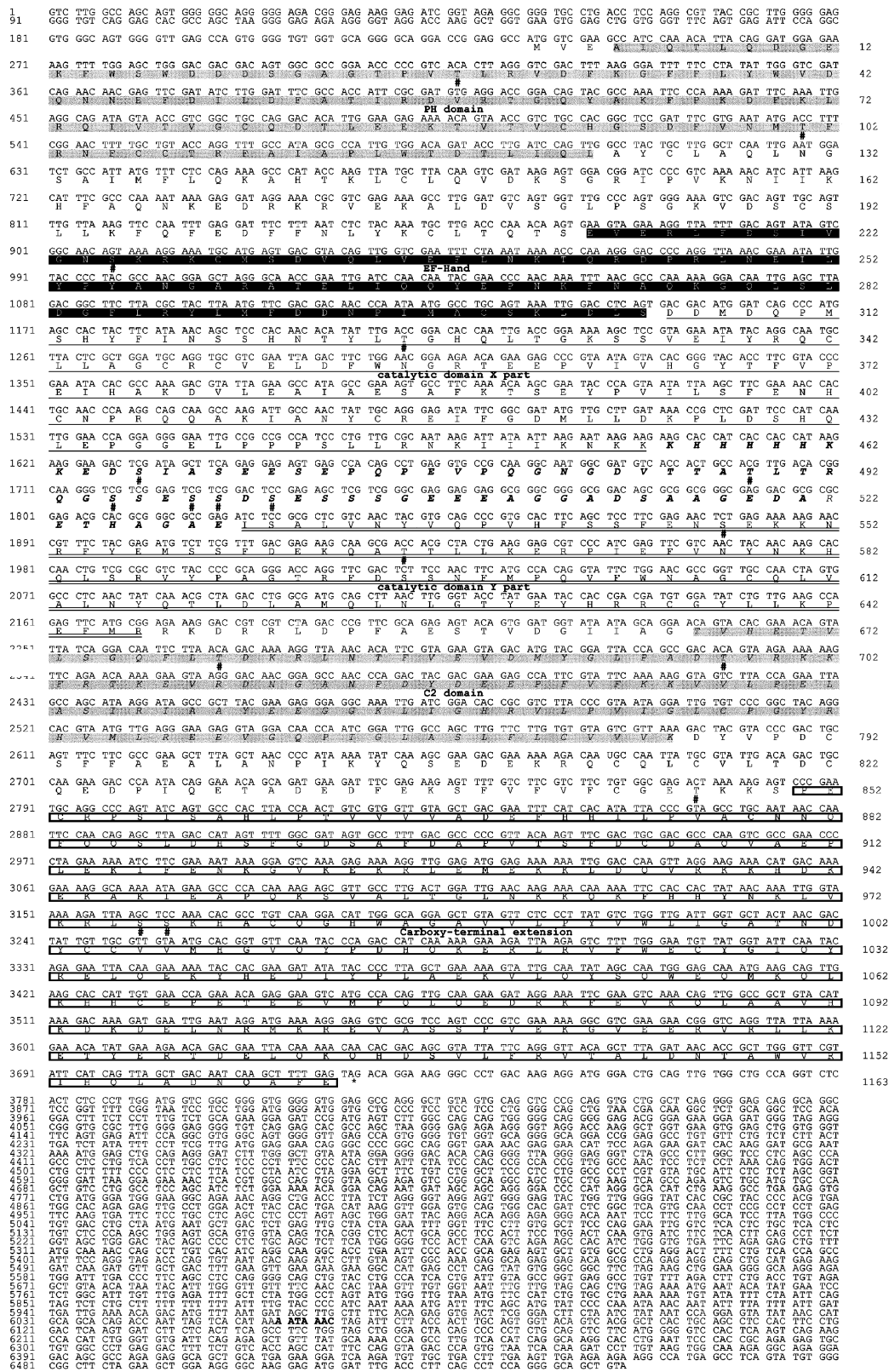
##### Expression developmental of *SLPLC-β*

To gain some insight into the potential role of *SLPLC-β*, its transcriptional activity was determined, in the male antennae, by real-time qPCR during the pupal and adult stages. *SLPLC-β* expression was detected at the beginning of pupal stage (at day 3), then reached a maximum at the end of this stage (day 9) and was maintained at this level throughout the adult life (Figure 5).

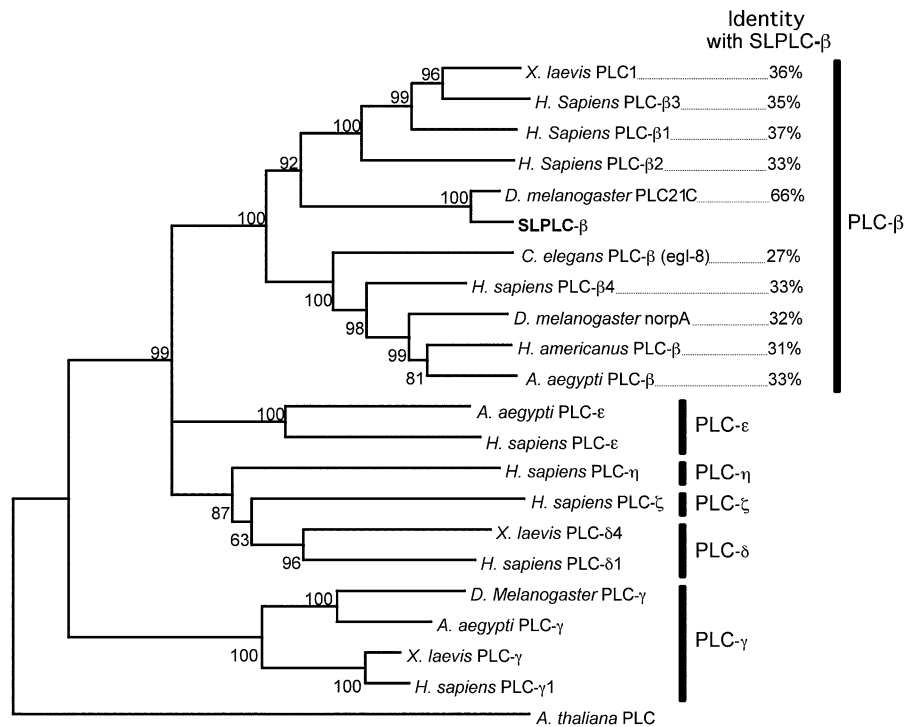
## Discussion

### Molecular cloning and primary structure of an antennal *SLPLC-β*

A partial cDNA fragment encoding a putative PLC was isolated by bioinformatic analysis of *S. littoralis* male antennal



**Figure 1** Nucleotide and deduced amino acid sequences of *SLPLC-β*. Nucleotide numbers are given on the left and amino acid numbers on the right. The PKC phosphorylation sites are designed by the symbol #. A polyadenylation signal (AATAAAA) in the 3'-UTR is indicated in bold type. The PH domain is shaded and the EF-hand motifs are indicated in black box. The X part of catalytic domain is underlined and the Y part is double-underlined. The C2 domain is written in italic type and shaded. The CTE is boxed and the XY linker sequence is written in bold italic type.

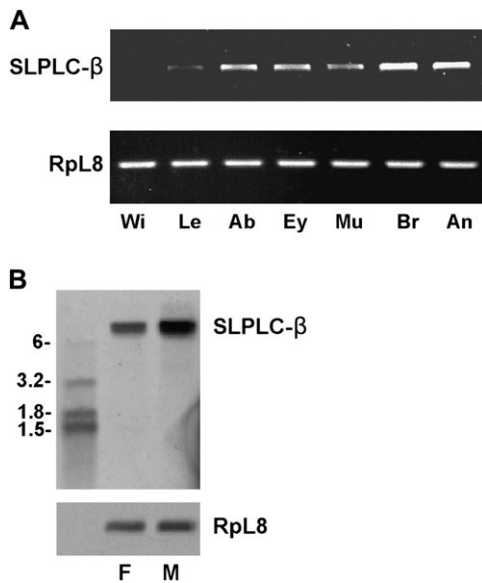


**Figure 2** Phylogenetic analysis of SLPLC-β. Bioinformatic analysis comparing *Spodoptera littoralis* PLC-β with other PLC isoforms was performed using MEGA version 4 (Tamura et al. 2007) with the following sequences (accession numbers given in brackets): *Xenopus laevis* PLC1, PLC-δ4, PLC-γ (NM\_001087700, NM\_001096677, AB287408), *Homo sapiens* PLC-β1, PLC-β2, PLC-β3, PLC-β4, PLC-ε, PLC-η, PLC-ζ, PLC-δ1, PLC-γ1 (NM\_015192, NM\_004573, NM\_000932, NM\_000933, AF\_190642, BC113950, NM\_033123, NM\_006225, NM\_002660), *Drosophila melanogaster* PLC21C, norpA, PLC-γ (M60453, NM\_080330, D29806), *Caenorhabditis elegans* PLC-β (egl-8) (AF188477), *Homarus Americanus* PLC-β (AF128539), *Aedes aegypti* PLC-β, PLC-ε, PLC-γ (XM\_001653754, XM\_001653081, XM\_001649088), *Arabidopsis thaliana* PLC (D50804), and *S. littoralis* PLC-β (SLPLC-β) (EU649777). The number at each branch point represents the bootstrap probability that 2 lineages join together to form a cluster. The identity of vertebrate and invertebrate PLC-βs versus *S. littoralis* PLC-β is expressed as a percentage of the SLPLC-β sequence.

EST library. Using a 5′–3′-RACE PCR-based strategy, we then succeeded in cloning the full cDNA of *SLPLC*. A phylogenetic tree of the PLC family was then inferred by comparing the amino acid sequence of *SLPLC* with those of known PLCs from vertebrates and invertebrates. The analysis of this tree provided evidence that *SLPLC* belongs to the cluster of PLC-β subtypes and shares most identity with *D. melanogaster* PLC21C. The polypeptide SLPLC-β contains all the structural features common to the PLC-β subfamily with the presence of an N-terminal PH domain followed by EF-hand motifs, a bipartite X/Y domain, a single C2 domain, and a CTE sequence (Rebecchi and Pentylala 2000).

Because the PH domain lacks any obvious catalytic properties and presents a high affinity for PIP<sub>2</sub>, it is suggested that this domain functions as an adaptor necessary to the anchorage of the enzyme to the membrane surface, leading to a progressive mode of PIP<sub>2</sub> hydrolysis by the active site (Drin and Scarlata 2007). The EF-hand motifs are capable of linking to fatty acids, and this interaction is also considered as critical for the catalytic activity (Rebecchi and Pentylala 2000; Drin and Scarlata 2007). It is known that PLC-βs are the major effectors of the G $\alpha_q$  subunit of heterotrimeric G proteins, and it has been reported that the C2 domain strongly links to activated G $\alpha_q$  subunit (Drin and Scarlata 2007; Oude

Weernink et al. 2007). Consequently, it is admitted that this domain is involved in the activation process of PLC-βs through its association with the G $\alpha_q$  protein. Within other identified domains of PLC-βs, there are residues and motifs that are highly conserved and known to be critical for function. The SLPLC-β catalytic domain consists in X and Y regions that contain residues H<sup>321</sup>, N<sup>322</sup>, E<sup>351</sup>, D<sup>353</sup>, H<sup>366</sup>, E<sup>400</sup>, K<sup>448</sup>, and S<sup>531</sup> shown to be essential for ligand discrimination, enzymatic activity, or calcium binding which is a required catalytic cofactor (Ellis et al. 1998; Rebecchi and Pentylala 2000). The X and Y regions of SLPLC-β are joined by a sequence (X/Y linker) to form 2 halves of the catalytic site in a distorted triose phosphate isomerase  $\alpha/\beta$  barrel configuration. It has been proposed that the X/Y linker exerts an inhibitory effect on the activity of PLC-βs until it is displaced by electrostatic repulsion upon membrane binding, triggering the unmasking of the catalytic site (Rebecchi and Pentylala 2000; Drin and Scarlata 2007). Finally, PLC-β isoforms are distinguished from other PLCs by a CTE domain that contains sequences important to membrane binding, nuclear localization, and their stimulation by the G $\alpha_q$  subunit. This domain is rich in basic amino acids that participate to the formation of helical structures which contribute to the association of PLC-β with the cell's particulate fraction



**Figure 3** Tissue expression of *SLPLC-β* in the adult. **(A)** One microgram of total RNAs was extracted from wings (Wi), legs (Le), abdomens (Ab), eyes (Ey), thoracic muscles (Mu), brains (Br), and antennae (An) of 2-day-old adult males and reverse-transcribed. *SLPLC-β* and *RpL8* cDNAs were amplified with the couple of primers SIF, SIR and RpF, RpR, generating 763 bp and 570 bp fragments, respectively. Amplification products were loaded on 1.5% agarose gels and visualized with ethidium bromide. **(B)** Fifteen micrograms of total RNAs from antennae of 2-day-old adults were analyzed by northern blot and hybridized with *SLPLC-β* and *RpL8* DIG-labeled probes. RNA sizes (kb) of RNA ladder (Roche) are given on the left.

(Kim et al. 1996; Rebecchi and Pentylala 2000). The CTE domain of *SLPLC-β* (residues 850–1116) is also highly basic, with an isoelectric point of 10.4. A sequence analysis program (Geneworks; Intelligenetics) predicted the secondary structure of this region to be an  $\alpha$ -helix, with residues K<sup>915</sup>, K<sup>920</sup>, K<sup>923</sup>, K<sup>925</sup>, K<sup>931</sup>, K<sup>932</sup>, K<sup>938</sup>, K<sup>939</sup>, K<sup>942</sup>, K<sup>944</sup>, K<sup>946</sup>, K<sup>952</sup>, K<sup>961</sup>, K<sup>962</sup>, and K<sup>964</sup> forming a positively charged surface on the helix. Several of these residues, K<sup>915</sup>, K<sup>920</sup>, and K<sup>923</sup>, are required for activation by  $G\alpha_q$  and appear to serve other regulatory roles (Kim et al. 1996). It is noteworthy that the C-terminal domain of *SLPLC-β* contains several consensus protein kinase C (PKC) phosphorylation sites suggesting that *SLPLC-β* could be regulated by PKC. This hypothesis was supported by the identification of a PKC in the antennal olfactory sensilla of the moth *A. polyphemus* (Maida et al. 2000). In addition, it has been demonstrated that mammalian PLC- $\beta$ s are phosphorylated by the PKC, and there are functional consequences of this phosphorylation for the interaction between PLC- $\beta$  and  $G\alpha_q$  (Rebecchi and Pentylala 2000).

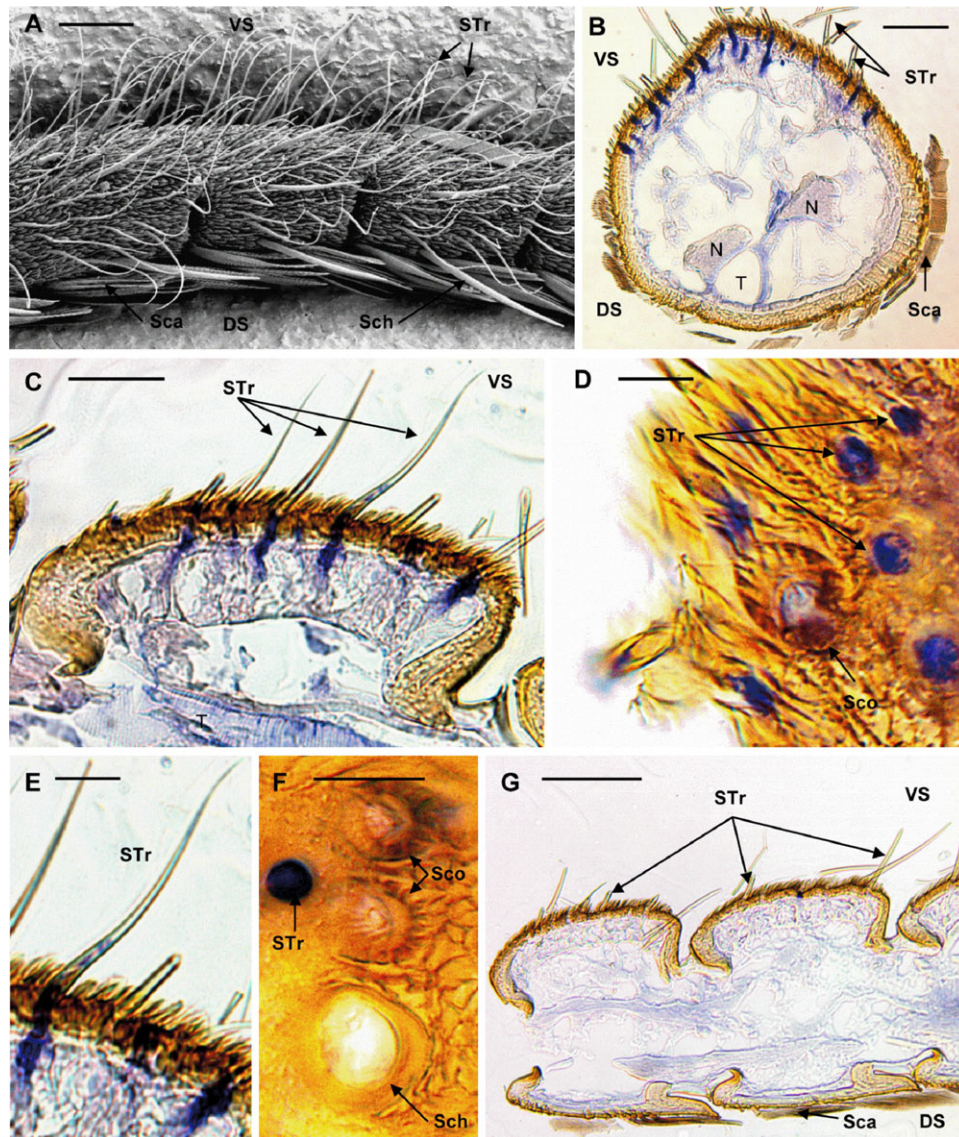
#### Tissue distribution and expression pattern in the antennae of *SLPLC-β*

Important clues about the functional role of genes may be gained from their spatiotemporal expression patterns. As

the majority of vertebrate and invertebrate PLC isoforms, *SLPLC-β* showed a wide tissue repartition that could reflect a pleiotropic function of this protein through its involvement in different transduction pathways regulating various physiological processes in insects. More precisely, the expression in antennae, eye, muscle, and brain suggests that this enzyme plays a role in the functioning of olfactory, visual, and muscular systems as well as in the activity of higher nervous centers in moths. This extended action of PLC- $\beta$  has already been described in *Rattus norvegicus*, *C. elegans*, and *H. americanus* (Homma et al. 1989; Xu and McClintock 1999; Bastiani et al. 2003), and the contribution of *SLPLC-β* in different sensory modalities would be consistent with the demonstration of a requirement of the *norpA* gene encoding PLC- $\beta$  in *D. melanogaster* visual and olfactory transduction processes (Riesgo-Escovar et al. 1995; Hardie et al. 2003). The high expression of *PLC-β* in *S. littoralis* brain is emphasized with indirect evidence of its presence throughout the *D. melanogaster* and *Apis mellifera* central nervous system and notably in several distinct neuropil regions, the antennal lobes, and the mushroom bodies which are known to ensure the olfactory information processing in insects (Shortridge et al. 1991; Müller 1999; Nässel 1999; Tsuchimoto et al. 2004). It has been even proposed that PLC- $\beta$  protein participates to signaling pathways modulating the activity of olfactory centers as reported in long-term memory processes in mice (Dong et al. 2009). Such central functions could be assigned to PLC- $\beta$  in *S. littoralis* by establishing the cartography of its expression in the brain.

In situ hybridization enabled us to determine the fine structure of *SLPLC-β* expression pattern in the male antennae. *SLPLC-β* expression was observed in both long and short *sensilla trichodea*, whereas no expression was observed in *sensilla coeloconica* and *sensilla chaetica*. The hybridization signals were located at the base of *sensilla trichodea* and inside the *sensilla* hair itself, which could correspond to the labeling of soma and dendrite of ORNs. Such a labeling has already been observed after in situ hybridization for other olfactory genes in *Manduca sexta*, *Mamestra brassicae*, and *S. littoralis* antennae (Rogers et al. 2001; Jacquin-Joly et al. 2002; Chouquet et al. 2008). In *S. littoralis* male, the sensitivity of ORNs housed in the different types of olfactory sensilla has been characterized by single sensillum recordings. ORNs located in long *sensilla trichodea* are tuned to pheromone compounds and ORNs that innervate short *sensilla trichodea* respond preferentially to plant odors, but also to pheromone molecules (Ljungberg et al. 1993; Anderson et al. 1995; Quero et al. 1996). In *sensilla coeloconica*, ORNs are involved in the detection of plant-related compounds, at least in *B. mori*, whereas *sensilla chaetica* are known to house mechanosensory and taste receptor neurons (Pophof 1997; Shields and Hildebrand 2001). Our hybridization results demonstrated that *SLPLC-β* expression was restricted to olfactory sensilla.





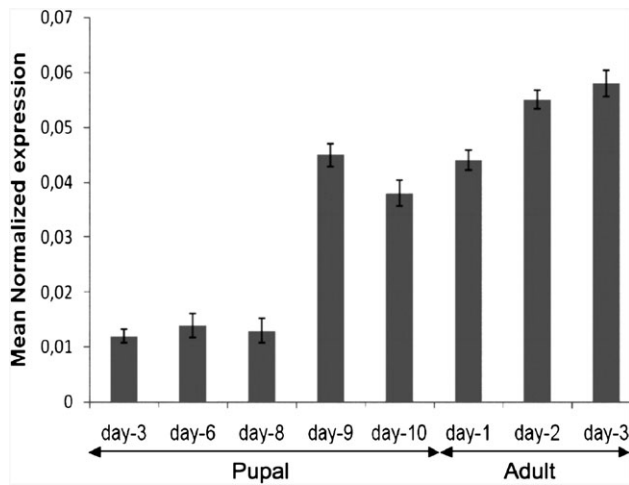
**Figure 4** Expression patterns of *SLPLC-β* after in situ hybridization on sections of *Spodoptera littoralis* male antennae. **(A)** Scanning electronic micrograph of male antennal segments with the localization of scales (Sca) on dorsal side (DS), *sensilla chaetica* (Sch) and *sensilla trichodea* (STr) on ventral side (VS) (courtesy of Dr Gnatzy). **(B)** Transversal section with labeling restricted to the ventral side carrying the olfactory sensilla. Trachea (T) and antennal nerve (N) are shown. **(C)** Longitudinal section of ventral side of an antennal segment showing intense labeling of *sensilla trichodea*. **(D)** Section through the cuticle with unlabelled *sensilla coeloconica* (Sco). **(E)** Detail of a *sensillum trichodeum* showing strong labeling at the base and inside the sensillar hair. **(F)** Section through the cuticle with unlabelled *sensilla coeloconica* (Sco) and *sensilla chaetica* (Sch). **(G)** Longitudinal section of antennae hybridized with sense *SLPLC-β* probe, showing absence of labeling. Scale bars: 50  $\mu\text{m}$  in A and F; 10  $\mu\text{m}$  in B, C, E, and G; 5  $\mu\text{m}$  in D and F. This figure appears in color in the online version of *Chemical Senses*.

#### Implication of *SLPLC-β* in the pheromone transduction pathway

The specific expression of *SLPLC-β* in *S. littoralis sensilla trichodea*, apparently at least in ORNs tuned to pheromone compounds, leads us to hypothesize that this protein participates to or modulate the pheromone transduction in moths. This hypothesis is supported by the immunolocalization of a PLC- $\beta$  subtype in homogenates of isolated pheromone-sensitive sensilla of *A. polyphemus* (Maida et al. 2000). Additionally, *SLPLC-β* was not only detected in male antennae

but also in female antennae where its presence is in agreement with the ability of females to detect their own pheromone compounds (Ljungberg et al. 1993). The expression level of antennal *SLPLC-β* was stronger in males than in females, and this sex difference may result from a higher number of pheromone-sensitive sensilla on the male antennae (Ljungberg et al. 1993).

Bioinformatic analysis of the *S. littoralis* male antennal EST library using sequences from public database allowed us to isolate a cDNA clone whose sequence partially encoded a  $G\alpha_q$  subunit known to specifically activate the



**Figure 5** Developmental analysis of *SLPLC-β* expression in male antennae. Total RNAs were extracted from male antennae during the nymphal and adult stages and reverse-transcribed. The synthesized cDNAs were analyzed by real-time PCR using the SIF and SIR primers for *SLPLC-β* and the RpFq and RpRq primers for *RpL8* reference gene.

PLC-β (Rebecchi and Pentylala 2000). Therefore, it is very likely that this subunit is also involved in insect pheromone transduction. Such a hypothesis is consistent with the detection of a  $G\alpha_q$  protein in olfactory *sensilla* preparations of the moth *Antheraea pernyi* (Laue et al. 1997). In addition, in situ hybridization experiments localized the expression of the  $G\alpha_q$  subunit in the pheromone-sensitive *sensilla trichodea* of the moth *M. brassicae* (Jacquin-Joly et al. 2002). In mammals, pheromone information in vomeronasal receptor neurons is mediated through the stimulation of PLC-β by a  $G\alpha_q$  subunit (Wekesa et al. 2003). In lobsters, several studies revealed the presence of a PLC-β associated with a  $G\alpha_q$  protein in olfactory aesthetascs which are distributed on the first antennae of male and serve to the detection of female sex pheromones (Fadool et al. 1995; Xu and McClintock 1999; Munger et al. 2000).

The importance of PLC-β in pheromone signaling is in concordance with the rapid increase in  $IP_3$  observed after odorant stimulation (Breer et al. 1990; Kaissling and Boekhoff 1993; Hatt and Ache 1994; Boekhoff et al. 1994). In vertebrates, the incubation of microvillar membranes from female vomeronasal organ (VNO) with male pheromones resulted in an increase in  $IP_3$  production (Wekesa et al. 2003). In addition, recent biochemical and electrophysiological data highlighted the participation of DAG in the pheromone transduction process. In mammals, the electrical response of VNO neurons to pheromones depends on the PLC activity, and it requires the activation of DAG-gated channel belonging to the superfamily of canonical transient receptor potential channels (TRPC) (Leypold et al. 2002; Stowers et al. 2002; Lucas et al. 2003). In moths, perfusion of a DAG-analogue stimulated a cationic current in *S. littoralis* ORNs in vitro that was similar to the mammalian TRPC2 current (Lucas and Pézier 2006), and extra-

cellular recordings from pheromone-sensitive *sensilla* demonstrated that DAG was able to evoke ORN firing activity in *B. mori* and *A. polyphemus* (Maida et al. 2000; Pophof and van der Goes van Naters 2002).

All these data support the idea that  $G\alpha_q$ -activated PLC-β protein and the subsequent production of second messengers participate to the pheromone signal transduction in moths.

#### Implication of PLC-β in the establishment of olfactory *sensilla*

Finally, our results revealed an antennal expression of *SLPLC-β* during the whole pupal and adult stages. The pupal antenna undergoes a morphological transformation to form a functional adult antennae, and this morphogenesis is marked by the differentiation of olfactory *sensilla* with an important neuronal reorganization (Franco et al. 2007). In *D. melanogaster*, molecular experiments demonstrated that the *PLC21C* gene was highly expressed in the brain throughout the larval and adult periods. It has been even proposed that the PLC21C protein was required for the maturation of central nervous system via signaling pathways controlling the proliferation and differentiation of neuronal cells (Shortridge et al. 1991). In mammals, numerous studies provided molecular and cellular evidence for the involvement of PLC-β-linked cascades regulating neurite outgrowth and synaptic plasticity in the developing sensory and motor cortex (Ruiz de Azúa et al. 2006; Koh et al. 2008; McOmish et al. 2008). Taken all these data into consideration, *SLPLC-β* likely plays a crucial role in the formation of antennae during the pupal stage necessary for the establishment of the neuronal architecture of adult olfactory *sensilla*. This interpretation is consistent with the increased expression level of *SLPLC-β* during the pupal stage, a period during which antennal neurons are progressively engaged into their differentiation. A maximal expression of *SLPLC-β* was also detected at the end of pupal stage and was maintained during the adult period. This expression pattern of *SLPLC-β* was similar to that of olfactory genes such as odorant-binding proteins and ORs (Vogt et al. 1989; Sakurai et al. 2004; Ishida and Leal 2005) and might be correlated to a massive synthesis of *SLPLC-β* protein which would be essential to ensure an olfactory function in adults.

Finally, our results provide the molecular characterization of a PLC-β potentially involved in signaling cascades responsible for the establishment and/or the functioning of the olfactory system in moths.

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