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Molecular identification and differential expression of sensory neuron membrane proteins in the antennae of the black cutworm moth *Agrotis ipsilon*

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ABSTRACT

The insect sensory neuron membrane proteins (SNMPs) SNMP1 and SNMP2 are transmembrane domain-containing proteins and are homologs of the vertebrate CD36 transmembrane proteins. It has been suggested that SNMPs play a significant role in insect chemoreception. Previous studies have demonstrated that SNMP1 is expressed in the pheromone-sensitive olfactory receptor neurons (ORNs), whereas SNMP2 is expressed in the supporting cells. In this study, we identified two full-length SNMP transcripts, *AipsSNMP1* and *AipsSNMP2*, in the black cutworm moth *Agrotis ipsilon* (Hufnagel). The qRT-PCR results indicated that the *AipsSNMP1* and *AipsSNMP2* transcripts were expressed significantly higher in the antennae than in other tissues of both sexes. The expression of *AipsSNMP1* and *AipsSNMP2* in the antennae from different development stages of both sexes was investigated and was shown to begin to express in the pupae stage from 3 days before emergence and then increased dramatically at the day of the emergence, and the high expression levels were maintained during the following 4 days after the emergence in both sexes. The mating status had no effect on the expression levels of the *AipsSNMP1* and *AipsSNMP2* transcripts. Consistent with previous *in situ* hybridization studies in other Lepidoptera insects, our immunolocalization results at protein level demonstrated that both AipsSNMP1 and AipsSNMP2 were expressed in pheromone-sensitive sensilla trichodea but with a completely different expression profile. AipsSNMP1 is more uniformed and highly expressed along the membrane of the ORN dendrites, whereas AipsSNMP2 is widely distributed at the bottom of the sensilla trichodea and highly localized in the sensillum lymph. Our studies provide further detailed evidence for the involvement and general functional role of insect SNMPs in the detection of sex pheromones and general odorant molecules.

1. Introduction

Feeding, courtship and mating are fundamental for insects, and most insects rely on their sensitive antennae that express specific olfactory proteins to detect survival- and reproductive-related chemical cues from the environment (de Bruyne and Baker, 2008; Zhou, 2010; Sachse and Krieger, 2011; Leal, 2013). Pheromones and plant volatiles diffuse into the sensilla via the multipores that penetrate the cuticular surface (Steinbrecht, 1997). When pheromones and plant volatiles enter the sensillum lymph, the antennae-enriched binding proteins (odorant binding proteins (OBPs) and chemosensory proteins (CSPs)) capture these semiochemicals and transport them across the aqueous lumen to the membrane-bound chemosensory receptors (odorant receptors (ORs) and ionotropic receptors (IRs)) (Vogt and Riddiford, 1981; Wanner et al., 2004; Benton et al., 2009; Kaissling, 2009; Zhou, 2010). Subsequently, the odorant molecules are rapidly degraded by odorant-degrading enzymes (ODEs) (Vogt and Riddiford, 1981; Leal, 2013). Moreover, another olfactory protein family, the sensory neuron membrane proteins (SNMPs), is recently suggested to play a significant role in insect chemoreception. Insect SNMPs are two transmembrane domain-containing proteins that are homologs of the vertebrate CD36 transmembrane protein family, which is comprised of receptors for lipoproteins and transporters of cholesterol and lipids in vertebrates (Febbraio and Silverstein, 2007; Levy et al., 2007). Non-SNMP members of the CD36 family have been also reported in insects with the same function and structure as vertebrate CD36; for example ninaD and croquemort in *Drosophila melanogaster* (Kiefer et al., 2002; Franc et al., 1996). To date, two SNMP subfamilies have been identified in insects, the SNMP1 and SNMP2 subfamilies. The insect SNMP1 and SNMP2 proteins were first identified in the pheromone-sensitive hairs of the wild silk moth *Antheraea polyphemus* (Rogers et al., 1997)

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and tobacco hornmoth *Manduca sexta* (Robertson et al., 1999; Rogers et al., 2001a), respectively. Since their discovery, SNMP1 and SNMP2 have been identified in at least four insect orders, including Lepidoptera, Coleoptera, Hymenoptera and Diptera (Rogers et al., 1997, 2001a; Nichols and Vogt, 2008; Forstner et al., 2008; Vogt et al., 2009; Li and Qin, 2011; Liu et al., 2013).

Members of the insect SNMP1 subfamily are expressed in the pheromone-sensitive olfactory receptor neurons (ORNs) (Rogers et al., 2001b; Forstner et al., 2008); however, the SNMP2 proteins are expressed in the supporting cells rather than the ORNs (Forstner et al., 2008). Several functions of insect SNMPs in odorant detection have been predicted, including: (1) SNMPs function as docking proteins that capture the pheromone binding protein (PBP)-pheromone complexes and off-load the pheromone to the pheromone receptors (Rogers et al., 2001b; Nichols and Vogt, 2008); (2) SNMPs specifically bind sex pheromone or PBP-pheromone complexes as novel pheromone receptors (Vogt et al., 1988; Rogers et al., 1997, 2001b); (3) SNMP proteins interact with the OBP/odorant complexes, stabilizing them at the membrane surface (Rogers et al., 1997); (4) SNMPs form a heterodimer with the OR proteins, the SNMP-OR heterodimer binds the pheromones (Rogers et al., 2001b); (5) SNMP proteins function like the OBPs to capture and deliver the odorants to the ORs (Rogers et al., 2001b). However, the only specific function of SNMPs was demonstrated in *D. melanogaster* in which SNMP1 (Protein ID: NP_650953) acts as a signaling component and is necessary for the detection of the aggregation pheromone *cis*-vaccenyl acetate (cVA) *in vitro* and *in vivo* (Benton et al., 2007; Jin et al., 2008).

In this study, we identified two full-length SNMP transcripts, *AipsSNMP1* and *AipsSNMP2*, in the black cutworm moth *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae), which is a destructive pest affecting many crops worldwide (Rings et al., 1975; Clement et al., 1982). To investigate the molecular basis of the *A. ipsilon* SNMPs in odorant perception, we performed extensive expression profiling of *AipsSNMP1* and *AipsSNMP2* transcripts among different tissues of both sexes, in the antennae at different developmental stages and in the antennae of both sexes before and after mating by quantitative real-time PCR at the transcript level. The specific subcellular distribution of AipsSNMP1 and AipsSNMP2 proteins in the various olfactory sensilla was also investigated using immunocytochemistry techniques. The possible functions of the SNMPs in moth odorant detection are discussed. Our studies provide further evidence for the involvement of insect SNMPs in the detection of sex pheromones and general odorant molecules.

2. Materials and methods

2.1. Animals and tissue collection

The *A. ipsilon* larvae were reared in the laboratory on an artificial diet, the main components of which were wheat germ, casein and sucrose. The adult moths were given a 20% honey solution after emergence. The field-collected adults were infused into the colony each year to avoid inbreeding effects. The laboratory colony was maintained at 24 ± 0.5 °C, 75 ± 5% relative humidity (RH) and a photoperiod of 16:8 (L:D) h.

For the *SNMP* transcript cloning and gene expression analysis, the pupae were sexed and were maintained in separate test tubes until adulthood. Different tissues (male antennae, female antennae, proboscis, labial palp, heads without antennae, thoraxes, abdomens, legs, wings, male accessory glands and female pheromone glands) from two- or three-day-old adult moths were collected. The antennae were excised from the female and male moths at different developmental stages (1 day, 3 day and 5 day before emergence, 0–7 days after emergence, mated and unmated). The tissues were excised, immediately frozen in liquid nitrogen, and stored at −80 °C or used directly.

2.2. RNA extraction and cDNA synthesis

The total RNA was extracted from the antennae or other tissues using the SV total RNA isolation system kit (Promega, Madison, WI, USA) and were quantified using 1.1% agarose gel electrophoresis and a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The single-stranded cDNAs were synthesized using the SuperScript™ III Reverse Transcriptase system (Invitrogen, Carlsbad, CA, USA). For the *SNMP* transcript cloning, the 5′ and 3′ RACE-Ready cDNA was synthesized from the male antennae RNA using the SMARTer™ RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) in accordance with the manufacturer's instructions.

2.3. SNMP transcript fragment cloning using degenerate PCR

The degenerate primers were designed using the conserved regions of the *SNMP1* and *SNMP2* genes from *M. sexta* (GenBank AF323589 and AF323588), *Heliothis virescens* (GenBank AJ251959 and AM905328), *Cnaphalocrocis medinalis* (GenBank JN867063 and JN867064), *A. polyphemus* (GenBank U95026 and AM905329), *Ostrinia nubilalis* (GenBank HM044389 and HM044392), and *Ostrinia furnacalis* (GenBank HM044390 and HM044393) (Table 1). The PCR was performed using 200 ng male antennal cDNA and 0.5 U *Ex Taq* DNA polymerase (Takara, Kyoto, Japan). The cycling conditions were as follows: initial denaturation at 95 °C for 3 min; 36 cycles at 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. The PCR products were gel purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA), subcloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA, USA), and the cDNA was sequenced using an ABI3730XL automated sequencer (Applied Biosystems) with standard M13 primers.

Table 1
Nucleotide primers used in this article.

Primer name	Sequence (5′–3′)	Position (bp)
Cloning SNMP fragments		
SNMP1-F	GYCARHTDAARAAGGAAATGGC	101–122
SNMP1-R	TTSAGCATBTTCACRAABGTYTT	1321–1343
SNMP2-F	GACSCYTTCCTNAGGGTCAA	824–843
SNMP2-R	GGYACBGARGCRATGGCBGG	1412–1431
5′- and 3′- RACE PCR		
SNMP1-GSP1	CGGAGCGAACTCAGTCCTCGCACAG	567–591
SNMP1-GSP2	CGTGCATGAAGGCTCCTATGTTCGCC	1052–1077
SNMP2-GSP1	CCTTCCGCTGCTCTCATGGTGGGT	940–963
SNMP2-GSP2	CCACGACGGCTGCCTCCTGATG	1357–1378
Full-length SNMP1 and SNMP2 cloning		
SNMP1-F	ATGGCGATGGCAAAGGAGCTGA	1–22
SNMP1-R	TTATATGTTGATCTTGGCCGGCTC	1546–1569
SNMP2-F	ATGTTCGGAAAATATGCCAAACTGT	320–344
SNMP2-R	TCAATTTCCTTTATTAACCATAGTAACGC	1854–1882
SYBR-Green qRT-PCR		
SNMP1-F	GAGCCAAGCCTATCCTTA	230–247
SNMP1-R	TCGTCCTCATTGTCTTCA	297–314
SNMP2-F	TCACTACCTCACTTCTAC	1424–1441
SNMP2-R	CTTATGCTTCTCCTTGTC	1490–1507
β-actin-F	GATGTTGACATTCGTAAG	859–876
β-actin-R	ATCTTGATCTTCATTGTG	972–989
RPS3-F	AAGTCCATGAAGTTCGTA	293–310
RPS3-R	GTACTCCTTGTCTTAGCA	378–395

F, forward primer; R, reverse primer; RPS3, ribosomal protein S3. Designation of oligonucleotide mixtures: H = A/C/T, S = C/G, B = C/G/T, R = A/G, Y = C/T, D = A/G/T, N = AGCT.