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Gene encoding the prothoracicotropic hormone of a moth is expressed in the brain and gut

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ABSTRACT

The molts of lepidopteran insects are typically controlled by the brain-derived prothoracicotropic hormone (PTTH) that stimulates ecdysteroidogenesis in the prothoracic glands (PGs). We report here that the larvae and pupae of the moth *Sesamia nonagrioides* can molt without brain (PGs must be present), suggesting that there might be a secondary source of PTTH. We addressed this issue by characterizing spatial and temporal expression patterns of the *PTTH* gene. To this end we identified a major part of the corresponding cDNA. Protein deduced from this cDNA fragment consisted of 128 amino acids and showed 48–85% homology with the matching regions of PTTHs known from other Lepidoptera. Quantification of *PTTH* expression in major body organs of the last instar larvae revealed high expression in the brain (fading in post-feeding larvae) and considerable expression in the gut (with a maximum in post-feeding larvae). The content of *PTTH* message in the gut was enhanced after decapitation. It is concluded that the molts of *S. nonagrioides* larvae are driven by *PTTH* gene expression in the gut.

1. Introduction

It is generally accepted that the molting process in caterpillars is initiated when the brain-derived prothoracicotropic hormone (PTTH) stimulates ecdysteroid biosynthesis in the prothoracic glands (PGs) (Gilbert et al., 2000). The PTTH of Lepidoptera was first identified in *Bombyx mori* (Kataoka et al., 1987; Kawakami et al., 1990) and later in the representatives of Saturnidae (Sauman and Reppert, 1996; Sehnal et al., 2002), Sphingidae (Shionoya et al., 2003), and Noctuidae (Xu et al., 2003; Xu and Denlinger, 2003; Wei et al., 2005; Xu et al., 2007) as a glycoprotein secreted by two pairs of neurosecretory brain cells.

Sesamia nonagrioides is a noctuid that completes larval development in a variable number of instars, dependent on the photoperiod. Larvae reared at 25 °C under long day conditions (16:8 h light:dark) pupate after five (mainly the males) or six (mainly the females) larval instars while those grown under short day conditions enter diapause and can undergo more than five supernumerary larval molts after the 6th instar (Eizaguirre and Albajes, 1992). Our recent work (Pérez-Hedo et al., 2010) demonstrated that the debrained or decapitated last instar larvae molted – some of them more than once – in spite of the brain absence. The removal of PGs by body ligation applied across the mesothorax prevented molting, proving that the presence of PGs was essential. These simple experiments revealed that the function of PGs in the caterpillars of *S. nonagrioides* was independent of the brain, in contrast to all Lepidoptera examined so far. It is possible that the PGs are regulated by another neuropeptide, for example by the diapause hormone whose prothoracicotropic activity was revealed in *B. mori* (Watanabe et al., 2007), or that PTTH is derived from a source outside the head (Sakurai et al., 1991). This paper examines the latter possibility.

The first step of the present work was to verify molt independence of the brain and dependence on the PGs in *S. nonagrioides* caterpillars of different ages. The second step was to identify *PTTH* cDNA and to examine expression of the *PTTH* gene in different larval tissues at several time points.

2. Material and methods

2.1. Insects and their rearing

The larvae of *S. nonagrioides* (Lepidoptera: Noctuidae) came from a culture maintained in the Laboratory of Entomology of the UdL-IRTA research center. After every 3–4 generations, the culture was boosted by insects collected in the maize fields of central Catalonia. The larvae were reared individually at 25 ± 0.5 °C and 16:8 h (light:dark) photoperiod on a semiartificial diet (Eizaguirre and Albajes, 1992). Their age was measured in days after the preceding ecdysis; for example, L6d0 marks newly ecdysed larvae of the 6th instar, L6d1 larvae 24 h after ecdysis, etc.

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2.2. Decapitated larvae and isolated abdomens

To confirm the importance of brain and PGs, the larvae were ligated behind the head or across the mesothorax, respectively, and the anterior body part was cut off. The head-ligated (decapitated) larvae were deprived of the brain and other head organs but retained their PGs which are located in prothorax, whereas isolated abdomens obtained by ligation across the mesothorax lacked both the brain and the PGs. The ligations were carried out with larvae a half day after ecdysis into the 4th, 5th, and 6th instar, and at days 1, 5, 7 and 9 (prepupae) of the 6th instar, respectively. The operated insects were kept for 5 h at 6 °C to reduce mobility during the initial phase of wound healing. Afterwards they were returned to the standard rearing conditions in vials supplied with small amounts of food that sustained sufficient air humidity. The insects were checked daily for 3 weeks. At least 10 larvae were used for each treatment.

2.3. RNA isolation

The following tissues were dissected under insect saline (Novák, 1966) at days 2, 5, 7, and 9 after ecdysis into the 6th instar: brain (Br), subesophageal ganglion (SbG), thoracic ganglia (ThG), abdominal ganglia (AbG), whole body except for the central nervous system and the digestive tract (carcass, Car), whole body except the brain (LwB), fat body (Ft), and gut (Gut). Dissected tissues were quickly immersed in liquid nitrogen and then held at -80° C until use. Total RNA was isolated with the acid phenol method (Chomczynski and Sacchi, 1987), precipitated with isopropanol and re-suspended in RNase-free diethylpyrocarbonate-treated water. The RNA was quantified and its quality assessed by absorbance measurements at λ260/λ280 nm with the Nanodrop ND-1000 spectrophotometer. Total RNA (1 μg) was treated with Turbo DNA-free DNase (AMBION, Austin, TX) according to the manufacturer's protocol in order to eliminate any traces of genomic DNA.

2.4. cDNA synthesis and PCR

The first-strand cDNA was synthesized from 1 μg brain RNA with random hexamer primers and the SuperScriptTM III First-Strand Synthesis System kit (Invitrogen, Carlsbad, CA, USA) following the recommended protocol. Primers PTTH-F1 and PTTH-R1 (Table 1), which were designed from the conserved regions of *PTTH* cDNAs known so far (Fig. 1), were employed to amplify the *PTTH* cDNA of *S. nonagrioides*. A set of specific primers PTTF-F2 and PTTH-R2 (Table 1) based on the identified cDNA sequence was used in subsequent PCR reactions carried out in the PTC-100 thermocycler (MJ Research, Waltham, MA, USA). The reaction mixtures of 25 μl contained 1x buffer, 200 μM dNTP mix, 2.0 Mm MgCl2, 400 nM of each primer, 1U of Taq polymerase (BIOTOOLS, Madrid, Spain) and 0.5 μl of the cDNA solution. The initial denaturing step of 3 min at 95 °C was followed by 35 cycles of 20 s at 95 °C, 1.30 min at 65 °C with a −0.2 °C change per cycle, and 1.30 min 72 °C; the reaction was concluded with 5 min at 72 °C. PCR product (about 390 bp) was separated by electrophoresis in 1.2% agarose gel and subsequently extracted with the QIAquick PCR purification kit (QIAGEN, Düsseldorf, Germany).

2.5. Sequencing and phylogenic analysis

PCR products were sequenced with the PTTH-F1 and PTTH-R1 primers (Table 1) using the BigDye Terminator Sequencing kit v3.1 (Applied Biosystems, Foster City, CA, USA)) and the ABI-3130 capillary electrophoresis system. Sequence data were edited with the Sequencing software (Applied Biosystems) and aligned with the cDNA sequence of other Lepidoptera using the ClustalW program (Chenna et al., 2003). The Neighbor-Joining method was employed for the construction of a phylogenetic tree (Tamura et al., 2007), validating the clustering structure with a bootstrapping test (10000 replicates).

2.6. Quantitative RT-PCR (qPCR) analysis

Real-time qPCR assays included end-point RT PCR and were run in the ABI-7500 device (Applied Biosystems). Sesamia-specific PTTH primers (qPTTH-Fw and qPTTH-Rv; Table 1) were designed from the identified cDNA sequence with the Primer Express v2.0 software (Applied Biosystems). The *16S rRNA* was quantified in parallel as an internal control used to normalize expression of the *PTTH* gene; the primers (Table 1) corresponded to conserved regions of the 16S rRNA in Lepidoptera. The qPCR was done with 5 μl mixtures containing 200 nM of the *PTTH* or *rRNA* primers and 1x SYBR Green Master mix (Applied Biosystems) and subjected to the following thermal profile: 10 min at 95 °C, 40 cycles of 15 s at 93 °C and 1 min at 60 °C, followed by quick denaturation at 95 °C plus a slow 5 min ramp to 30 °C. Three independent reactions were run with each tissue sample. The ΔΔCt method (Yuan et al., 2006) was used to normalize the PCR results and to quantify the mRNA contents.

2.7. Statistical analysis

Analysis of variance was run using SAS/STAT user's guide version 9.1 (SAS Institute, Cary, NC, USA) to test differences among the treatments. The comparisons were made using the Duncan method. The t- tests between treatments were run only when the ANOVA F-test suggested significant differences.

3. Results

3.1. Development of decapitated larvae and isolated abdomens

Decapitated larvae molted despite the absence of brain as a PTTH source. Decapitation also removed the corpora allata, which are the source of juvenile hormone (JH) preventing metamorphosis (Goodman and Granger, 2008). Due to JH elimination, all

Table 1
Primers used for amplification and quantification of *S. nonagrioides PTTH* cDNA and rRNA.

Primer name	Sequence 5′ → 3′	Length	T°m	Amplicon size
PTTH-F1	CCGTTAGTTTGTGTGATAGTATG	23	50.8	387 bp
PTTH-R1	CAGGTGCAGGGAGGATCCGG	20	65.8	
PTTH-F2	TTAGTCCCAAGGGTGATGGC	20	58.4	309 bp
PTTH-R2	TGGCTTGATTATGATGCTTCC	21	57.9	
qPTTH_Fw	AGATTGGCTCGAGACAGTGAATT	23	65.0	83 bp
qPTTH_Rv	GGATCAGGTTGAATGGAATCCAT	23	66.8	
qrRNA_Fw	ATTACGCTGTTATCCCTAAGGTAA	24	60.6	112 bp
qrRNA_Rv	GGTGACAGAAAAATATGGAGAACTT	25	62.2	