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Dynamics of diapause hormone and prothoracicotropic hormone transcript expression at diapause termination in pupae of the corn earworm, *Helicoverpa zea*

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ABSTRACT

Both diapause hormone (DH) and ecdysone (E) are capable of terminating pupal diapause in members of the *Helicoverpa*/*Heliothis* complex. In this study we examine how the transcript encoding prothoracicotropic hormone (PTTH), the neuropeptide that stimulates the prothoracic gland to produce E, and the transcript encoding DH respond to developmental changes, as well as environmental and hormonal cues that can trigger the termination of diapause. In nondiapausing individuals PTTH and DH transcripts are abundant from pupation until adult eclosion, while in pupae that enter diapause PTTH transcripts are undetectable and abundant DH transcripts are present only briefly after pupation. Injection of E can break diapause at either 18 or 21 °C, but DH is effective in breaking diapause only at the higher temperature. Transfer of pupae to a diapause-terminating temperature of 25 °C, injections of 1 nmol DH or 75 ng E at 21 °C, and injections of 500 ng E at 18 °C, are all accompanied by a simultaneous elevation of mRNAs encoding both PTTH and DH, although the rate of PTTH mRNA increase is consistently more rapid than that of DH. Subthreshold doses of E or injections of distilled water elicit a temporary rise in PTTH and DH transcripts but do not lead to diapause termination. The results suggest that these two hormonal systems work together in the cascade of events leading to diapause termination, producing a sophisticated control system that is finely tuned and responsive to subtle temperature changes in the overwintering environment.

1. Introduction

The hormonal mechanism that regulates diapause termination in pupae of the *Helicoverpa*/*Heliothis* complex of pest species presents an interesting puzzle. Most larval and pupal diapauses are thought to be the simple consequence of a shut-down in the synthesis and/or release of prothoracicotropic hormone (PTTH), the brain neuropeptide that stimulates the prothoracic gland to synthesize the steroid hormone, ecdysone [3]. The classic view is that the absence of PTTH results in failure of the prothoracic gland to produce ecdysone, hence development is halted. Consistent with this view are numerous experiments showing that pupal diapause can routinely be terminated with exogenous ecdysteroids. But, the diapause of *Helicoverpa*/*Heliothis* species can also be terminated by diapause hormone (DH) [10,14–16], a neuropeptide first described as an inducer of diapause in embryos of the commercial silkmoth *Bombyx mori* (reviewed in [12]), a response that is the exact opposite of its effect in these moth pupae. The story is further complicated by the observation that diapausing pupae of *Helicoverpas zea* [5] and *Helicoverpa punctiger* [1] appear to become independent of the brain (the source of PTTH) shortly after pupation and do not require the presence of the brain for diapause termination. The discovery that DH can terminate diapause in these pupae suggests the possibility that DH may have subsumed the role of PTTH in terminating diapause in this case, but whether PTTH is actually involved in the *Helicoverpa*/*Heliothis* diapause has not been determined. It is also evident that the response of diapausing pupae to DH stimulation is temperature dependent: for example, in *H. armigera* DH can only exert this effect at temperatures above 23 °C [15], while in *H. zea*, the threshold for DH action is approximately 21 °C [14].

The molecular structures of DH and PTTH in *H. zea* have been reported [4,11], thus enabling us to now evaluate the responses of these two genes to diapause-terminating stimuli. In this study we monitor the expression patterns of these two genes in both nondiapausing and diapausing pupae, and we then break diapause using elevated temperatures as well as injections of 20-hydroxyecdysone and DH to document the expression patterns of these two genes when diapause is broken. We provide evidence that transcripts encoding both PTTH

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and DH are elevated at diapause termination and that their elevation is synchronous rather than sequential. Further experiments compare the capacity of DH and 20-hydroxyecdysone to terminate diapause at different temperatures. The two hormones have distinct temperature-sensitivity profiles, suggesting that they both contribute to the diapause response but that they do so using mechanisms that do not completely overlap.

2. Materials and methods

2.1. Insects

H. zea eggs were obtained from North Carolina State University (Raleigh, NC) in March 2009 and were maintained in our laboratory since then. For colony maintenance and nondiapause insect rearing, *H. zea* was cultured in a constant temperature room at 25 °C with a photoperiod of 16 h light and 8 h dark (16:8, L:D) throughout its life cycle. Groups of newly hatched larvae were fed artificial diet (Bio-Serv, NJ) in 5 1/2 oz cups for 4 days and then transferred individually to 32-cell trays. Fresh diet was supplied to larvae every 5 days until pupation. Adults were fed 10% sucrose solution in a 30 cm × 30 cm × 30 cm plastic cage covered with cheese cloth at the top for eggs collection.

For diapause induction, third instar larvae were transferred from the 25 °C culture room to a constant temperature room at 18 °C with a photoperiod of 8 h light and 16 h dark (8:16, L:D). Fresh diet was supplied every week until pupation. Pupae were collected and placed in Petri dishes until used. The diapause status was verified by checking the position of dark eye spots on the heads of 1–2 week-old pupae [6]. Diapause incidence was over 90% under these rearing conditions.

2.2. H. zea diapause termination bioassay

Diapausing pupae at different stages were employed for the diapause termination bioassay. Two-week-old diapausing pupae were used to examine the diapause termination effects of diapause hormone and 20-hydroxyecdysone at 21 or 18 °C. To elucidate the effect of 25 °C on diapause termination pupae were first held at 18 °C for 12 months. Diapause hormone and 20-hydroxyecdysone were dissolved in distilled water and injected into the test groups using finely drawn glass capillaries. Controls were injected with 5 μl distilled water. Diapause termination percentages were recorded on the designated dates, followed by brain and subesophageal ganglion (SG) dissection.

2.3. Brain and subesophageal ganglion dissection and RNA extraction

To compare DH and PTTH transcripts in nondiapausing and diapausing pupae, both brains and SGs were dissected in 0.75% NaCl solution. For Northern blots brains and SGs from fifty pupae were collected and for quantitative PCR brains and SGs from fifteen individuals were sampled for each biological replicate. Collected brains and SGs were immediately homogenized manually in a 1.5 ml tube using a plastic pestle in 1 ml Trizol® reagent (Invitrogen, Carlsbad, CA). Homogenized samples were stored at −70 °C until used. Total RNA was extracted according to the manufacturer's manual for Trizol® reagent.

2.4. Northern blot analysis

Northern blots were performed to investigate expression patterns of DH and PTTH genes. For nondiapausing individuals held at 25 °C, the period between pupation and adult eclosion was 8 days, and samples were collected each of those days. Pupal diapause greatly extends the pupal stage, and samples of diapausing pupae reared at 18 °C, were collected at 1–7 days, 2 weeks, and 1, 2, and 6 months. All pupae were dissected at the same time during the photophase (5–7 h after the onset of the photophase).

Based on the reported DH-PBAN mRNA (GenBank ID: U08109) and PTTH mRNA (GenBank ID: AY172670) for *H. zea*, primers, including ZDP1 and ZDP3 for DH, PTTHP1 and PTTHP4 for PTTH, were designed and synthesized to obtain DH and PTTH fragments. Since heat shock protein cognate 70 (hsc70) was nearly equally expressed in both diapausing and nondiapausing pupae of *H. zea* [13], hsc70 was used as a control gene for Northern blots. Primers C70P1 and C70P2 based on hsc70 mRNA sequence (GenBank ID: GQ389712) were also synthesized to obtain a 702-bp hsc70 cDNA fragment for probe labeling. Primer sequences are shown in Table 1. 1.5 μg total RNA from nondiapausing pupae Br-SG complexes were reverse transcribed into cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and Oligo $(dT)_{20}$ according to the manufacturer's instructions. The corresponding fragments from DH, PTTH, and hsc70 cDNAs were PCR amplified, purified, and cloned into pCR®2.1-TOPO® vector for sequencing at the Plant-Microbe Genomic Facility, Ohio State University, to verify that the correct fragments were obtained. These plasmids were diluted 200-fold and were used as templates for a second round of PCR. These PCR products were further purified and used for probe labeling. DIG-High Prime (Roche, Indianapolis, IN) was employed to label probes of DH, PTTH, and hsc70 according to the user's manual.

Total RNAs from samples were isolated as described above. 5 μg RNA from each sample was separated on 1% agarose gel with 0.41 M formaldehyde and was transferred to Amersham Hybond-N+ membrane (GE Healthcare, Buckinghamshire, UK) using a TurboBlotter™ Rapid downward transfer system (Whatman, Piscataway, NJ). The membranes with transferred RNA were cross-linked by ultraviolet and kept at −20 °C until used.

Membranes and corresponding probes were co-incubated in polyethylene hybridization bags at 37 °C overnight, twice washed in 2× SSC with 0.5% SDS at room temperature, and washed twice again in 0.5× SSC with 0.5% SDS at 68 °C. The procedures including membrane blocking, antibody incubation, washing for signal detection, and X-ray film exposure were performed according to the supplier's instructions (Roche, Indianapolis, IN).

2.5. Quantitative real-time PCR

To further investigate expression levels of DH and PTTH genes after various treatments, RNA reverse transcription combined with quantitative real-time PCR were performed. Three biological replicates were collected for each sample. Brains with SGs from fifteen pupae were dissected and pooled for each biological replicate. Total RNA was extracted from each sample as indicated above and 1.5 μg RNA was first treated with DNase I, Amplification Grade (Invitrogen, Carlsbad, CA), to eliminate possible genomic DNA contamination in the RNA samples, then reverse transcribed using a SuperScript™ III first strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) according to the instructions. The synthesized cDNAs were stored at −20 °C until used.

Multiple candidate genes including cytoplasmic actin A3a1 (GenBank ID: AF286060.1), hsc70, GTP-binding protein alpha subunit (GenBank ID: AY957405.1), elongation factor 1-alpha (GenBank ID: U20136.1), thymosin beta-like protein (GenBank ID: DQ875231.1), ribosomal protein S7 (GenBank ID: DQ875215.1), and ribosomal protein L27 (GenBank ID: DQ875214.1) were selected and examined as reference genes. Among these genes, we found that the most stable gene was ribosomal protein S7 (RpS7). Thus different primer pairs were designed and synthesized for qPCR. Standard curves were constructed using serially diluted plasmid