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Transcription factor fork head regulates the promoter of diapause hormone gene in the cotton bollworm, *Helicoverpa armigera*, and the modification of SUMOylation

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ABSTRACT

The transcription factor fork head (FoxA) plays important roles in development and metabolism. Here, we cloned a fork head gene in *Helicoverpa armigera*, and found that the fork head protein is mainly located in the nucleus. This fork head gene belongs to the FoxA subfamily of the Fox transcription factors. The diapause hormone and pheromone biosynthesis-activating neuropeptide (DH-PBAN), which are two well-documented insect neuropeptides that regulate insect development and pheromone biosynthesis, are encoded by a single mRNA. In the present study, fork head was shown to bind strongly to the promoter of *H. armigera* DH-PBAN gene, and regulate its promoter activity. Furthermore, the effect of SUMOylation of the FH protein on the regulation of Har-DH-PBAN gene was investigated, and we show that the SUMO can modify Har-FH protein and cause down-regulation of DH-PBAN gene expression. These results suggest that SUMOylated FH plays a key role in insect diapause in *H. armigera*.

1. Introduction

Environmental conditions are not always suitable for insect development, and some adverse environmental changes even challenge insect survival. As a response, many insects have evolved an adaptation to survive seasonally recurring adverse conditions, such as a programmed developmental arrest in winter. In insects, the developmental arrest is called diapause, which is induced by photoperiod, temperature, and nutrients, and then these signals are transduced into humoral factors (Denlinger et al., 2005). In the cotton bollworm, *Helicoverpa armigera* (Har), an agriculturally important noctuid moth, pupal diapause is induced by incubating larvae at low temperature and short day illumination, whereas high temperature and long day illumination causes pupal development toward adult. The embryonic diapause of the silkworm, *Bombyx mori* is known to be induced by a neuropeptide hormone, diapause hormone (DH), which is a 24-amino acid neuropeptide secreted from the subesophageal ganglion (SG) (Yamashita, 1996). The cDNA and gene encoding DH were first cloned from *B. mori* (Sato et al., 1993; Xu et al., 1995), and the structural analysis of the DH cDNA and gene showed that neuropeptide pheromone biosynthesis-activating neuropeptide (PBAN), which controls sex pheromone biosynthesis in females of lepidopteran species, was also encoded in the cDNA. Hereafter, the cDNA and gene are designated as DH-PBAN.

DH-PBAN cDNA or genes have been identified in 15 species including *B. mori* (Sato et al., 1993), *Helicoverpa zea* (Ma et al., 1994), *Helicoverpa assulta* (Choi et al., 1998), *Mamestra brassicae* (Jacquin-Joly et al., 1998), *Agrotis ipsilon* (Duportets et al., 1999), *Spodoptera littoralis* (Iglesias et al., 2002), *Heliothis virescens* (Xu and Denlinger, 2003), *Manduca sexta* (Xu and Denlinger, 2004), *H. armigera* (Zhang et al., 2004b), *Adoxophyes* sp (Choi et al., 2004), *Samia cynthia ricini* (Wei et al., 2004), *Plutella xylostella* (Lee and Boo, 2005), *Spodoptera exigua* (Xu et al., 2007), *Clostera anastomosis* (Jing et al., 2007) and *Antheraea pernyi* (Wei et al., 2008). Although the function of Bom-DH is well defined, the function of DH-like peptide in other species is unclear. In a study of DH function in *H. armigera* pupal diapause, Zhang et al. (2004a,b) found that Har-DH could not induce diapause but functions in the regulation of pupal development through activating the prothoracic glands (PGs) to synthesize ecdysone. Recently, *B. mori*

Abbreviations: SG, subesophageal ganglion; DH-PBAN, diapause hormone and pheromone biosynthesis-activating neuropeptide; Har, *Helicoverpa armigera*.

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DH was also demonstrated to stimulate PGs to produce ecdysone (Watanabe et al., 2007).

DH-PBAN cDNAs and genes have been characterized from a number of insect species, but little is known about the regulatory mechanisms at work in DH-PBAN promoter regions. In *B. mori*, the transcription factor POU-M2 plays an important role in the regulation of DH-PBAN promoter activity (Zhang et al., 2004a). In *H. armigera*, the transcription factor Ap-4 can bind to the proximal cis-element E-box of the DH-PBAN promoter (Hu et al., 2010). However, POU-M2 cannot effectively activate Har-DH-PBAN promoter, though POU-M2 can bind to the POU-binding site of the DH-PBAN promoter (Zhang et al., 2004a; Zhang and Xu, 2009). By promoter sequence analysis of Har-DH-PBAN promoter, several potential sites for transcription factors were predicted, including three fork head sites (Hong et al., 2006).

The fork head (FH) gene was first found to be required in *Drosophila melanogaster* embryos for the proper formation of terminal structures (Weigel et al., 1989). Subsequently, a number of FH genes were identified in various organisms, and "FOX (fork head box) proteins" was used as the new root symbol of FH transcription factors (Carlsson and Mahlapuu, 2002). The FOX proteins play roles in development, differentiation, proliferation apoptosis, stress resistance and metabolism. In response to harsh environmental conditions, *Ceanorhabditis elegans* larvae can undergo developmental arrest called dauer (lifespan extension); and the FOX transcription factor DAF-16 is well-known to participate in this process (Van der Horst and Burgering, 2007). DAF-16 influences the expression of several downstream genes, which are involved in many different cellular processes, such as cell-cycle inhibition, oxidative-stress resistance, apoptosis and metabolism. As a result of these gene changes regulated by DAF-16, the lifespan of *C. elegans* can be prolonged.

The dauer in *C. elegans* and diapause in insects share a common characteristic: an extremely low metabolic rate. Therefore, it is plausible that FH may participate in the regulation of insect development or diapause. Sim and Denlinger (2008) clearly demonstrated that FH mediates the diapause response in the mosquito, *Culex pipiens*, when RNAi was used to knock down its gene expression. In *H. armigera* DH-PBAN gene, three predicted FH sites are present in its promoter, suggesting that FH may play an important role in the regulation of pupal development or diapause through activating Har-DH-PBAN gene (Hong et al., 2006). The post-translational modification SUMOylation is essential for many cellular activities (Geiss-Friedlander and Melchior, 2007; Gill, 2005; Johnson, 2004), such as modifying transcription factors, serving to decrease transcription factor activity in most cases (Gill, 2003). In *D. melanogaster*, protein SUMOylation functions in development (Mauri et al., 2008; Talamillo et al., 2008). From a library of suppressive subtraction hybridization (SSH), we found a SUMO gene that was up-regulated during the pupal diapause initiation of *H. armigera* (Bao and Xu, 2011). After analyzing Har-Fork head (Har-FH) protein sequence, we found a putative SUMO acceptor site. Therefore, we wondered whether SUMO can modify Har-FH and affect its transcriptional activity.

In the present paper, we report the cloning and characterization of a cDNA encoding the transcription factor FH in *H. armigera* (Har-FH) and its developmental expression and binding to the DH-PBAN gene promoter. These results suggest that Har-FH does participate in the regulation of Har-DH-PBAN gene expression. Furthermore, the effect of SUMOylation on the FH protein in the regulation of Har-DH-PBAN gene was investigated, and our results show that SUMO can modify Har-FH protein and result in a down-regulation of DH-PBAN gene expression, suggesting that SUMOylated FH plays a key role in insect diapause.

2. Materials and methods

2.1. Animals

H. armigera larvae were reared on an artificial diet at 20 °C with an L14:D10 photoperiod (nondiapause type) or an L10:D14 photoperiod (diapause type). All nondiapause-type pupae developed toward adults, and more than 90% of diapause type individuals entered diapause. The developmental stages were synchronized at each molt by collecting new larvae or pupae. All tissues were dissected in insect saline containing 0.75% NaCl and stored at −80 °C until use.

2.2. RNA extraction and RT-PCR

Total RNA was extracted from the subesophageal ganglion (SG) using the acid-guanidine method (Chomczynski and Sacchi, 2006). One microgram of total RNA was reverse transcribed at 37 °C for 1 h in a volume of 25 μl with the M-MLV reverse transcription system (Promega, Madison, USA). One microliter of the reverse transcription product was added to 50 μl of the PCR reaction system, and amplification was performed with the degenerate primers FHF (5′-TCT CGT CCT TGA A(A/G)C G(C/T)T TCT-3′) and FHR (5′-CTA (C/T) CA GTT CAT CAT GGA TCT G-3′), which were designed according to the conserved fork head cDNA sequences from *D. melanogaster* (Weigel et al., 1989) and *B. mori* (Mach et al., 1995). PCR was performed under the following conditions: 30 s at 94 °C, 30 s at 45 °C, 30 s at 72 °C with 30 cycles, and then 10 min at 72 °C.

2.3. The rapid amplification of cDNA ends (RACE)

Based on the partial sequence of Har-FH cDNA amplified by the degenerate primers FHF and FHR, the specific primers GSPFH5 (5′-GCA GGA AGC AGC CGT TCT CGA A-3′) and GSPFH3 (5′-CTT CTA CCG ACA GAA CCA GCA G-3′) for the 5′- and 3′-cDNA ends respectively, were designed. The 5′- and 3′-RACE-ready cDNA was synthesized using the SMART™ RACE kit (Clontech) according to the manufacturer's protocol. PCR was performed with 5′- and 3′-RACE-ready cDNA, primers GSPFH5 and UPM for 5′-RACE, or GSPFH3 and UPM for 3′-RACE, under the following conditions: 3 min at 94 °C; 30 cycles of 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C; and 5 min at 72 °C. The PCR products were separated on a 1.5% agarose gel, purified, ligated into the pMD18-T vector (Takara), and amplified in *E. coli*. The sequencing reaction was run by the Invitrogen Company (Guangzhou, China).

2.4. Har-FH polyclonal antibody generation and western blot analysis

Har-FH ORF was amplified with the two primers FH-PF (5′-CGC GGA TCC ATG ATC TCG CAG AAG CTG TCG-3′) and FH-PR (5′-CCC AAG CTT TAC TGT CAC AAG GGC GGC TGC G-3′), which contain the restriction sites BamHI and HindIII, respectively. The PCR product was excised with BamHI and HindIII, and then subcloned into the pET28a vector (pET-Har-FH). The recombinant pET-Har-FH protein was expressed in BL21 cells induced by IPTG. The *E. coli* pellet was solubilized in 6 M urea in 50 mM Tris–HCl buffer, pH 8.0, followed by Ni-NTA column purification. Purified recombinant Har-FH protein was used to generate polyclonal antibodies in rabbit.

The brain and SG or brain-SG complexes of pupae were homogenized in ice-cold phosphate-buffered saline (PBS), followed by centrifugation at 12,000 g for 20 min at 4 °C. The protein extract was quantified using the Bradford method (Bradford, 1976) and stored at −80 °C until use. The protein extracts (20 μg of each sample) were separated using 12% SDS–PAGE and transferred onto