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Short Communication

Bombyx mori cathepsin D expression is induced by high temperature and H_2O_2 exposure

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ABSTRACT

Cathepsin D is involved in the metamorphosis of the silkworm, *Bombyx mori*. Here, we show the expression profile of *B. mori* cathepsin D (*BmCatD*) in the fat body during exposure to stressors, such as high temperature and H_2O_2. Exposure of larvae in the fifth instar stage to high temperature (28 °C) led to accelerated metamorphosis and shortened larval stage compared to control larvae grown at 23 °C. Concomitantly, the expression level of *BmCatD* mRNA was greatly increased during exposure to high temperature. We also detected significantly elevated H_2O_2 levels in the hemolymph of larvae treated with high temperature. To confirm that oxidative stress induces *BmCatD* expression, *B. mori* larvae were injected with H_2O_2. As predicted, we observed increased expression of *BmCatD* following H_2O_2 exposure. Based on these results, we conclude that *BmCatD* expression is induced by high temperature and H_2O_2 exposure and that this stress-induced *BmCatD* expression leads to early metamorphosis.

Introduction

Insect metamorphosis is carried out via programmed cell death (PCD) (Riddiford, 1985). During the course of metamorphosis, proteolytic enzymes play vital roles in the PCD of obsolete organs (Rabossi et al., 2004; Zhao et al., 2005; Gui et al., 2006; Yang et al., 2006, 2007; Lee et al., 2009). The examination of insect larval tissues showed that tissues such as the silk gland and gut are completely histolyzed via PCD during metamorphosis (Shiba et al., 2001; Lee et al., 2002; Uhlirova et al., 2003; Gui et al., 2006), and other tissues, such as the fat body, undergo reorganization with PCD (Rizki, 1978; Rabossi et al., 2004; Gui et al., 2006; Lee et al., 2009).

B. mori cathepsin B (*BmCatB*) is involved in regulating PCD of the fat body during *B. mori* metamorphosis (Xu and Kawasaki, 2001; Lee et al., 2009). We recently showed that *B. mori* cathepsin D (*BmCatD*) is required for PCD of both larval fat body and gut during *B. mori* metamorphosis (Gui et al., 2006). As might be expected, the expression of *BmCatB* and *BmCatD* is increased by the metamorphosis promoting 20-hydroxyecdysone and repressed by a juvenile hormone analog, which functions to maintain the larval state (Gui et al., 2006; Lee et al., 2009). RNA interference of *BmCatB* or *BmCatD* causes arrest of the larval–pupal transformation, indicating its requirement for metamorphosis (Gui et al., 2006; Lee et al., 2009). Additionally, previous work has shown that *BmCatD* is induced by baculovirus infection (Gui et al., 2006).

The change in expression of *BmCatD* in response to varying environments is still poorly understood. Therefore, we sought to determine if *BmCatD* expression shows any change under stress conditions such as high temperature and oxidative stress, and to determine if any observed changes are correlated with metamorphosis. To test this, we reared silkworms at either 23 °C (control) or 28 °C (high temperature) and compared the expression of *BmCatD* during the fifth larval instar stage. We also looked for changes in *BmCatD* expression when fifth-instar larvae were injected with H_2O_2. Our results showed that *BmCatD* expression can be induced by high temperature and H_2O_2 exposure and that this stress-induced *BmCatD* expression leads to early metamorphosis.

Materials and methods

Silkworms

The silkworm, *Bombyx mori*, used in this study was an F_1 hybrid, Baekok-Jam (Gui et al., 2006; Lee et al., 2009). Silkworms were reared on fresh mulberry leaves at 23 °C (control) or 28 °C (high temperature). Spinning and wandering occurred on day seven of the fifth instar larval stage and pre-pupation and pupation occurred two and three days thereafter, respectively. We have also designated the following

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stages: day one of the fifth larval stage occurs after ecdysis, the spinning stage begins when larvae start spinning and the pupal stage begins after pupation.

RNA extraction and northern blot

Total RNA extraction and northern blot analysis were performed as previously described (Gui et al., 2006; Lee et al., 2009). Fat bodies of *B. mori* were collected on ice and washed twice with phosphate-buffered saline (PBS; 140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and pH 7.4). Total RNA was isolated from the fat body using a Total RNA Extraction Kit (Promega, Madison, WI, USA). Harvested total RNA (5 μg/lane) was separated on a 1.0% formaldehyde agarose gel, transferred onto a nylon blotting membrane (Schleicher & Schuell, Dassel, Germany) and hybridized at 42 °C with a probe in hybridization buffer containing 5× SSC, 5× Denhardt's solution, 0.5% SDS, and 100-μg/ml denatured salmon sperm DNA. *BmCatD* cDNA (Gui et al., 2006) was labeled with [α-^{32}P]dCTP (Amersham, Arlington Heights, IL, USA) using the Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, CA, USA) and used as probes for hybridization. After hybridization, the membrane filter was washed three times, each for 30 min in 0.1% SDS and 0.2× SSC at 65 °C, and then exposed to autoradiography film. Images of northern blots were analyzed using a computerized image analysis system (Alpha Innotech Co., San Leandro, CA, USA). The Alpha Imager 1220 (ver. 5.5) was used to aid analysis. The integrated density value was used to determine the density of each band. The relative levels of mRNA are calculated as the mean of three measurements normalized to the expression of control (100%).

Collection of hemolymph

Hemolymph was collected in cold test tubes by cutting the legs of *B. mori* larvae. The hemolymph was centrifuged at 10,000×*g* for 10 min to pellet hemocytes and cell debris. The recovered supernatant was used for experimentation.

Measurement of hydrogen peroxide

Measurement of H_2O_2 was performed as described previously (Kim et al., 2008). H_2O_2 concentration was measured by colorimetric quantitative determination of hydrogen peroxide in hemolymph using a BIOXYTECH® H_2O_2-560™ Assay (OXIS International, Inc., Foster City, CA, USA). This assay is based on the oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) by hydrogen peroxide under acidic conditions. The ferric ion binds to a xylenol orange indicator dye to form a stable colored complex, which can be measured at 560 nm. Each experiment was performed in triplicate. Hydrogen peroxide concentrations are expressed as μM ml^{-1} hemolymph.

Injection experiments

Injection was performed as described previously (Gui et al., 2006). To induce oxidative stress, 20 μl of H_2O_2 (25 μM or 50 μM) was injected into larvae on the first day of the fifth instar. Control larvae of the same developmental stage were injected with PBS (20 μl/larva). Injected larvae were reared on fresh mulberry leaves at 23 **°C**. Fat bodies from all treated larvae were collected at one-day intervals post-treatment, washed twice with PBS, and subjected to total RNA extraction and northern blot analysis as described above.

Results and discussion

BmCatD is induced by high temperature

To examine whether *BmCatD* expression is increased by a high-temperature stimulus, fifth instar larvae were reared at 28 °C while controls were maintained at 23 °C. We first analyzed whether high-temperature stress affects development of *B. mori* and found that the period from the beginning of the fifth instar to the end of the pupal stage is shortened by approximately 4 days when larvae were reared at 28 °C (Fig. 1A). At 28 °C, the fifth larval stage averaged 6.2 days, approximately one day shorter compared to controls. Additionally, larvae reared at 28 °C began spinning on day seven of the final instar, leading to decreased pupal weight (Fig. 1B).

Next, we analyzed whether *BmCatD* expression is induced during high-temperature stress. We performed northern blot analyses on the fat bodies of larvae and found that the *BmCatD* expression increased

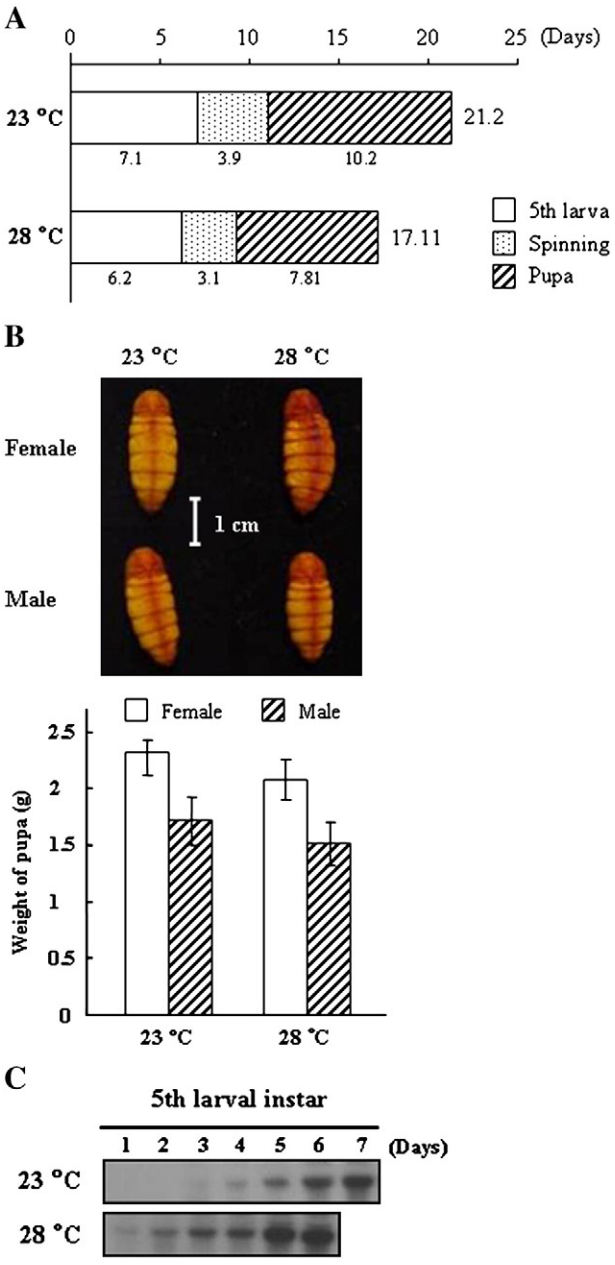

Fig. 1. Effects of high temperature on *B. mori* development. (A) Developmental period from the fifth instar to pupal stage. The period is indicated at the bottom of each stage. (B) Pupal weight measurements of high temperature treated (28 °C) and control (23 °C) fifth instar larvae (mean ± SD of three measurements; $n = 15$). The scale bar corresponds to 1 cm. (C) Northern blot analysis of *BmCatD* transcripts in the fat body of high temperature treated (28 °C) and control (23 °C) larvae. The larval period is indicated on the top of each lane.