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20-hydroxyecdysone and juvenile hormone analog prevent precocious metamorphosis in recessive trimolter mutants of *Bombyx mori*

Hua-Bing Wang [a,1], Saheb Md. Ali [a,b], Minoru Moriyama [a,2], Masashi Iwanaga [a], Hideki Kawasaki [a,*]

[a] Faculty of Agriculture, Utsunomiya University, 350 Mine, Utsunomiya, Tochigi 321-8505, Japan
[b] United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan

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ABSTRACT

The trimolter mutants of *Bombyx mori* have four instead of five larval instars of normal tetramolters. Here, we show that the tetramolter was induced in the recessive trimolter European No.7 mutant (rt-E7) by application of either the juvenile hormone analog (JHA) or 20-hydroxyecdysone (20E). However, treatments with JHA or 20E did not change the number of larval instars of the dominant trimolter Si Chuan mutant (DT-SC). Krüppel-homolog 1 (Kr-h1) is an early JH-response gene that mediates the anti-metamorphic action of JH. In the wing disc of tetramolter *B. mori*, Kr-h1 RNAs decreased shortly after ecdysis to the fifth instar, while pupal specifier gene, Broad Complex Z1 (BR-Z1) RNAs slightly increased and coincided with the onset of metamorphic competence of wing discs. Analysis of the developmental profile of Kr-h1 in the wing disc of rt-E7 showed that its transcript slightly increased from 12 to 24 h and gradually decreased between 24 and 72 h in the fourth (last) larval instar, while Kr-h1 mRNA decreased rapidly between 12 and 72 h in DT-SC. In addition, the expression of BR-Z1 in DT-SC during the early fourth (last) larval instar is relatively higher than that in rt-E7. These results indicated that the occurrence of pupal commitment of the wing disc in DT-SC was much earlier than that in rt-E7. In the early fourth larval instar of rt-E7, feeding on 20E or treatments with exogenous JHA caused up-regulation of Kr-h1, suppressed premature induction of BR-Z1, and then induced an additional larval instar. By contrast, in DT-SC mutant, since pupal commitment immediately occurred after third ecdysis, precocious metamorphosis was not successfully rescued. The results suggest that Kr-h1 and BR-Z1 involved in the prevention of precocious metamorphosis in recessive trimolter mutants by application of 20E and JHA. The result indicated that Kr-h1 and BR-Z1 expression reflected larval–pupal transition of the recessive trimolter of *B. mori*.

1. Introduction

The postembryonic development of insects is punctuated by a series of molts. The number of larval instars varies over broad limits, since it requires specific physiological adaptations; there has been evolutionary pressure for reducing the number of molts (Sehnal, 1985). The number of larval instars is genetically fixed; for example, in Lepidoptera, *Manduca sexta* and *Bombyx mori* typically have five larval instars; in cyclorrhaphous Diptera, some Hymenoptera, and Coleoptera, there are three larval instars (Sehnal, 1985; Kingsolver, 2007).

Molting is associated with environmental conditions such as nutrition, temperature, photoperiod and rearing density. In most, though not in all insects, the number of larval molts varies under the influence of environmental conditions (Wigglesworth, 1972; Kingsolver, 2007). It has been ascertained that temperature, light, and nutritional conditions can change the manifestation of molting (Nijhout, 1975; Pipa, 1976; Kingsolver, 2007). *B. mori* normally has five larval instars with developmental commitment to metamorphosis occurring early in the 5th (final) instar. However, the number of molting in several mutants varies from two to six (Tajima, 1964; Gu et al., 1995; Mitsuoka et al., 2001). Among these mutants, the trimolter mutants undergo precocious metamorphosis at the end of the fourth larval instar (Gu et al., 1995; Mitsuoka et al., 2001). The tetramolter can be induced from recessive trimolter mutants by application of either JHA or 20E (Komori, 1981; Gu et al., 1995; Mitsuoka et al., 2001). Our previous studies showed that feeding on 20E-induced extra larval ecdysis by maintaining substantial levels of ecdysteroid in the hemolymph

* Corresponding author. Tel.: +81 28 649 5455; fax: +81 28 649 5401.
E-mail address: kawasaki@cc.utsunomiya-u.ac.jp (H. Kawasaki).
[1] Present address: Department of Agricultural and Environmental Biology, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan.
[2] Present address: National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan.

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and that application of JHA enhanced the PTTH-secreting activity of the brain (Mitsuoka et al., 2001). However, the molecular basis of these effects remains unclear.

It is generally accepted that ecdysteroids trigger molting, while JH determines the nature of the molt (Riddiford, 1994). Exposure to JH leads to repetition of larval stages, while the decline of JH during the early stage of the final instar switches the imaginal discs to pupal commitment (Kremen and Nijhout, 1989, 1998; Riddiford, 1994). The molecular mechanism through which the decline of JH induces metamorphosis has remained poorly understood. Recently, methoprene-tolerant (Met), Kr-h1, and BR-C are the best known transducers of the JH signal. Met is a candidate for the JH receptor (Konopova and Jindra, 2007). In the larval stage, Met regulates the expression of the anti-metamorphic gene, Kr-h1, and inhibits expression of BR-C that promotes pupal development (Minakuchi et al., 2009). Kr-h1 is an early JH-responsive gene and mediates the anti-metamorphic action of JH in *Drosophila melanogaster* and *Tribolium castaneum* (Pecasse et al., 2000; Minakuchi et al., 2008, 2009; Zhang et al., 2011). RNAi-mediated knockdown of *T. castaneum* Kr-h1 in the larval stage caused a precocious larval–pupal transition (Minakuchi et al., 2009). It has been suggested that the amount of *TcKr-h1* transcript is dependent on the JH level and the expression of Kr-h1 gene is maintained by JH in the larval stage (Minakuchi et al., 2009). Two Met orthologs and a *Kr-h1* ortholog have been identified from *B. mori* (Shinoda et al., unpublished). BR-C, one of the 20E-induced early transcription factors, is expressed specifically during larval–pupal metamorphosis under the control of 20E and JH (Bayer et al., 1996; Zhou and Riddiford, 2002; Riddiford et al., 2003). BR-C is necessary for metamorphosis and the role of BR-C in the larval–pupal transition has been studied in *D. melanogaster* (Zhou and Riddiford, 2002), *B. mori* (Uhlirova et al., 2003; Wang et al., 2010), and *T. castaneum* (Suzuki et al., 2008; Parthasarathy et al., 2008).

In Lepidoptera, the dynamics of the growth that produces pupal structures are best understood for wing discs (Kawasaki, 1988; Nijhout et al., 2007; Truman and Riddiford, 2007; Nijhout and Grunert, 2010). Lepidoptera wing discs are the early forming imaginal discs which show complete metamorphosis (Nijhout, 1976; Kremen and Nijhout, 1989, 1998; Zhou et al., 1998; Truman et al., 2006). In *B. mori*, pupal commitment of wing discs occurs within 16 h after the last larval ecdysis (Obara et al., 2002; Koyama et al., 2004). In this study, we showed that feeding on 20E or treatment with JHA prevented the precocious metamorphosis of recessive trimolter rt-E7 at the fourth larval instar and induced an additional larval instar. However, treatments with JHA or 20E did not change the number of larval instars of dominant trimolter DT-SC. To identify the molecular mechanisms that underlie molt cycle and larval–pupal metamorphosis, we compared expression profiles of Kr-h1 and BR-Z1 in the wing disc of tetramolter larvae, rt-E7 and DT-SC.

2. Materials and methods

2.1. Insects

The tetramolter strain N124 × C124, the recessive trimolter strain European No.7 (rt-E7) and the dominant trimolter strain Si Chuan (DT-SC) of *B. mori* were used in the present study. The spontaneous recessive rt-E7 mutant (Takahashi and Mitarai, 1949) was mapped at 3.0 cM of the silkworm genetic linkage group 6 (Banno et al., 2005). The spontaneous dominant DT-SC mutant (Tanaka, 1919) was also located at 3.0 cM of linkage group 6 (Chikushi, 1972; Banno et al., 2005). They were maintained in our lab and reared on fresh mulberry leaves at 25 °C under a 12 h light–12 h dark photoperiod.

2.2. Application of 20-hydroxyecdysone

20-hydroxyecdysone (20E, Sigma Chemical Company) was dissolved in distilled water to a concentration of 20 ppm and then applied on the fresh mulberry leaves. The trimolter larvae were fed these leaves from 0 to 72 h after the third ecdysis.

2.3. Application of methoprene

JHA (Methoprene, Zoecon Corporation) was diluted in ethanol to a concentration of 1 mg/ml and topically applied to the larvae. Methoprene was applied along the dorsal surface as described in previous studies (Mitsuoka et al., 2001). Larvae were treated with 2 μg methoprene daily during the first 3 days of fourth larval instar.

2.4. cDNA preparations

Wing discs were collected and washed three times in excessively cold phosphate-buffered saline (PBS) and then frozen and stored at −80 °C. Total RNA was isolated from wing discs using RNAiso (Takara, Japan). First-strand cDNA was synthesized from 1 μg total RNA in a 20 μl reaction mixture using ReverTra Ace (Toyobo, Japan).

2.5. Quantitative PCR

Quantitative PCR (quantitative RT-PCR: qPCR) was performed and analyzed as described previously (Wang et al., 2009). qPCR was conducted on an ABI7500 real-time PCR machine (Applied Biosystems) using FastStart Universal SYBR Green Master (Roche). Each amplification reaction was performed in a 20 μl qPCR reaction under the following conditions: denaturation at 95 °C for 10 min followed by 40 cycles of treatment at 95 °C for 10 s, 59 °C for 30 s, and 72 °C for 35 s. Ribosomal protein S4 (Bmrpl: GenBank accession no. NM_001043792) was used as a control gene. The data were normalized by determination of the amount of Bmrpl in each sample to eliminate variations in mRNA and cDNA quality and quantity. The transcript abundance value of each individual was the mean of three replicates. Primers were designed using Primer express 3.0 according to the manufacturer's protocol. The primers used are:

Kr-h1 (GenBank accession no. NM_001177861),
5′-CACTTCGCATCCAAATCATC-3′
and 5′-GATCGTGCGTGTGCTGTAAG-3′;
BR-Z1 (GenBank accession no. NM_001111334),
5′-CTTCAACCCGTCTAACTCCTACAACT-3′
and 5′-GGTCGCATCTGTAATCTTCTTGG-3′;
Bmrpl, 5′-GCCTCATAAAGGTGATGGGAAAG-3′
and 5′-TGATAAGTGGGTCTGGGTAGCG-3′.

3. Results

3.1. Developmental expression of Kr-h1 and BR-Z1 in the wing disc of the normal tetramolter

The transcript levels of Kr-h1 and BR-Z1 were determined in the wing disc of 4th and 5th instar of the normal tetramolter using qPCR. The transcript level of Kr-h1 was high at 12 h of the fourth instar, and gradually increased thereafter. It peaked at 72 h and then decreased when the larvae molt for the fourth time (Fig. 1A). While the transcript level of BR-Z1 was very low in the fourth larval instar (Fig. 1B).

Kr-h1 RNAs decreased shortly after ecdysis to the fifth larval instar and declined to much lower levels at 36–72 h, and then