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Expression of cuticular protein genes, *BmorCPG11* and *BMWCP5* is differently regulated at the pre-pupal stage in wing discs of *Bombyx mori*

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ABSTRACT

Through BLAST search of the genomic database of *Bombyx mori*, we found a clone, *BmorCPG11*, which has only putative BR-C binding sites in the 2 kb upstream region. Both *BmorCPG11* and *BR-Z2* were expressed in the cephalic region of the epidermis, differently from BR-Z1 and BR-Z4. *BR-Z2* transcripts increased by the addition of 20-hydroxyl-ecdysone (20E), which was slightly inhibited by cycloheximide. *BmorCPG11* expression was also induced by the addition of 20E, which was inhibited by cycloheximide. Both *BmorCPG11* and *BR-Z2* were induced by the 20E pulse treatment, but they were inhibited by the addition of cycloheximide. Both genes showed similar expression pattern in wing discs during the developmental stage and *in vitro* ecdysone responsiveness, and both showed relatedness. The result of a reporter assay demonstrated the strong relatedness of *BmorCPG11* and BR-Z2. The expression profiles of *BmorCPG11* and *BR-Z2* are different from those of *BMWCP5* and *βFTZ-F1*. The present findings showed different regulation of cuticular protein genes by ecdysone-responsive transcription factors at the pre-pupal stage of wing discs of *B. mori*.

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1. Introduction

We observed three different expression patterns of cuticular protein genes in the fifth larval instar in wing discs of *Bombyx mori* (Futahashi et al., 2008). These expression patterns correspond with ecdysone responsiveness (Wang et al., 2009b, 2010). Expression of *BMWCP2* (now classified as *BmorCPR99)* and *BMWCP5* (now classified as *BmorCPR92*) was induced by an ecdysone pulse through a transcription factor, βFTZ-F1, which bound to their upstream region and increased their promoter activity (Nita et al., 2009; Wang et al., 2009b). *BMWCP10* (now classified as *BmorCPR21*) has an EcRE upstream of its transcription start site, and the binding of EcR and activation of its promoter was demonstrated (Wang et al., 2010). Thus, the expression of the cuticular protein genes of *B. mori* was regulated by ecdysone-responsive transcription factors.

Broad complex (BR-C) functioned together with βFTZ-F1 (Wang et al., 2009b) or EcR (Wang et al., 2010) to enhance the promoter activity of *BMWCP5* (Wang et al., 2009b) or *BMWCP10* (Wang et al., 2010) respectively. Therefore, BR-C is suggested to function together with other ecdysone-responsive transcription factors and induces the expression of pupal specific genes (Wang et al., 2009b). BR-C is required for the pupal expression of cuticular protein genes (Zhou and Riddiford, 2002; Bayer et al., 2003; Uhlirova et al., 2003). However, the BR-C of insects consists of four isoforms that are expressed differently in different space and time (Emery et al., 1994; Mugat et al., 2000; Ijiro et al., 2004; Nishita and Takiya, 2004; Reza et al., 2004; Nishita and Takiya, 2006); the mechanism of such expression has not been clarified.

The recent determination of the entire genome sequence of insects has yielded many findings. Regarding cuticular protein genes, over 200 cuticular protein genes have been identified in *Anopheles gambiae* (Cornman et al., 2008) and *Bombyx mori* (Futahashi et al., 2008), and several distinct families of cuticular proteins have been recognized (Willis, 2010). Among these, three types of cuticular protein genes that have an R&R (Rebers and Riddiford, 1988) consensus sequence have been identified, and the R&R consensus sequence has been reported to bind with chitin (Rebers and Willis, 2001; Togawa et al., 2004). BMWCP2 and BMWCP5 have R&R consensus sequence and belong to hard cuticular protein, RR2, while BMWCP10 has R&R consensus sequence and belongs to soft cuticular protein, RR1. Other types of cuticular proteins that do not have an R&R consensus sequence have been reported not to bind with chitin, as observed in the case of CPF and CPFL (Togawa et al., 2007). Whether they contribute to the epi or procuticle regions remains to be determined.

The determination of insect genomic sequences also brought about the comprehensive analysis of cuticular-protein gene expression (Togawa et al., 2008) and the analysis of its regulation by transcription factors (Wang et al., 2009a, 2009b, 2010). Cuticular protein genes have different expression profiles (Togawa et al., 2008) and regulatory systems by ecdysone-responsive transcription factors (Wang et al.,

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2009b, 2010). From this, insect cuticular protein genes are suggested to be suitable material for clarifying the regulatory mechanism of ecdysone-responsive transcription factors.

The insect cuticle is made up of two cuticular layers, the epicuticle and the procuticle. The latter can be divided into the regions secreted before ecdysis (exo-cuticle) and that secreted later (endo-cuticle) (Wigglesworth, 1972). The exo- and endo-cuticle are thick; they are constructed by chitin and cuticle proteins with R&R residues (Rebers and Riddiford, 1988), and other types of cuticular protein have been suggested to construct or fill the space in the three cuticle layers. However, the sequence of the production of cuticular proteins is unclear. In the present study, we showed two cuticular protein genes that were expressed at the pre-pupal stage but have different expression profiles. The expression pattern of *BmorCPG11* resembled BR-Z2, and its promoter activity was regulated by BR-Z2, which is different from that of *BMWCP5*, which is regulated by βFTZ-F1. The present findings would help understand the regulatory mechanism of cuticular protein genes by ecdysone-responsive transcription factors and cuticle construction by cuticular proteins.

2. Materials and methods

2.1. Experimental animals and sample preparation

The larvae, hybrids of the N124 and C124 strains of *B. mori*, were reared at 25 °C. Wandering occurred on day 6 of the 5th larval instar, pupation occurred 3 days thereafter, and adults eclosed 10 days after pupation. The day of the fourth ecdysis was designated as V0, the beginning of wandering, as W0, and the day of pupation, as P0. The time at three days after the beginning of wandering was designated as W3. The W3 stage was divided into three different stages, W3 early (W3E), W3 mid (W3M), and W3 late (W3L). The W3 stages are determined on the time and visible shortening of the length of the leg.

Epidermis, wing discs and silk glands were prepared by carefully removing fat body, muscle, and trachea under a microscope. RNA was extracted and reverse-transcribed to cDNA for use in RT-PCR and qRT-PCR. The same sets of cDNA were used for the qRT-PCR experiment of different genes.

2.2. BLAST search of genomic sequences of cuticular protein genes

The cDNA sequences of *BmorCPG11* were used for BLAST search analysis. BLAST search was operated using the genomic database of *B. mori* (http://kaikoblast.dna.affrc.go.jp/). The binding sites of BR-C were identified through the sequences referring to previous studies (von Kalm et al., 1994) and a website (http://www.genomatix.de/en/index.htmL).

2.3. Wing disc culture

Wing discs of larvae at the V4 and W2 stages were prepared for the *in vitro* culture. Wing discs were cultured according to the method of Kawasaki (1989) at 25 °C in Grace's medium (Invitrogen). 20-Hydroxyl-ecdysone (20E) and cycloheximide were applied to the culture medium at a concentration of 2 μg/mL and 50 μg/mL, respectively. The concentration of 50 μg/mL cycloheximide inhibits protein synthesis in our culture system. V4 wing discs were cultured in a medium containing 20E with or without cycloheximide. Cycloheximide was added from the start of culture. For ecdysone pulse treatment, wing discs of the W2 stage were incubated 12 h in the medium containing 20E and then transferred to a hormone-free medium. Cycloheximide was added at the time when transferred to hormone free medium.

2.4. Plasmid construction

All nucleotide positions of the *BmorCPG11* gene given below are numbered with respect to the putative transcription start site as +1(http://www.fruitfly.org/seq_tools/promoter.htmL). The promoter region spanning from -1951 to +46 bp was amplified with a forward primer (5′-CCCGGTACCAGCATTATCCCTTTGTCGTTATTTC-3′) and a *BmorCPG11* reverse primer (5′-CCCGCTAGCCTTTCGTCGTTTAGCGAATGTTAAG-3′) using *B. mori* P50 strain genomic DNA as the template. The amplified DNA fragment was digested with *Kpn I* and *Nhe I* and then ligated into the *Kpn I*/*Nhe I* sites of the luciferase reporter plasmid pGL3/basic to generate *CPG11-1951*. The deletion construct was obtained by PCR using the forward primer *CPG11-1581*: 5′-CCCGGTACCATTCGTATACAATTTTAGGCTCCTTC-3′ combined with the *BmorCPG11* reverse primer. The restriction enzyme sites are underlined. The PCR products were digested with *Kpn I* and *Nhe I* and then subcloned into a pGL3-basic vector to generate *CPG11-1581*. The *Renilla* luciferase vector, *PhRG-hsp*, was made by ligation of *Kpn I* and the *Hind III* fragment in *hsp-LacZ* (Tomita et al., 2001) into *PhRG-B* (Promega). *Renilla* luciferase activity was used to normalize the data.

2.5. Quantitative reverse transcription-PCR

Quantitative reverse transcription-PCR was performed on an ABI7500 real-time PCR machine (Applied Biosystems) using Fast Start Universal SYBR Green Master (Roche) according to the manufacturer's protocol. The data were normalized in each sample using ribosomal protein S4 as an internal standard (rpl), which is expressed at the same level consistently in all the organs examined (data not shown), to eliminate variations in mRNA and cDNA quality and quantity. The conditions for the qRT-PCR amplification reaction were as follows: denaturation at 95 °C for 10 min followed by 40 cycles of treatment at 95 °C for 10 s and at 60 °C for 1 min. The transcript abundance value of each individual was obtained as the mean of three replicates.

Seven pairs of primers were designated by software Primer3 (http://frodo.wi.mit.edu/). The specificity of the primers was confirmed using NCBI BLAST (BLASTN) algorithms. The primers used were

ATTAATGAGATCGTCCTTACTCCTG-3′ and 5′-CTCGCTTGTCTTGTTTCTTAGGTTC-3′ for *BmorCPG11*;
5′-CTTCAACCCGTCTAACTCCTACAACT-3′ and 5′-GCACAGTACCTTCCCGCATAGT-3′ for *BR-C Z2* (*BR-Z2*);
5′-GATTCACAATCCACCGTATCACC-3′ and 5′-CCATCATGCGTTACCAAGTACG-3′ for *rpl*;
5′-AAATTTATTCTCTCAAGTGGATTGG-3′ and 5′-GTAAATGATCCAAAACCAGCATAAC-3′ for *βFTZ-F1*;
5′-ATTATACCCCAGTATTGAGTCATGC-3′ and 5′-GCGAAGCATGTATATACGAAGAATG-3′ for *BMWCP5*;
5′-CTTCAACCCGTCTAACTCCTACAACT-3′ and 5′-GGTCGCATCTGTAATCTTCTTGG-3′ for *BR-C Z1* (*BR-Z1*); and
5′-GACGGAATGGCGAGCACATC-3′ and 5′-AGCGGCTGGAGGTGTTGCTG-3′ for *BR-C Z4* (*BR-Z4*).

The GenBank accession numbers of these genes are as follows: *BmorCPG11*: BR000432, *BMWCP5*: AB047483, *βFTZ-F1*: D10953, *BmBR-C Z1*: AB113084-5, *BmBR-C Z2*: AB113086-7, *BmBR-C Z4*: AB113088, and *rpl*: nm_ 001043792.

2.6. Site-directed mutagenesis

A single mutation (-1279/-1275), *BR-Z2* mut, was performed with the Quick-ChangeTM Site-Directed Mutagenesis Kit (Stratagene) using the *BmorCPG11-1581* plasmid as the template. *Pfu* DNA polymerase was used to synthesize the mutated promoter followed

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