Expression patterns of hypothetical cuticular protein genes in *Bombyx mori*

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Abstract

The study was conducted to clarify the expression patterns of *Bombyx mori* hypothetical cuticular protein genes, CPH1, CPH2 and CPH30 expressed in wing discs at pre-pupal stage. The expression of CPH1 and CPH2 was observed from the W3 early stage and peaked at pupation when the ecdysteroid titer declined. Whereas CPH30 transcripts increased gradually after the beginning of wandering and peaked at W3 late stage and then decreased rapidly from P0 when the ecdysteroid titer decreasing. CPH30 was induced by the edysone pulse, and its expression peaked 18h after transfer to a hormone free medium. CPH1 and CPH2 were also induced by the edysone pulse, and their expression peaked 24 h after transfer to a hormone free medium. The peak of CPH1 and CPH2 expression was observed later than that of CPH30 both in *vivo* and *in vitro*. Genes, CPH1, CPH2 and CPH30 transcripts were not observed after the 20E pulse treatment in the presence of cycloheximide. Transcripts of CPH30 increased by the addition of 20E in V4 wing discs. On contrary, transcripts of CPH1 and CPH2 were not observed after the addition of 20E in V4 wing discs which is different from the case of CPH30. The expression profiles of CPH1 and CPH2 are different from those of CPH30. The present findings suggested different regulation of hypothetical cuticular protein genes at the pre-pupal stage in wing discs of *Bombyx mori*.

Keywords: Hypothetical cuticular protein, edysone, wing disc, gene expression, *Bombyx mori*.

1. Introduction

Ecdysone signaling functions through the edysone responsive transcription factors, and several transcription factors function each other, and they activate the target genes [1]. Through edysone signaling insect regulates their development; molting, metamorphosis [2]. Thus, according to the edysone fluctuation in the hemolymph, different transcription factors are produced, and bring about insect metamorphosis. In the final larval instar of *Bombyx* early gene E74B was expressed from the early stage, followed by the expression of BR-C isoforms, BHR3, BHR4, E75A and βFTZ-F1 [3]. Recent genomic research revealed that over two hundred cuticle protein genes were identified in *B. mori* [4], which may enable to construct the different types of cuticle in different stage and different space. DHR38 [5,6] and BR-C [7] were reported about their relation to cuticle protein gene expression. Thus, the stage specific expression of the cuticle protein genes was determined by edysone-responsive transcription factors. βFTZ-F1 has been suggested to be a regulator responsible for the stage-specific expression of cuticle protein genes during the prepupal stage [8,9]. After being expressed, βFTZ-F1 has also been found to positively regulate the pupal cuticle protein gene, Edg84A, during the mid- to late prepupal period [10]. βFTZ-F1 was required for normal larval cuticle production (Yamada et al., 2000) [11]. Early responsive gene, Broad-Complex, has been reported, and some target genes of BR-C [12,13,14] and stage and tissue specific isoforms of BR-C [15,16] have been identified in Drosophila melanogaster. BR-C also has been reported to be critical for specifying pupal program and for suppressing both larval and adult program in *Manduca sexta* [2] and *Tribolium castaneum* [17, 18]. However, the regulatory mechanism by which BR-C controls the expression of cuticle protein genes at a molecular level is not well understood. Several cuticle protein genes with different developmental profiles and hormonal responses in wing disc of *B. mori* were reported. As for example BMWCp2, 3, 7, 8, and 9 were observed only at the stage of pupal wing formation. BMWCp1, 4, 5, and 6 are expressed twice, during pupation and the mid-pupal stage [19]. All of them were expressed after an edysone pulse *in vivo* and induced by the removal of 20-hydroxyedysone after exposure to it *in vitro* [20, 21].
In the present study we selected three hypothetical cuticular protein genes for the examination of the developmental expression and their responsiveness to ecdysone and ecdysone pulses, since we found the relatedness of expression to the transcription factor BR-Z2 and βFTZ-F1. These results suggest that CPH1 and CPH2 could be regulated by βFTZ-F1 and CPH30 could be regulated by BR-Z2 in a stage specific manner.

2. Materials and methods

2.1 Experimental animals

A hybrid strain of B. mori was reared at 25 ºC in a 12 h light: 12 h dark photoperiod. Larvae began wandering on six day of the fifth larval instar, pupation occurred 3 days thereafter, and adults enclosed 10 days after pupation. The periods (in days) corresponding to the developmental stages of the fourth to fifth larval ecdysis, wandering, pupation, and eclosion were designated as V0, W0, P0, and A0, respectively. The three days before pupation were designed as W1-W3. The W3 stage was divided into three different sub-stages, W3 early (W3E), W3 mid (W3M), and W3 late (W3L) (Fig: 1). The W3 sub-stages were determined on the time and visible shortening of the length of the leg.

Fig 1: Life cycle of Bombyx mori showing developmental stages and W3 stage was divided into three different sub-stages, W3 early (W3E), W3 mid (W3M), and W3 late (W3L). The W3 sub-stages were determined on the basis of time and visible shortening of the length of the leg.

2.2 In vitro culture of wing discs

Wing discs of larvae at the V4 and W2 stages were prepared for the In vitro culture. For wing disc preparation, the fat body and trachea were carefully removed under a microscope. The culture was carried out according to a previous report [12] at 25 ºC under sterile conditions. In vitro induction was conducted at various times following administration of 2 µg/ml 20E to V4 wing discs and after cessation of a 12 h pulse of 2 µg/ml 20E to discs from W2. The necessity of protein synthesis for induction was tested in the cultured discs by administration of 50 µg/ml cycloheximide from the start of culture (V4) or at the time of 20E removal (W2).

2.3 RNA sample preparation and first-strand cDNA synthesis

To determine the expression levels of the cuticular protein genes, total RNA were extracted at distinct stages from wing discs with an isogen reagent (Nippon Gene) and quantified by spectrophotometry at 260 nm. One microgram of total RNA was used to synthesize first-strand cDNA using ReverTra Ace (Toyobo, Japan) according to the manufacturer’s instructions.

2.4 Real-Time PCR

Real-Time PCR was conducted on an ABI7500 real-time PCR machine (Applied Biosystems) using the FastStart Universal SYBR Green Master (Roche) according to the manufacturer’s protocol. Each amplification reaction was performed in a 25 µl qRT-PCR reaction under the following conditions: denaturation at 95 ºC for 10 min followed by 40 cycles of treatment at 95 ºC for 10 sec and at 60 ºC for 1 min. 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. Each pair of primers was designed using Primer3 software (http://frodo.wi.mit.edu/). The specific primers for each gene were listed below.

5/-CGTGAGAGAAACAACTATCG-3' and 5'-GGTTCGACATCGAGAAC-3' for CPH1;
5'-CCGAAGTGCACGAGAAC-3' and 5'-CGAGTCCGTGGCTTAGT-3' for CPH2;
5'-TCATGCAGTCTTTGAGTTATCCTC-3' and 5'-CCTTAGACGTTGCTACTTCC-3' for CPH30;
5'-GATTCACAATCCACCGTATCACC-3' and 5'-CCATCATGCGTTACAGAGTACC-3' for rpl.

The accession numbers of the used genes were as follows: CPH1: BR000451, CPH2: BR000452, CPH30: BR000495 and rpl: NM_001043792.

3. Results

Expression of CPH1 and CPH2 was observed from the W3E stage and peaked at pupation when the ecdysteroid titer decreased; it then suddenly decreased after pupation (Fig: 2A, 2B).
Fig 2: Developmental expression of hypothetical cuticular protein genes of CPH1 (A), CPH2 (B), CPH30 (C). RNA was extracted from wing discs and reverse-transcribed to cDNA for use in Real-Time PCR. Values represent the mean ± S.E.M. of results from three independent experiments.

Fig 3: Expression by ecdysone pulse treatment of CPH1 (A), CPH2 (B) and CPH30. RNA was extracted from wing discs and reverse-transcribed to cDNA for use in Real-Time PCR. Level of mRNA after ecdysone pulse treatment. Wing discs of the W2 stage were incubated 12 h in a medium containing 2 mg/ml 20E and then transferred to a hormone-free medium with (open triangle) or without (closed triangle) cycloheximide (50 mg/ml) for the indicated time. Values represent the mean ± S.E.M. of results from three independent experiments. Asterisks indicate p<0.05 significance by the student’s t-test.
CPH1 and CPH2 was also induced by the ecdysone pulse, and its expression peaked 24 h after transfer to a hormone free medium (Fig: 3A, 3B). The peak of CPH1 and CPH2 expression was observed later than that of CPH30 both in vivo and in vitro. The CPH1 and CPH2 transcript was not observed after the 20E pulse treatment in the presence of cycloheximide. It was not observed either after the addition of 20E in V4 wing discs, which is different from the case of CPH30. Cuticular protein gene, CPH1 and CPH2 showed a similar expression pattern with βFTZ-F1.

Transcripts of βFTZ-F1 was increased from the W3E stage, peaked at the P0 stage, increased 6 h after 20E removal, and peaked at 24 h In vitro [20]. The timing of peak expression was similar to that of CPH1 and CPH2. CPH1 and CPH2 were not expressed by the addition of 20E in the V4-stage wing discs or by the ecdysone pulse treatment in the presence of cycloheximide. This CPH1 and CPH2 responsiveness is same to that of βFTZ-F1 shown in previous reports [20, 21]. After the beginning of wandering CPH30 transcripts increased gradually and peaked at W3 late stage (Fig: 2C), which is similar to the expression of BR-Z2 [25].

A similar expression peak was induced by the ecdysone pulse in vitro (Fig: 3C). CPH30 transcripts increased 6 h after removal of 20E, peaked at 18 h, and then decreased. An increase of CPH30 transcripts was not observed after 20E pulse treatment in the presence of cycloheximide (Fig: 3C), which indicates that the induction of CPH30 requires 20E-inducible factors. To determine whether the induction of CPH30 mRNA was mediated directly or indirectly by 20E, wing discs were cultured with 20E in the presence or absence of the protein translation inhibitor cycloheximide, and CPH30 mRNA was assessed by real-time PCR. CPH30 transcripts increased after 20E addition. Induction was not observed in the presence of cycloheximide. Thus, CPH30 gene was upregulated by the 20E addition (Fig: 4).

The expression pattern of CPH30 resembled that of BR-Z2. Their transcripts increased from stage W0 and continued to increase until stage W3L peak. The ecdysteroid titer in the hemolymph started to increase at around stage W0. The expression pattern of CPH30 gene resembled that of BR-Z2 [20], which may be regulated by BR-Z2. The developmental expression and ecdysone-responsiveness of CPH30, CPH1 and CPH2 resembled BR-Z2 and βFTZ-F1 respectively. The expression of CPH30, CPH1 and CPH2 was compared schematically in Fig: 5. As observed, CPH30 transcripts appeared, increased and peaked earlier than those of CPH1 and CPH2.

**Fig 4:** Expression after of 20E addition of CPH30. RNA was extracted from wing discs and reverse-transcribed to cDNA for use in Real-Time PCR. Level of mRNA of the ecdysone treatment. V4 wing discs were incubated for the indicated time in a medium containing 2 μg/ml 20E with (open triangle) or without (closed triangle) cycloheximide (50 μg/ml). Values represent the mean ± S.E.M. of results from three independent experiments. Asterisks indicates p<0.05 significance by the student’s t-test.

### 4. Discussion
In the present study, we analyzed the expression and regulation of CPH30 and CPH1 and CPH2 in wing disc using real-time PCR assay system. In Bombyx wing disc some of cuticular protein genes were expressed in the early fifth larval stage, while most cuticular protein genes were expressed at pre-pupal stage [4]. Among the cuticular protein genes that are expressed at the pre-pupal stage, BmorCPR23 and BmorCPR34 were induced by E74A and BHR4 respectively [23]. The present paper reported cuticular protein genes that were expressed at the pre-pupal stage, but were expressed differently from BmorCPR23 and BmorCPR34. The expression profile of CPH30 was similar to that of BR-Z2 [23] and the induce CPH30 by BR-Z2 and CPH1 and CPH2 by βFTZ-F1 respectively.

BR-C isoforms expression in the epidermis were reported in B. mori [26, 27, 28]. BR-C isoforms bind to different DNA sequences, which would bring about the different target genes including cuticular protein genes. BR-Z2 transcription was induced by 20E in the existence of cycloheximide suggested direct induction by 20E. The induction of BR-Z2 was also observed by ecdysone pulse treatment, which was inhibited by cycloheximide addition. Thus, BR-Z2 is induced by ecdysone directly and pulse treatment through other factors. Transcripts
of BR-Z2 showed peak at W3L stage, when the hemolymph expression profile of CPH1 and CPH2 was similar to that of βFTZ-F1[23]. From this, it is suggested that the possibility to ecdysoid titer decreased after its peak [25]. CPH30 expression showed similar profile to that of BR-Z2, except that CPH30 was not induced by 20E in the medium containing cycloheximide. In correspondence with expression of BR-Z2, CPH30 expression peaked at W3L stage. Together with the result of the expression in the wing disc, the strong relatedness of CPH30 and BR-Z2 is suggested. Thus, the expression of cuticular protein gene CPH30 indicates to be regulated by BR-Z2 transcription factor.

βFTZ-F1 transcription was neither induced by 20E nor in the existence of cycloheximide [21]. CPH1 and CPH2 were also not induced by 20E in V4 wing disc cultured system. The induction of βFTZ-F1 was observed by ecdysone pulse treatment, which was inhibited by the addition of cycloheximide [23]. This result of pulse treatment indicated the existence of other factors affecting the expression of βFTZ-F1. Thus, βFTZ-F1 is induced by pulse treatment through other factors.

βFTZ-F1 is inducible by ecdysone pulse [23]. Transcripts of βFTZ-F1 showed peak at P0 stage, when the hemolymph ecdysoid titer decreased [23]. CPH1 and CPH2 expression showed similar profile to that of βFTZ-F1. βFTZ-F1 was suggested to function in the induction of CPH1 and CPH2, since βFTZ-F1 was inducible by decline in the ecdysoid titer and was induced by a 6-h exposure to 20E followed by 6 h in the hormone-free medium [24]. βFTZ-F1 has been reported to bind to the upstream of a target gene [10, 29] and the transcriptional activity by the βFTZ-F1 binding on the cuticle protein gene has recently reported with BMWCP2 [9]. From these reports and the present findings, it is suggested that βFTZ-F1 functions as the primary factor in response to the ecdysone pulse and binds with or recruits other factors, resulting in the regulation of the stage specific expression of target genes, such as CPH1 and CPH2. In correspondence with expression of βFTZ-F1, CPH1 and CPH2 expression peaked at P0 stage. Together with the result of the expression in the wing disc, the strong relatedness of CPH1 and CPH2 and βFTZ-F1 is suggested. Thus, the expression of cuticular protein gene CPH1 and CPH2 indicates to be regulated by βFTZ-F1 transcription factor.

Fig 5: Schematic representation of Ecdysteroid titer [30], and cuticular protein genes expressed in wing discs of B. mori in the late fifth larval instar.

5. Conclusion
The developmental expression and hormonal responsiveness of cuticular protein gene CPH30 were found strong relatedness with transcription factor BR-Z2 and cuticular protein genes CPH1 and CPH2 with transcription factor βFTZ-F1. It may be suggested that the expression of CPH1 and CPH2, and CPH30 genes might be regulated by ecdysone responsive transcription factor βFTZ-F1 and BR-Z2 respectively but through mutagenesis and promoter assay analysis using gene gun and a transient reporter assay system the hypothetical cuticular protein genes regulation will be elucidated more details in future.

6. References


