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Developmental expression and hormonal responsiveness of cuticular protein genes at the prepupal stage in wing discs of *Bombyx mori*

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Abstract
The present study was conducted to clarify the developmental expression and hormonal responsiveness of cuticular protein genes of *Bombyx mori* expressed in wing discs at prepupal stage. The cuticular protein genes that showed the same expression timing with each transcription factor were selected and examined their ecdysone responsiveness. Hence, two cuticular protein genes *CPH33* and *CPR93* were considered for the study. *CPH33* transcripts rapidly increased from W2, remained at a high level until the W3M stage, and then decreased from the W3L stage. *CPR93* transcripts showed a different expression profile. They increased from the W3M stage and peaked at the W3L stage, then decreasing from P0. *CPH33* and *CPR93* were induced by the ecdysone addition and by the ecdysone pulse also. The developmental expression and ecdysone responsiveness of *CPH33* and *CPR93* resembled *BHR4* and *E74A* respectively. Thus, ecdysone-responsive transcription factors regulated different cuticular protein genes and determined their expression timing.

Keywords: *CPH33, CPR93*, transcription factors, ecdysone, wing disc, *Bombyx mori.*

1. Introduction
Insect steroid hormone ecdysone participates in ecdysis by activating the ecydsone-signaling cascade and inducing ecdysis with a high concentration [1]. Ecdysone signaling functions through ecdysone-responsive transcription factors, and several transcription factors interact each other and activate the target genes [2]. Recently, based on the whole genome sequence, the comprehensive identification of cuticular protein genes has been attempted in *Apis mellifera* [3], *Drosophila melanogaster* [4], *Anopheles gambiae* [5], and *Bombyx mori* [6], and over 200 cuticular protein genes were identified in *A. gambiae* and *B. mori*. These cuticular protein genes show different developmental profiles [6, 7] and the involvement of ecdysone-responsive transcription factors have been reported [8, 9, 10].
It was reported that ecdysone-responsive transcription factors determined the expression of cuticular protein genes [8, 9, 10]. βFTZ-F1 increased the promoter activity of the cuticular protein gene, *BMWCP2*, which was expressed around pupation [11] when the ecdysteroid titer decreased after its peak [12]. Moreover, it's bound upstream of the promoter region of *BMWCP2* [11]. BR-C Z2 activated the *BmorCPG11* promoter independently from other ecdysone responsive transcription factors [10]. BR-C isoforms were expressed in a stage- and tissue-specific manner [13, 14]. BR-C has also been reported to be critical for specifying pupal program and for determining the pupal program and suppressing both the larval and adult programs in *D. melanogaster, M. sexta* [15] and *T. castaneum* [16]. Therefore, it is suggested that the regulation of cuticular protein genes by BR-C is restricted in pupal cuticular protein genes.
In addition to above transcription factors, participation of following factor was identified. BHR3 regulated *BmorCPH5*, and their transcription peak was observed earlier than that of *BMWCP2* [8]. Thus, transcription factors bind to upstream of the target cuticular protein genes and bring about the stage-specific expression of cuticular protein genes in wing discs and epidermis. These studies had been conducted by using a genomic database.
The present study showed two cuticular protein genes that were expressed at the pre-pupal stage but have different expression profiles. The expression pattern of *CPH33* resembled BHR4 which is different from that of *CPR93*. Developmental expression and ecdysone responsiveness data would help to understand the regulatory mechanism of cuticular protein genes by ecdysone-responsive transcription and cuticle construction by cuticular proteins.

2. Materials and Methods

2.1 Insect and developmental stages

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 eclosed 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). The W3 sub-stages were determined on the time and visible shortening of the length of the leg [8].

2.2 *In vitro* culture

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 [17] 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 BLAST search

The cDNA sequences of *CPH33* and *CPR93* were used for BLAST search analysis to obtain their upstream sequences. BLAST search was carried out using the genomic database of *B. mori* (http://kaikoblast.dna.affrc.go.jp/). According to previous studies [8, 18, 19, 20, 21], BHR4-binding site (G/AG/AGTCA) and E74A binding site were searched to the upstream of *CPH33* and *CPR93* respectively.

2.4 cDNA synthesis

To determine the expression levels of the cuticular protein genes and transcription factors, 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 Rever Tra Ace (Toyobo, Japan) according to the manufacturer's instructions.

2.5 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 Real-Time 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′-CTCAGTAAGGCAACATGATCG-3′ and
5′-GTACGGCTTCGTGTGTTCTG-3′ for *CPH33;*
5′-GTGAATACTCCCTCCTGCAACC-3′ and
5′-AATATCACAGATCGAGCAGACTTC-3′ for *CPR93;*
5′ -GTGTTTTCCGTCGCATACAG-3′ and
5′-GGTTGGCGTGTTCAAGGTAG-3′ for *BHR4*;
5′-GCACCACCTATCGAGATAAAGC-3′ and
5′-CTGCCCGTTGTTTTGTAAATG-3′ for *E74A;*
5′-GATTCACAATCCACCGTATCACC-3′ and
5′-CCATCATGCGTTACCAAGTACG- 3′ for *rpl*

The accession numbers of the used genes were as follows**:** *CPH33*: BR000500, *CPR93*: AB047482, *BHR4*: NM_001043550: *E74A:* DQ471939 and *rpl*: NM_001043792.

3. Results

We found cuticular protein gene, *CPH33 and CPR93*, which showed distinctive expression profile from that of other cuticular protein genes in the fifth larval stage of *B. mori*. Transcripts of *CPH33* showed an expression peak at W3M stage. *CPR93* showed peak at W3L stage. Therefore, the expression profiles of *CPH33* were examined and compared them with those of *CPR93* transcripts, which showed a peak slightly later than those of *CPH33. CPH33* transcripts rapidly increased from W2, remained at a high level until the W3M stage, and then decreased from the W3L stage (Fig. 1A). *CPR93* transcripts showed a different expression profile. They increased from the W3M stage and peaked at the W3L stage, then decreasing from P0 (Fig. 1B).

To elucidate the regulation of cuticular protein gene by ecdysone-responsive transcription factors we searched for the ecdysone-responsive transcription factors whose developmental expression resembled that of cuticular proein genes by real-time PCR. The developmental expression of ecdysone-responsive transcription factors was examined in wing discs. We found ecdysone-responsive transcription factors, BHR4 and E74A, which showed distinctive expression profile from those of other ecdysone-responsive transcription factors as described below. *BHR4* transcripts increased after the W2 stage, peaked at the W3E stage and remained at a high level until the W3M stage, and then decreased (Fig. 1C). The transcripts of *E74A* increased from the W3M stage, peaked at W3L, and then decreased rapidly from P0 (Fig. 1D). Thus, the developmental expression of *CPH33* and *CPR93* resembled *BHR4* and *E74A* respectively.

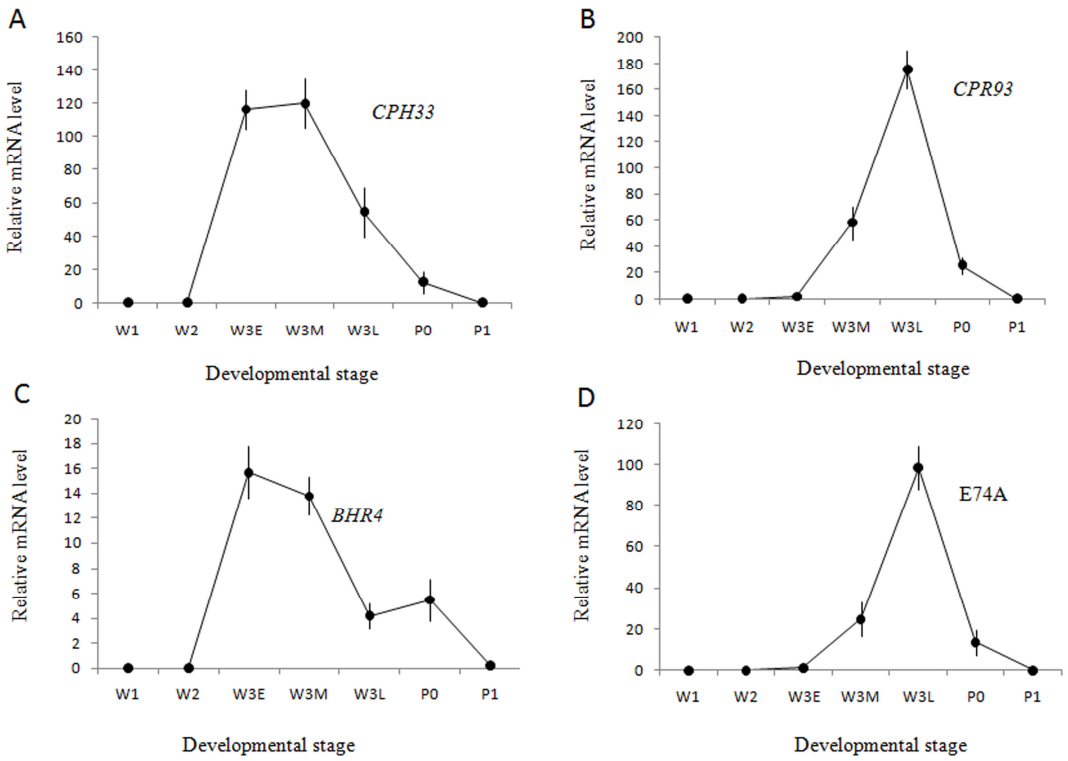

Fig 1: Developmental expression of cuticular protein genes and ecdysone-responsive transcription factors of *CPH33* (A), *CPR93* (B), *BHR4* (C) and *E74A* (D). RNA was extracted from wing discs and reverse-transcribed to cDNA for use in Real-Time PCR. Values represent the mean ± S.E.M. of results from three independent experiments.

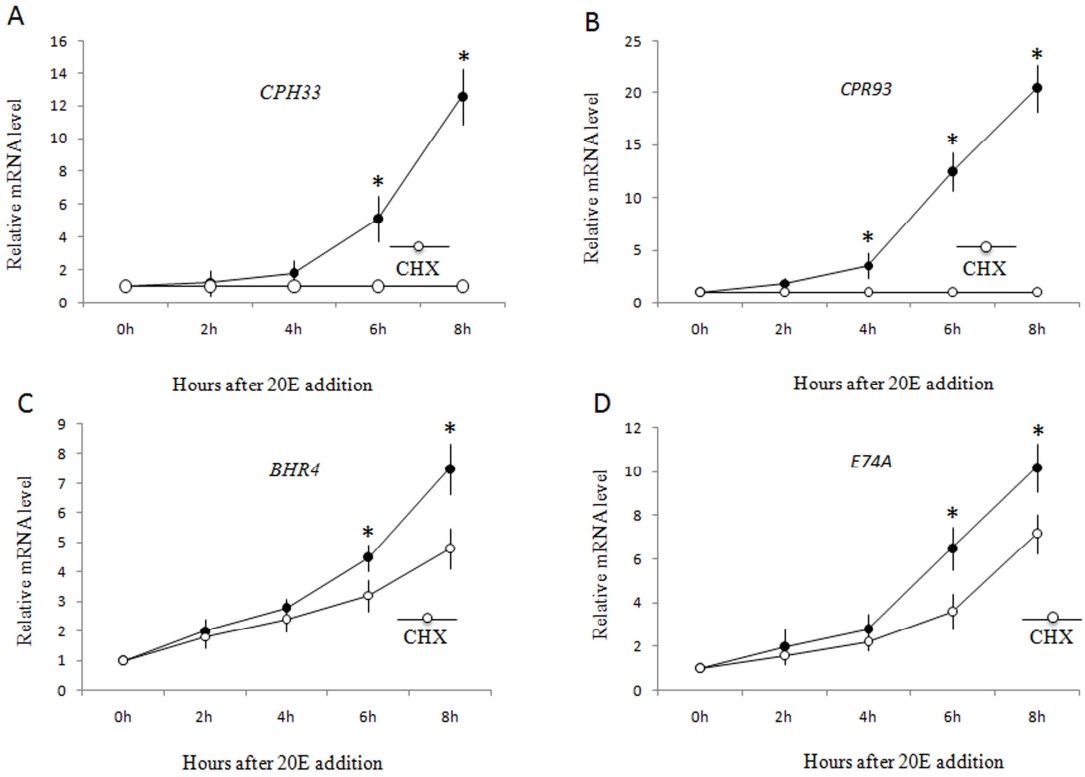

Fig 2: Expression after of 20E addition of *CPH33* (A), *CPR93* (B), *BHR4* (C) and *E74A* (D). RNA was extracted from wing discs and reverse-transcribed to cDNA for use in Real-Time PCR. Level of mRNA of the ecdysone treatment. V4 wing discs were incubated for the indicated time in a medium containing 2 µg/ml 20E with (open circle) or without (closed circle) 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.

Then we investigated whether cuticular protein genes showing different expression timing show different ecdysone responsiveness, which resembled those transcription factors that showed similar expression timing. Two kinds of responsiveness were tested. One is the response to the ecdysone addition; the other is that of the ecdysone removal after its treatment (ecdysone pulse treatment). The transcripts of *CPH33* and *CPR93* increased by the addition of 20E (Fig. 2A, 2B) but did not increase by the addition of cycloheximide to the medium containing 20E.The result suggests the existence of factors that activated the transcription of these cuticular protein genes.*CPH33* transcripts were induced 6 h after 20E removal and peaked at 12 h, while *CPR93* transcripts gradually increased 12 h after hormone removal, then rapidly increased, and peaked at 18 h (Fig. 3A, 3B).

BHR4 and *E74A* transcripts showed expression peaks at W3E and W3L, respectively (Fig. 1C, 1D). We investigated the ecdysone responsiveness of these ecdysone-responsive transcription factors to clarify what determine the expression timing of these transcription factors. Transcripts of *BHR4* and *E74A* increased by the addition of 20E and were slightly inhibited by the addition of cycloheximide in the 20E-containing medium (Fig. 2C, 2D).The results suggest that both ecdysone-responsive transcription factors were induced directly by 20E and indirectly by other factors. *BHR4 and E74A* transcripts were induced by ecdysone pulse, which were not observed by the addition of cycloheximide. *BHR4* and *E74A* transcripts showed expression peak at 12 (Fig. 3C) and 18h (Fig. 3D) after 20E removal, respectively. Thus, different ecdysone-responsive transcription factors showed different ecdysone-responsiveness and the ecdysone responsiveness of *CPH33* and *CPR93* resembled *BHR4* and *E74A* respectively.

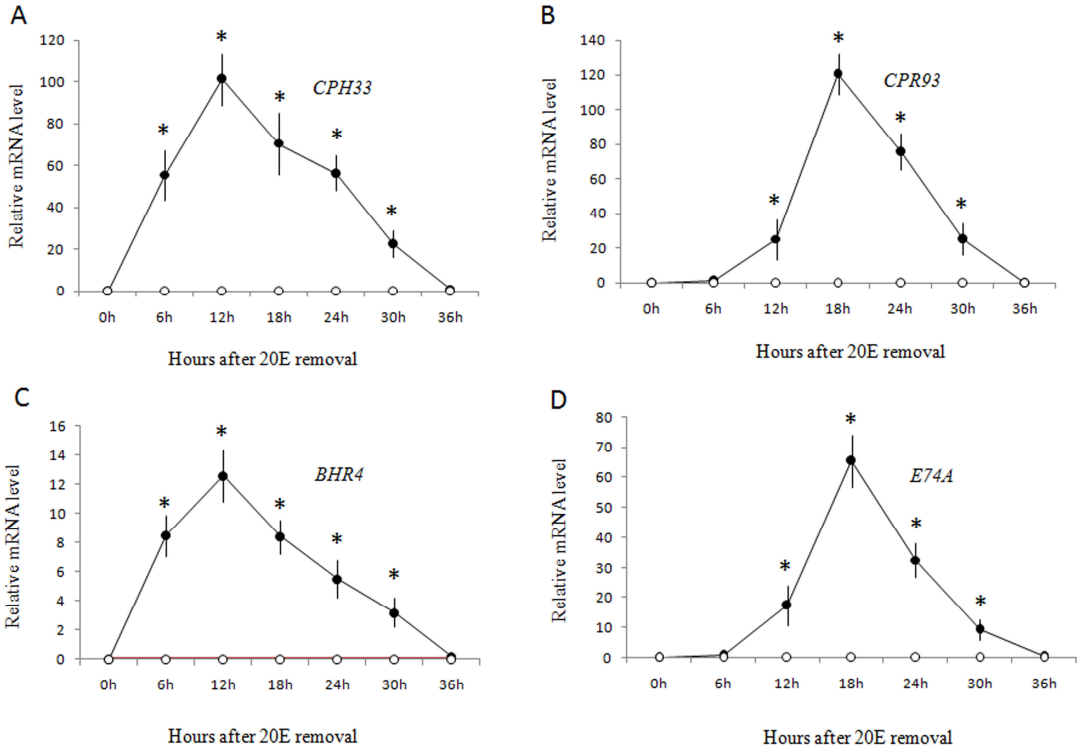

Fig 3: Expression by ecdysone pulse treatment of *CPH33* (A), *CPR93* (B), *BHR4* (C) and *E74A* (D). RNA was extracted from wing discs and reverse-transcribed to cDNA for use in Real-Time PCR. Level of mRNA after ecdysone pulse treatment. Wing discs of the W2 stage were incubated 12 h in a medium containing 2 µg/ml 20E and then transferred to a hormone-free medium with or without cycloheximide (50 µg/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.

4. Discussion

Through the comparison of the ecdysone responsiveness of transcription factors, in the present study, we found that *BHR4* and *E74A* were induced both by 20E addition and the ecdysone pulse, as previously reported in *D. melanogaster* [22], *M. sexta* [23] and *B. mori* [24].

BHR4 induction by ecdysone pulse was rapid, which suggests that *BHR4* could be inhibited by the high concentration of BHR3 as observed in *M. sexta* homolog, *MHR4* [25], but could be induced when the level of *BHR3* becomes low. This result suggests that the factor bringing about the induction by the ecdysone pulse is BHR3 and that the decrease of BHR3 brings about the induction of *BHR4,* and the disappearance of BHR3 as well as the appearance of BHR4 induces *E74A*, as previously reported [2,26]. The present result indicated the possibility that BHR4 induces *E74A*, as previously observed in *Drosophila* DHR3 [26], since they are induced after *BHR4* increases and require factors for their induction.

BHR4 was the first gene that was induced by the ecdysonepulse (Fig. 3C), which suggests that BHR4 is involved in the induction of subsequent ecdysone-responsive transcription factors. The fluctuation of these transcription factors and the ecdysone responsiveness support the previous researches of the BHR3 homolog, DHR3 [2, 20], and *E74A* is inducible by ecdysone [22] and the ecdysone pulse [23], and an expression peak at this stage suggests that it is produced by the interaction with BHR4 [2]. A previous report [2] and the recent results suggested that *BHR4* is induced by the decrease of BHR3 and then induces *E74A* and *βFTZ-F1* [8].

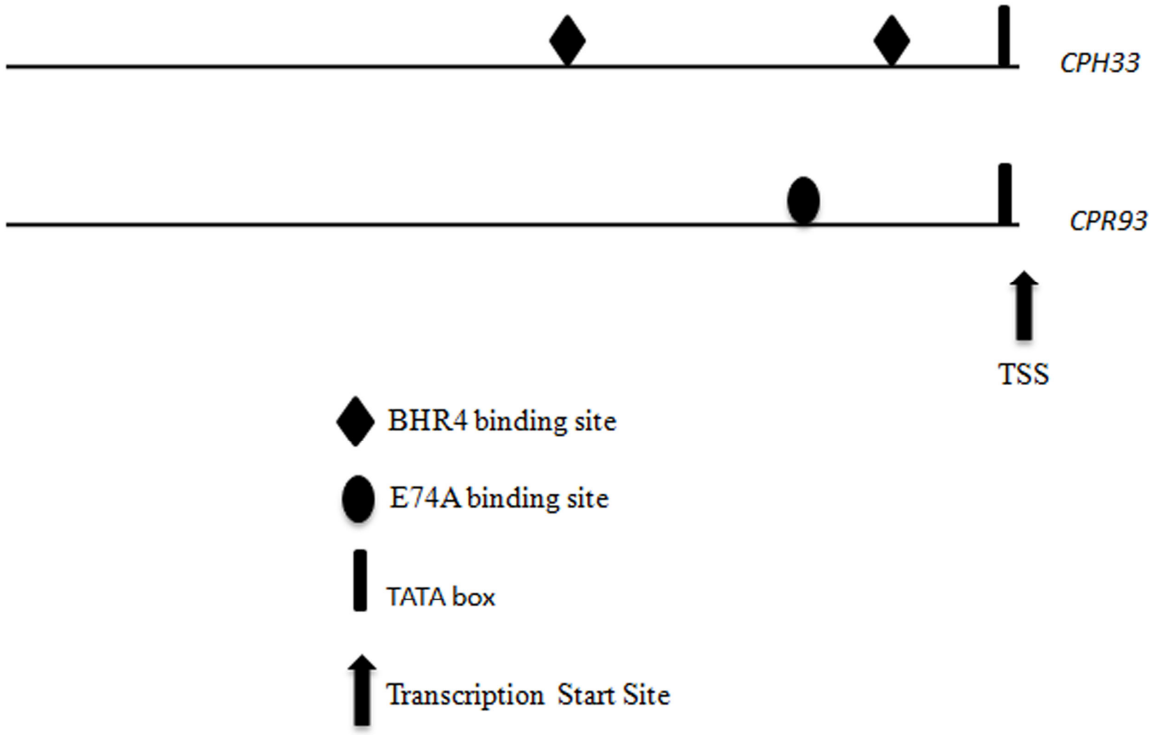

Fig 4: Schematic representation of the putative binding sites of the ecdysone-responsive transcriptional factor located on the upstream of indicated cuticular protein genes. The BHR4 and E74A binding sites are shown. Bars indicate 2kb upstream region from the transcription start site.

Cuticular protein genes expression are regulated by those transcription factors whose developmental expression and the ecdysone responsiveness resembled and those transcription factors binding sites are located in the 2kb upstream sequences of specific cuticular protein genes[8,9,10], proved through genomic information and a transient reporter assay system. In the present study the BHR4 and E74A binding sites are located in the upstream promoter region of *CPH33* and *CPR93* respectively (Fig. 4).

The expression profile of *CPR93* resembled that of *E74A, CPR93* transcripts showed expression peak at the W3L stage, and showed resemblance of ecdysone-responsiveness to that of *E74A*. The expression profile of *CPH33* resembled that of *BHR4* that showed expression peak at W3E and W3M, and ecdysone responsive showed resemblance to that of *BHR4*. Two potential BHR4 binding sites found in the 5/ flanking region of the *CPH33* gene (Fig.4). Whether this gene is the target of BHR4 remains to be elucidated.

5. Conclusion

Recent studies have shown that BR-Z2 [10], and βFTZ-F1 [11] regulate different cuticular protein genes. Thus, these ecdysone-responsive transcription factors are suggested to regulate their target genes, and the series of their expression would bring about insect metamorphosis. Based on these studies, we are trying to clarify the regulatory mechanism of cuticular protein genes and the present findings added new evidences to previous results. The present findings suggest that cuticular protein genes are expressed successively according to their regulating transcription factors, resulting in a continuous series of cuticular protein production, which enable to construct the epi-, exo-, and endo-cuticle. These different types of cuticle proteins are combined and form the pupal cuticle, and the present findings suggest that ecdysone-responsive transcription factors determine the location where cuticular protein genes are expressed.

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