

Transcriptional Profiling Shows that BHR4 and E74A Are the Regulators of *CPH33* and *CPH34* Gene Expression

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Abstract. Our endeavor is to identify with the basic system that regulates sequentially expressed cuticular protein genes around pupation. The present study was conducted to clarify the regulatory mechanism of a cuticular protein gene of *Bombyx mori*, *BmorCPH33*, whose expression in wing discs was atypical from others and peaked at wandering stage day 3 (W3) early to W3 late stage when ecdysteroid began to decrease before pupation. We compared the expression profiles of *BmorCPH33* and *BmorCPH34* to clarify their regulatory mechanism. Developmental profile of *BmorCPH33* resembled *BHR4*, therefore we compared the expression profile of this transcription factor with that of *BmorCPH33*. *BHR4* transcripts increased by the addition of 20E. *BmorCPH33* expression was also induced by the addition of 20E, which was inhibited by cycloheximide. Both *BHR4* and *BmorCPH33* transcripts were induced after 6h 20E removal after exposure to 20E, but they were inhibited by the addition of cycloheximide. Thus, the results showed relatedness of *BmorCPH33* and *BHR4*. Through this culture system it is suggested that *BHR4* is a regulator of insect metamorphosis. The expression profiles of *BmorCPH34* resembled those of *E74A*, therefore we compared the expression profiles of this transcription factor with *BmorCPH34*. *BmorCPH34*, *E74A* transcripts were up-regulated by the 20E pulse treatment. The results showed the relatedness of *BmorCPH34* and *E74A*. 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*.

Keywords: *BHR4*; cuticular protein; ecdysone; ecdysone pulse; wing disc; *Bombyx mori*

1 Introduction

Expressions of most cuticular protein genes were induced by an ecdysteroid pulse; the expression required the existence and removal of 20E [1-3]. This condition is similar to that of the stage around the ecdysis. Few examples were reported to be up-regulated by 20E [4-6]. They were induced by the addition of ecdysone *in vitro*. Thus, cuticular protein genes, which have different developmental profiles, were induced by different types of ecdysone signal, and these things were described in a recent review [7]. The determination of insect genomic sequences also brought about the comprehensive analysis of cuticular-protein gene expression and the analysis of its regulation by transcription factors [8-17]. Cuticular protein genes have different expression profiles [8-17] and regulatory systems by ecdysone-responsive transcription factors [9-19]. From this, insect cuticular protein genes are suggested to be a suitable material for clarifying the regulatory mechanism of ecdysone-responsive transcription factors.

Expression of *CPR55*, *BMWCP2* and *BMWCP5* was induced by an ecdysone pulse through a transcription factor, β FTZ-F1, which bound to their upstream binding sites and increased their promoter activity [10, 16, 17, 20]. Recent experimental results proved that *CPR95* was regulated by *E74A* and *BmorCPG13* by BR-C Z2 through a reporter assay by using wing discs [9, 11]. Thus, it was proved that the expression of the cuticular protein genes of *B. mori* was regulated by ecdysone-responsive transcription factors. In the present study, we found a cuticular protein gene, *BmorCPH33*, which showed a distinctive expression profile. We tried to clarify the regulatory mechanism of *BmorCPH33* through the expression and culture system by using wing discs. The expression pattern of *BmorCPH33* resembled that of *BHR4*,

and its ecdysone responsiveness was related with BHR4, which is different from that of *BmorCPH34*, which was related with E74A. 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 Developmental Stages

The *B. mori* larvae were reared at 25 °C under a photoperiod of 12:12 (L:D) h. 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 Wandering stage day 1 (W1), Wandering stage day 2 (W2), Wandering stage day 3 (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. The newly emerged pupa was designated as P0 and the following consecutive days were designated as P1–P9.

2.2 In Vitro Culture of Wing Discs

The wing discs of larvae at the V4 and W2 stages were prepared for the *in vitro* culture. For wing disc preparation, the fat body, muscle, and trachea were carefully removed under a microscope. The culture was carried out according to a previous report at 25 °C under sterile conditions [21]. We looked at induction *in vitro* at various times following administration of 2µg/ml 20E to V4 wing discs and after cessation of a 12 h pulse of 2µg/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 and transcription factors, total RNA was 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 Quantitative RT-PCR

Table 1. List of Primers for qRT-PCR

Genes	Forward Primer	Reverse Primer
<i>BmorCPH33</i>	5'-CTCAGTAAGGCAACATGATCG-3'	5'-GTACGGCTTCGTGTGTTCTG-3'
<i>BmorCPH34</i>	5'-CAGTCTTCGGCAGATTGAG-3'	5'-GCTAGAGGAGCGTATCCAAAG-3'
<i>BHR4</i>	5'-GTGTTTTCCGTCGCATACAG-3'	5'-GGTTGGCGTGTTCAAGGTAG-3'
<i>E74A</i>	5'-GCACCACCTATCGAGATAAAGC-3'	5'-CTGCCCGTTGTTTTGTAAATG-3'
<i>Rpl</i>	5'-GATTCACAATCCACCGTATCACC-3'	5'-CCATCATGCGTTACCAAGTACG-3'

The wing discs were collected and washed three times in phosphate-buffered saline (PBS) buffer and then frozen and stored at -80°C. Total RNA was isolated from wing discs using ISOGEN (Nippongene, Japan). First-strand cDNA was synthesized from 1µg total RNA in a 20µl reaction mixture ReverTraAce (Toyobo, Japan). RT-qPCR was conducted on an ABI7500 real-time PCR machine (Applied Biosystems) using FastStart Universal SYBR Green Master (Roche) according to the manufacturer's protocol. Each amplification reaction was performed in a 25 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 sec and at 60 °C 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 to contain an intron in the genomic sequence using Primer3 software (<http://frodo.wi.mit.edu/>). The oligonucleotide primer sets used for RT-qPCR were listed in Table 1.

3 Results

3.1 Atypical Developmental Profile of *BmorCPH33* and *BmorCPH34* in Wing Discs

In the beginning, we found a cuticular protein gene, *BmorCPH33*, which showed an atypical expression profile from other cuticular protein genes. Transcripts of *BmorCPH33* showed expression peak at W3E-W3M. Therefore, we examined expression profiles of *BmorCPH33* comparing with *BmorCPH34* transcripts that showed peak slightly later from those of *BmorCPH33* in the beginning experiment. *BmorCPH33* transcripts rapidly increased after W2 and remained high level until W3M stage, then decreased from the W3L stage (Fig. 1A). *BmorCPH34* transcripts showed a different expression profile. They increased from W3M stage and peaked at W3L stage, then decreased from P0 (Fig. 1B). Thus, two cuticular protein genes showed different developmental profiles. Each cuticular protein genes were regulated by each ecdysone-responsive transcription factor (Ali et al.), therefore we selected transcription factors that showed similar developmental profiles to each cuticular protein genes, and examined their developmental profiles.

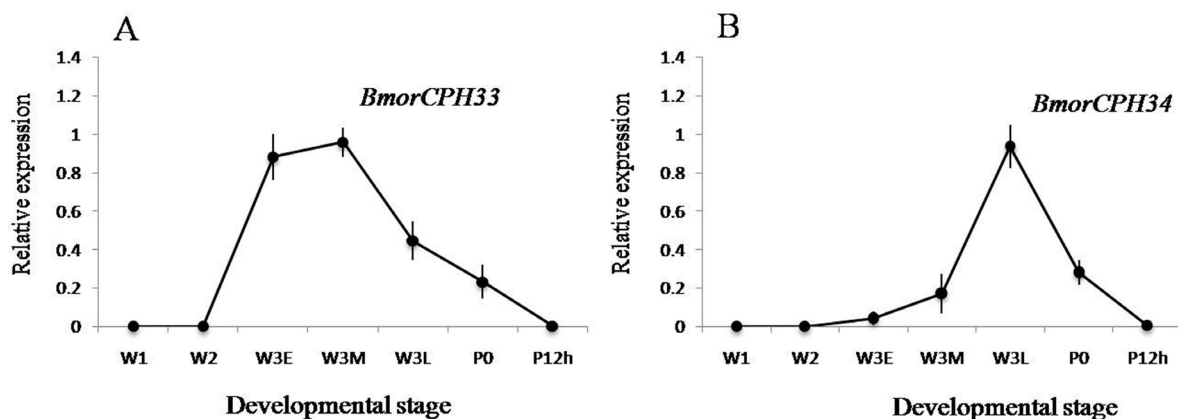


Figure 1. Developmental profile of cuticular protein genes. Each datum was calculated from three independent experiments. RNA was extracted from wing discs and reverse-transcribed to cDNA for use in qRT-PCR. (A), Level of *BmorCPH33* mRNA from W1 to 12 h after pupation. (B), Level of *BmorCPH34* mRNA from W1 to 12 h after pupation. The results are expressed as the mean \pm S. E. M.

3.2 Developmental Profile of *BHR4* and *E74A* Resembled that of *BmorCPH33* and *BmorCPH34* Respectively

BHR4 transcripts increased from W3E stage and remained high level until W3M stage then decreased (Fig. 2A). The developmental profile of *BmorCPH33* resembled that of *BHR4*. Transcripts of *E74A* increased from W3M stage and peaked at W3L then decreased rapidly from P0 (Fig. 2B). The developmental profile of *BmorCPH34* resembled that of *E74A*. Thereafter we tested the ecdysone responsiveness of these cuticular protein genes and transcription factors.

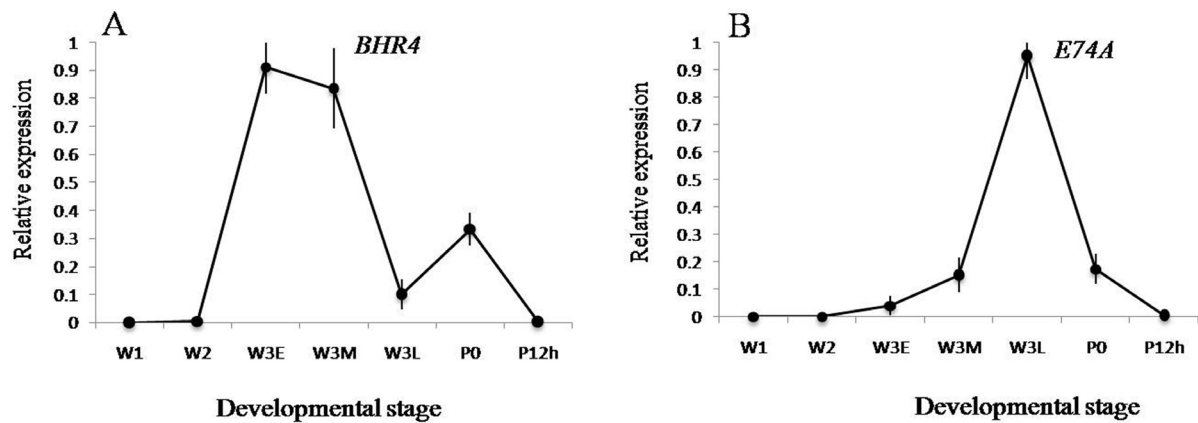


Figure 2. Developmental profile of ecdysone-responsive transcription factors. Each datum was calculated from three independent experiments. RNA was extracted from wing discs and reverse-transcribed to cDNA for use in qRT-PCR. (A), Level of *BHR4* mRNA from W1 to 12 h after pupation. (B), Level of *E74A* mRNA from W1 to 12 h after pupation. The results are expressed as the mean \pm S. E. M.

3.3 Ecdysone-Responsiveness of *BmorCPH33* and *BmorCPH34* Resembled that of *BHR4* and *E74A* Respectively

The transcripts of both cuticular protein genes increased by the addition of 20E (Fig. 3A, 3B) but did not increase by the addition of cycloheximide to the medium containing 20E. Transcripts of *BHR4* and *E74A* increased by the addition of 20E and slightly inhibited by the addition of cycloheximide in the 20E containing medium (Fig. 4A, 4B). The results suggest that both cuticular protein genes were induced by ecdysone through factors and transcription factors were directly by 20E and indirectly by other factors.

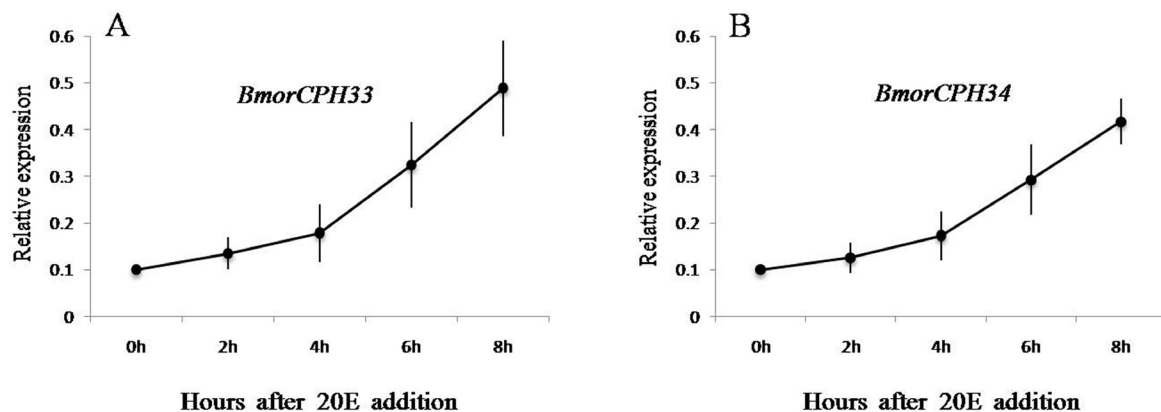


Figure 3. Ecdysone responsiveness of *BmorCPH33* (A) and *BmorCPH34* (B). Level of mRNA of the ecdysone treatment. V4 wing discs were incubated for the indicated time in a medium containing 2 μ g/ml 20E with (open circle) or without (closed circle) cycloheximide (50 μ g/ml). The results are expressed as the mean \pm S. E. M., and significance was $p < 0.01$ (*) by the student's t-test.

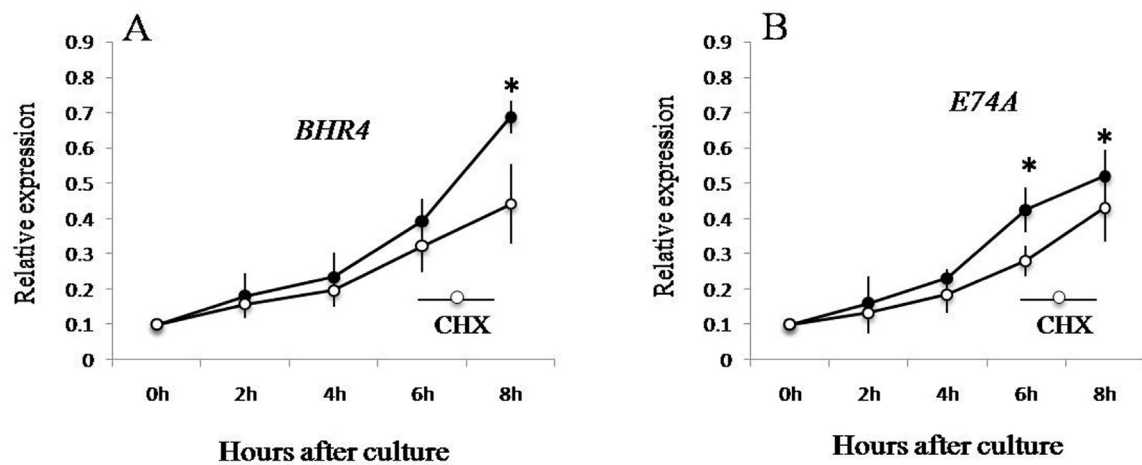


Figure 4. Ecdysone responsiveness of *BHR4* (A) and *E74A* (B). Level of mRNA of the ecdysone treatment. V4 wing discs were incubated for the indicated time in a medium containing 2 $\mu\text{g/ml}$ 20E with (open circle) or without (closed circle) cycloheximide (50 $\mu\text{g/ml}$). The results are expressed as the mean \pm S. E. M., and significance was $p < 0.01$ (*) by the student's t-test.

BmorCPH33 transcripts were induced 6 h after 20E removal and peaked after 12 h, while *BmorCPH34* transcripts gradually increased 12 h after hormone removal, rapidly increased after 12 h and peaked at 18 h (Fig. 5A, 5B). *BHR4* transcripts were induced 6 h after ecdysone removal (Fig. 6A), which resembles *BmorCPH33* transcripts. *E74A* showed similar expression pattern with *BmorCPH34* that showed peak (Fig. 6B) 18 h after 20E removal. From developmental profiles and ecdysone-responsiveness, *BmorCPH33* showed strong relatedness with *BHR4* and *BmorCPH34* showed it with *E74A*.

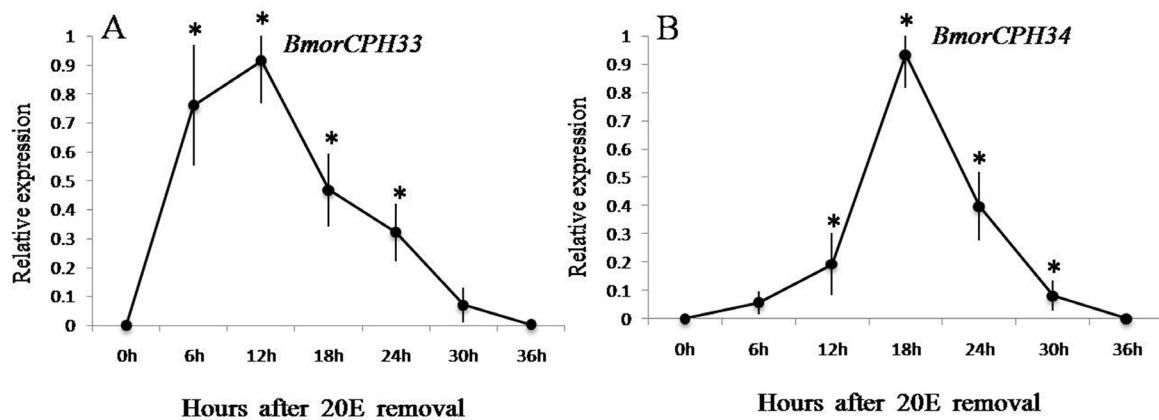


Figure 5. Effect of ecdysone pulse treatment of *BmorCPH33* (A), *BmorCPH34* (B). Level of mRNA after ecdysone pulse treatment. Wing discs of the W2 stage were incubated 12 h in a medium containing 2 $\mu\text{g/ml}$ 20E and then transferred to a hormone-free medium with (open circle) or without (closed circle) cycloheximide (50 $\mu\text{g/ml}$) for the indicated time. The results are expressed as the mean \pm S. E. M., and significance was $p < 0.01$ (*) by the student's t-test.

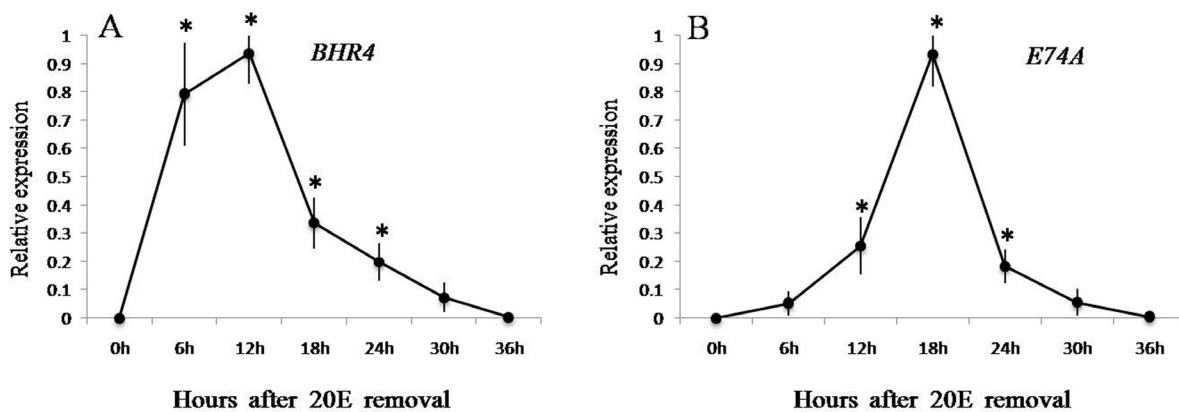


Figure 6. Effect of ecdysone pulse treatment of *BHR4* (B) and *E74A* (C). Level of mRNA after ecdysone pulse treatment. Wing discs of the W2 stage were incubated 12 h in a medium containing 2 μ g/ml 20E and then transferred to a hormone-free medium with (open circle) or without (closed circle) cycloheximide (50 μ g/ml) for the indicated time. The results are expressed as the mean \pm S. E. M., and significance was $p < 0.01$ (*) by the student's t-test.

4 Discussion and Conclusion

Current studies have showed that BR-C, E74A and β FTZ-F1 regulate different cuticular protein genes [9, 10, 11, 16, 17, 20]. 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 this study, we tried to clarify the regulatory mechanism of a cuticular protein gene that showed atypical expression profile. For this, we compared the expression profile of concerned *BmorCPH33* with that of *BmorCPH34* that is induced slightly later than *BmorCPH33*. In the present study, it is strongly suggested that *BmorCPH33* is regulated by *BHR4* and *BmorCPH34* is regulated by E74A in the *Bombyx* wing disc. The present results suggest that these ecdysone-responsive transcription factors determine the expression time of related cuticular protein genes, resulted in the space of these cuticular proteins in the cuticle layer of insect integument. Among cuticular protein genes, *BmorCPH33* is only one that showed expression peak at W3E and W3M stages in beginning experiments. The developmental expression profile of *BmorCPH33* and *BmorCPH34* resembled that of *BHR4* and *E74A*, respectively. Both *BmorCPH33* and *BmorCPH34* were upregulated by 20E but were inhibited by the addition of cycloheximide. Both were induced by the ecdysone pulse, but the induction of *BmorCPH33* was more rapid than that of *BmorCPH34*. Ecdysone-responsiveness of *BmorCPH33* and *BmorCPH34* resembled that of *BHR4* and *E74A* respectively. Ecdysone and ecdysone-pulse induction of cuticular protein genes revealed to depend on the transcription factors that regulate them. Ecdysone responsiveness of cuticular protein genes have been demonstrated by using *in vitro* wing disc culture system [20]. Together with these reports; the present findings suggested the regulatory mechanism of cuticular protein genes by ecdysone-responsive transcription factors. Through the comparison of ecdysone responsiveness of transcription factors, we found interesting evidences. *BHR4* and *E74A* were induced both by 20E addition and the ecdysone pulse, as previously reported [22, 23, 24]. The induction of *BHR4* was rapid, which is suggested that *BHR4* is inhibited by *BHR3* [25]. The present result offered the possibility of *BHR4* functions to induce *E74A* as previously observed [26].

E74A is inducible by ecdysone and the ecdysone pulse [22, 23]. The induction of an expression peak at this stage suggests that it is produced by the interaction of *BHR4* [27]. From the previous report and the present results, it is suggested that *BHR4* is induced by the decrease of *BHR3*, and then induce *E74A* [27]. If *BHR3* determines the timing of metamorphosis, ecdysone-pulse inducible cuticular protein genes construct the pupal cuticle layers. Therefore, *BmorCPH33* is suggested to be one of the first products for the pupal cuticle. Therefore, *BmorCPH33* is suggested to be involved in epicuticular layer in spite that R&R less cuticular proteins are suggested to construct epicuticular layer [28]. In contrast, *BmorCPH34* would construct procuticle according to its timing of production. Thus, the combination of cuticular layers would be determined, and the nature of the cuticle is determined like this.

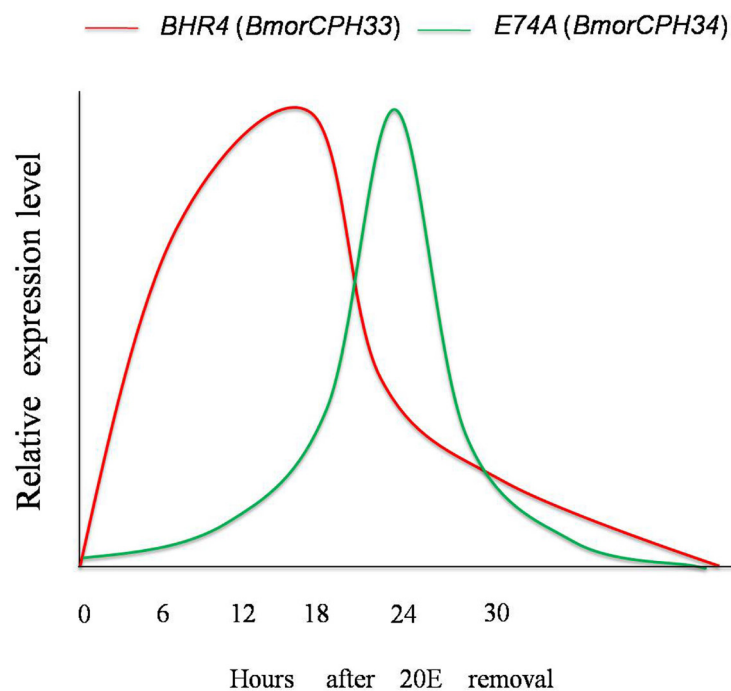


Figure 7. Schematic representation of ecdysone-responsive transcription factors and cuticular protein genes expressed in wing discs of *B. mori* after 20E removal. Relative mRNA expression levels of *BHR4*, *BmorCPH33*, *E74A* and *BmorCPH34* are indicated.

The present findings suggest that cuticular protein genes are expressed in series according to their regulating transcription factors, resulting in a continuous series of cuticular protein production, which enable to construct epi-, exo-, and endo-cuticle. These cuticular proteins are combined and form the pupal cuticle, and the present findings propose that ecdysone responsive transcription factors determine the location where cuticular protein genes are expressed. An RNAi-mediated knockdown would provide more definitive proof for the result. We hope to consider about RNAi in future experiment.

Conflict of interest: The authors declare that they have no conflict of interest

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