REGULATION OF TWEEDLE CUTICULAR PROTEIN GENE EXPRESSION AT THE PRE-PUPAL STAGE IN WING DISCS OF *BOMBYX MORI*

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ABSTRACT: The present study was undertaken to clarify the regulatory mechanism of *Bombyx mori* tweedle cuticular protein gene expressed in wing discs at pre-pupal stage with special emphasis to developmental expression and hormonal responsiveness. The tweedle cuticular protein genes *BmorCPT2* and *BmorCPT3* were selected for the study. The expression of *BmorCPT2* was observed from the W3E stage and peaked at pupation when the ecdysteroid titer declined. After pupation suddenly expression decreased. Whereas the transcripts of *BmorCPT3* increased from the W3M stage, peaked at W3L, and then decreased rapidly from P0 when the ecdysteroid titer decreasing. *BmorCPT3* was induced by the ecdysone pulse, and its expression peaked 18h after transfer to a hormone free medium. *BmorCPT2* was also induced by the ecdysone pulse, and its expression peaked 24 h after transfer to a hormone free medium. The peak of *BmorCPT2* expression was observed later than that of *BmorCPT3* both in vivo and in vitro. Both genes, *BmorCPT2* and *BmorCPT3* transcripts were not observed after the 20E pulse treatment in the presence of cycloheximide. Transcripts of *BmorCPT3* increased by the addition of 20E in V4 wing discs. On contrary, *BmorCPT2* transcript was not observed after the addition of 20E in V4 wing discs which is different from the case of *BmorCPT3*. The expression profiles of *BmorCPT2* are different from those of *BmorCPT3*. The present findings showed different regulation of tweedle cuticular protein genes at the pre-pupal stage in wing discs of *Bombyx mori*.

KEY WORDS: Tweedle cuticular protein, ecdysone, wing disc, gene expression, *Bombyx mori*.

Insect cuticle is composed of many kinds of cuticular proteins with different temporal and spatial patterns (Willis, 1996). Stage specific expression of the cuticle protein genes is induced by the fluctuation of hormones. Ecdysone up-regulates (Braquart et al., 1996; Noji et al., 2003) and down-regulates (Hiruma et al., 1991) or ecdysone pulse induces (Apple and Fristrom, 1991; Suzuki et al., 2002; Noji et al., 2003; Zhong et al., 2006) different ecdysone responsive cuticle protein genes (Riddiford, 1982; Bouhin et al., 1992; Krämer & Wolbert, 1998; Zhou & Riddiford, 2002). βFTZ-F1 has been suggested to be a regulator responsible for the stage-specific expression of cuticle protein genes during the prepupal stage (Kawasaki et al., 2002). The timing of βFTZ-F1 expression has been reported to be affected by DHR3 and Blimp-1 (Lam et al., 1997; White et al., 1997; Agawa et al., 2007). 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 (Murata et al., 1996). Studies with mutants have shown that βFTZ-F1 was required for normal larval cuticle production (Yamada et al., 2000). BmβFTZ-F1, an ortholog of βFTZ-F1 of the silkworm *Bombyx mori*, was expressed during larval, pupal molts and adult development, in coincidence with an ecdysone pulse (Sun et al., 1994; Nita et al., 2009). Although an earlier study proposed that BmβFTZ-F1 was a possible

factor directing the stage-specific expression of the peptide gene *BmACP-6.7* (Shiomi et al., 2000), but the detailed role of BmbFTZ-F1 in tweedle cuticular protein gene expression regulation has not been elucidated.

In *Manduca*, E74A was induced by the ecdysone pulse and was expressed before larval and pupal ecdysis (Stilwell et al. 2003). Target genes of E74 were identified by the confirmation of E74 binding to the upstream region of the L71-6 gene (Urness & Thummel 1995), or by the inhibition of the vitellogenine gene expression by E74B RNAi (Sun et al. 2005). A relationship of E74 to cuticular protein gene expression was suggested using E74 mutation (Fletcher & Thummel 1995).

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 (Ali et al., 2012a; Ali et al., 2012b; Ali et al., 2013). Cuticular protein genes have different expression profiles (Togawa et al., 2008) and regulatory systems by ecdysone-responsive transcription factors (Ali et al., 2013). From this, insect cuticular protein genes are suggested to be the suitable material to clarify the regulatory mechanism of ecdysone-responsive transcription factors.

The majority of cuticular proteins have the Rebers and Riddiford Consensus (R&R Consensus), which in an extended form is known to bind chitin (Rebers and Willis, 2001; Togawa et al., 2004; Willis et al., 2005). Proteins with R&R Consensus can be split into three groups, RR-1, RR-2, and RR-3, with some correlation to the type or region of the cuticle. Recently, other motifs of cuticular proteins have been reported. In *Drosophila melanogaster*, the Tweedle motif was found by identification of a body shape mutant (Guan et al., 2006). Because Tweedle proteins are predicted to form b-strands, and because a barrel structure formed by multiple b-strands provides an interface for aromatic residues to stack with and bind to chitin (Iconomidou et al., 1999; Hamodrakas et al., 2002), studies have postulated that Tweedle proteins interact directly with chitin (Guan et al., 2006). The present study was conducted on *Bombyx mori* tweedle cuticular protein genes, *BmorCPT2* and *BmorCPT3* expression and ecdysone responsiveness in wing discs at the pre-pupal stage to analyze their regulatory mechanism.

MATERIALS AND METHODS

Insects

The *B. mori*, larvae were reared at 25°C under a 12-h light: dark cycle. Larvae began wandering after the sixth day of the fifth larval instar, and pupation occurred 3 days later. The first day of the fifth larval instar was designated as V0 with the following feeding phases from V1 to V6 correspondingly until the beginning of wandering, which is W0. The following three days before pupation was designated as W1–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. The newly emerged pupa was designated as P0 and the following consecutive days were designated as P1–P9.

BLAST search of genomic sequences of cuticular protein genes

The cDNA sequences of *BmorCPT2* and *BmorCPT3* were used for BLAST search analysis. BLASTsearch was operated using genomic database of B. mori (http://kaikoblast.dna.affrc.go.jp/). The binding sites of βFTZ-F1 and E74 were identified through the sequences by referring to previous studies (Ueda & Hirose 1990; Urness & Thummel 1990) and a website (http://www.genomatix.de/en/index.html).

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 (Kawasaki, 1989) at 25 ºC under sterile conditions. We conducted *in vitro* induction 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).

RNA sample preparation and first-strand cDNA synthesis

To determine the expression levels of the CP genes and transcription factors, total RNA was extracted at distinct stages from wing discs using RNAiso (Takara, Japan) and quantified by spectrophotometry at 260 nm. First-strand cDNA was synthesized from 1 μg total RNA in a 10 μl reaction mixture using ReverTra Ace (Toyobo, Japan).

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). 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 specificity of the primers was confirmed using NCBI BLAST (BLASTN) algorithms. The primers used were

BmorCPT2: 5/-GTGGTACTCGCCTGTGTGG-3/
and 5/-GCCGCTGATAGAGGAAGAGC-3/
BmorCPT3: 5/-TTCTTGGTATTAGCTGCCGTTG-3/
and 5/-CTCCCGGCGCATTGTAAG-3/
Rpl: 5/-GATTCACAATCCACCGTATCACC-3/
and 5/-CCATCATGCGTTACCAAGTACG-3/
Gene Bank accession No. of these genes are follows;
BmorCPT2: BR000651, *BmorCPT3*: BR000652 *Rpl*: nm_001043792.

RESULTS

In the present study we examined the induction of cuticular protein *BmorCPT3* and *BmorCPT2* in several ways. We examined mRNA levels during normal development; we monitored induction in vitro at various times following administration of 20E to V4 wing discs and after cessation of a 12 h pulse of 20E to discs from W2. The necessity of protein synthesis for induction was tested in the cultured discs by administration of cycloheximide from the start of culture (V4) or at the time of 20E removal (W2).

BmorCPT3 transcripts increased gradually after the beginning of wandering W3M stage and peaked at W3 late stage (Fig. 1A) when the ecdysteroid titer decreasing. A similar expression peak was induced by the ecdysone pulse in vitro (Fig. 2A). *BmorCPT3* transcripts increased 12 h after removal of 20E, peaked at 18

h, and then decreased. An increase of *BmorCPT3* transcripts was not observed after 20E pulse treatment in the presence of cycloheximide (Fig. 2A), which indicates that the induction of *BmorCPT3* requires 20E-inducible factors. To determine whether the induction of *BmorCPT3* 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 *BmorCPT3* mRNA was assessed by real-time PCR. *BmorCPT3* transcripts increased after 20E addition (Fig. 3). Induction was not observed in the presence of cycloheximide. Thus, *BmorCPT3* gene was upregulated by the 20E addition (Fig. 3).

Previously (Ali et al., 2013) reported that the transcripts of *E74A* increased from the W3M stage, peaked at W3L, and then decreased rapidly from P0. Transcripts of *E74A* increased by the addition of 20E and were slightly inhibited by the addition of cycloheximide in the 20E-containing medium. E*74A* transcripts was induced by ecdysone pulse , which were not observed by the addition of cycloheximide. *E74A* transcripts showed expression peak at 18h after 20E removal (Ali et al., 2013). So, the expression pattern of *BmorCPT3* resembled that of *E74A*.

We compared the expression of *BmorCPT2* using the same conditions as above. Expression of *BmorCPT2* was observed from the W3E stage and peaked at pupation when the ecdysteroid titer decreased; it then suddenly decreased after pupation (Fig. 1B). *BmorCPT2* was also induced by the ecdysone pulse, and its expression peaked 24 h after transfer to a hormone free medium (Fig. 2B). The peak of *BmorCPT2* expression was observed later than that of *BmorCPT3* both in vivo and in vitro. The *BmorCPT2* 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 (data not shown), which is different from the case of *BmorCPT3*.

Cuticular protein gene, *BmorCPT2* showed a similar expression pattern with βFTZ-F1 (Ali et al., 2013); 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 (Ali et al., 2013). The timing of peak expression was similar to that of *BmorCPT2*. *BmorCPT2* was 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 *BmorCPT2* responsiveness is same to that of *βFTZ-F1* shown in previous reports (Sun et al., 1994; Zhong et al., 2006; Ali et al., 2013). Thus, the developmental expression and ecdysone-responsiveness of *BmorCPT3* and *BmorCPT2* resembled *E74A* and *βFTZ-F1*, respectively.

The expression of *BmorCPT3* and *BmorCPT2* was compared schematically in Fig. 4. As observed, *BmorCPT3* transcripts appeared, increased and peaked earlier than those of *BmorCPT2*.

As the developmental expression and ecdysone-responsiveness of *BmorCPT3* and *BmorCPT2* resembled *E74A* and *βFTZ-F1*, respectively. Then we performed searching upstream region of cuticular protein genes, *BmorCPT3* and *BmorCPT2* derived from wing disc ESTs to compare the upstream regulatory sequences and whether there is present or not E74A and βFTZ-F1 binding sites in the upstream promoter region of *BmorCPT3* and *BmorCPT2* respectively. These promoter region for different cuticular protein genes were performed and made clear the regulatory mechanism of cuticular protein genes by ecdysone responsive transcription factor (Ali et al., 2013, Ali et al., 2012a; Ali et al., 2012b). By this, we found *BmorCPT3* that has five putative E74A binding sites and *BmorCPT2* that has two putative βFTZ-F1 binding sites in the 2 kb upstream region (Fig. 5). From the above circumstances there is a possibility to regulate *BmorCPT3* by E74A and *BmorCPT2* by βFTZ-F1 in a stage specific manner in the wing discs of *Bombyx mori*.

DISCUSSION

Cuticle protein genes have been annotated and classified in Anopheles, *Drosophila, Tribolium, and Bombyx*, and many reports have revealed that the cuticle protein genes show a variety of spatial and temporal expression patterns. However, reports concerning the transcription and regulation of the transcriptional level are few. Therefore, in the present study, we analyzed the expression and regulation of *BmorCPT3* and *BmorCPT2* 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 (Futahashi et al., 2008). Among the cuticular protein genes that are expressed at the pre-pupal stage, *BmorCPH5* and *BmorCPR34* were induced by BHR3 and BHR4 respectively (Ali et al., 2013). The present paper reported cuticular protein genes that were expressed at the pre-pupal stage, but were expressed later than and differently from *BmorCPH5* and *BmorCPR34*.The expression profile of *BmorCPT3* was similar to that of E74A and the expression profile of *BmorCPT2* was similar to that of βFTZ-F1. From this, it is suggested that the possibility to induce *BmorCPT3* by E74A and *BmorCPT2* by βFTZ-F1 respectively.

E74A transcription was induced by 20E in the existence of cycloheximide suggested direct induction by 20E (Ali et al., 2013). The induction of *E74A* was also observed by ecdysone pulse treatment, which was inhibited by the addition of cycloheximide (Ali et al., 2013). This result of pulse treatment indicated the existence of other factors affecting the expression of *E74A*. Thus, *E74A* is induced by ecdysone directly and pulse treatment through other factors (Karim & Thummel, 1991; Stillwell et al., 2003). E74A is inducible by ecdysone (Karim & Thummel, 1991) and ecdysone pulse (Stillwell et al., 2003). Transcripts of *E74A* showed peak at W3L stage, when the hemolymph ecdysteroid titer decreased after its peak (Ali et al., 2013).The induction of expression peak at this stage suggests to be brought about by the interaction of BHR3 (White et al., 1997) and βFTZ-F1 from the previous study (Woodard et al., 1994; Broadus et al., 1999; Yamada et al., 2000). *BmorCPT3* expression showed similar profile to that of *E74A*, except that *BmorCPT3* was not induced by 20E in the medium containing cycloheximide. In correspondence with expression of E74A*, BmorCPT3* expression peaked at W3L stage. Together with the result of the expression in the wing disc, the strong relatedness of *BmorCPT3* and E74A is suggested. Thus, the expression of cuticular protein gene *BmorCPT3* indicates to be regulated by E74A transcription factor.

The expression profile and ecdysone responsiveness of *BmorCPT3* is similar to that of E74A but different from that of *BmorCPT2* which resembled to βFTZ-F1. Thus, the expression of these cuticular protein genes indicates different type of regulation by different transcription factors.

βFTZ-F1 transcription was neither induced by 20E nor in the existence of cycloheximide (Ali et al., 2013). *BmorCPT3* was 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 (Ali et al., 2013). 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 ecdysone pulse (Ali et al., 2013). Transcripts of *βFTZ-F1* showed peak at P0 stage, when the hemolymph ecdysteroid titer decreased (Ali et al., 2013). *BmorCPT2* expression showed similar profile to that of *βFTZ-F1*. In correspondence with expression of *βFTZ-F1, BmorCPT2* expression peaked at P0 stage. Together with the result of the expression in the

wing disc, the strong relatedness of *BmorCPT2* and *βFTZ-F1* is suggested. Thus, the expression of cuticular protein gene *BmorCPT2* indicates to be regulated by *βFTZ-F1* transcription factor.

BmβFTZ-F1 was suggested to function in the induction of *BmorCPT2*, since BmβFTZ-F1 was inducible by decline in the ecdysteroid titer and was induced by a 6-h exposure to 20E followed by 6 h in the hormone-free medium (Sun et al., 1994). In Drosophila, βFTZ-F1 has shown to positively regulate the pupal cuticle protein genes, *EDG84A* and *EDG78E*, during the mid to late prepupal period (Murata et al., 1996; Kayashima et al., 2005). A functional βFTZ-F1 binding site has been described in one adult cuticular-peptide gene of *B. mori* (Shiomi et al., 2000). βFTZ-F1 has been reported to bind to the upstream of a target gene (Murata et al., 1996; Shiomi et al.,2000), to form a complex with MBF1 and MBF2 (Liu et al., 2000), to recruit a coactivator (Zhu et al., 2007), and to interact with MHR4 (Hiruma and Riddiford, 2001). Furthermore, a mutation of βFTZ-F1 has also been reported to inhibit the expression of ecdysone responsive genes, resulting in a defect of pupal and adult morphogenesis (Broadus et al., 1999). The transcriptional activity by the βFTZ-F1 binding on the cuticle protein gene has recently reported with *BMWCP2* (Nita et al., 2009). 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 *BmorCPT2*.

From the recent result (Ali et al., 2013, Ali et al., 2012a; Ali et al., 2012b) of the cuticular protein gene expression regulation by ecdysone responsive transcription factors regarding binding sites position analysis in the upstream cuticular protein genes, it was reported that one functional BHR3 binding site was found in 2kb upstream promoter region of *BmorCPH5* and the expression pattern of *BmorCPH5* resembled to ecdysone responsive transcription factor BHR3. Through site-directed mutagenesis of the binding site and a transient reporter assay system it was proved that the ecdysone responsive transcription factor BHR3 regulated *BmorCPH5* gene expression. By the same experimental procedure proved *BMWCP9* regulation by βFTZ-F1, *BmorCPG11* by BR-Z2. In the present experiment there are five E74A binding sites found in the 2kb upstream region of *BmorCPT3* gene and two βFTZ-F1binding sites found in the 2kb upstream region of *BmorCPT2* gene (Fig. 5). The developmental expression and ecdysone responsiveness resembled to the tweedle cuticular protein genes with each of their binding site related transcription factors. It may be suggested that the expression of *BmorCPT2* and *BmorCPT3* genes was regulated by ecdysone responsive transcription factor βFTZ-F1 and E74A respectively.

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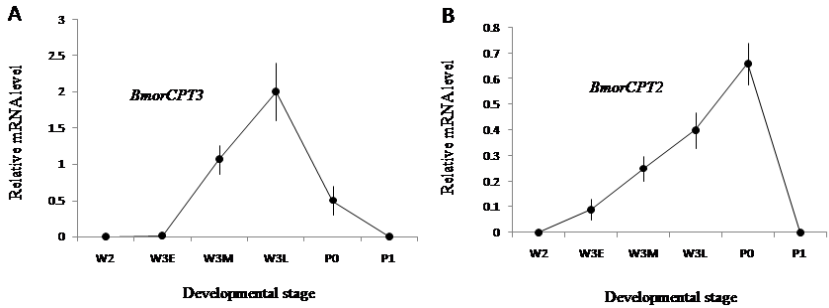

Figure 1. Developmental profile of cuticular protein genes. RNA was extracted from wing discs and reverse-transcribed to cDNA for use in Real-Time PCR. (A), Level of *BmorCPT3* mRNA from W1 to P1. (B), Level of *BmorCPT2* mRNA from W1 to P1. Values represent the mean ± S.E.M. of results from three independent experiments.

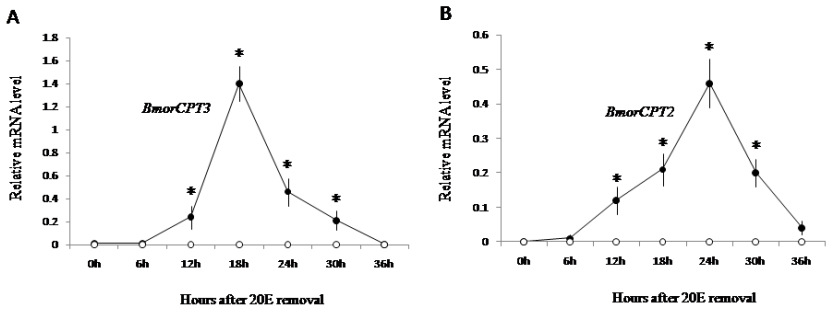

Figure 2. Effect of ecdysone pulse treatment of *BmorCPT3* (A) and *BmorCPT2* (B). RNA was extracted from wing discs and reverse-transcribed to cDNA for use in Real-Time PCR. Level of mRNA after ecdysone pulse treatment. Wing discs of the W2 stage were incubated 12 h in a medium containing 2 μg/ml 20E 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.

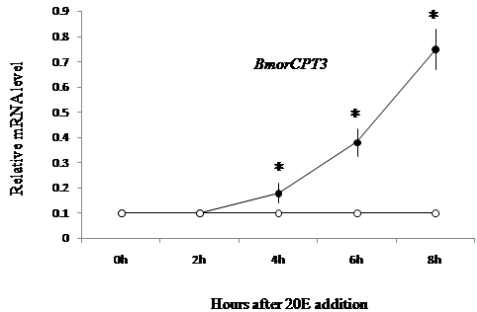

Figure 3. Effect of 20E addition of *BmorCPT3*. RNA was extracted from wing discs and reverse-transcribed to cDNA for use in Real-Time PCR. Level of mRNA of the ecdysone treatment. V4 wing discs were incubated for the indicated time in a medium containing 2 μg/ml 20E with (open circle) or without (closed 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.

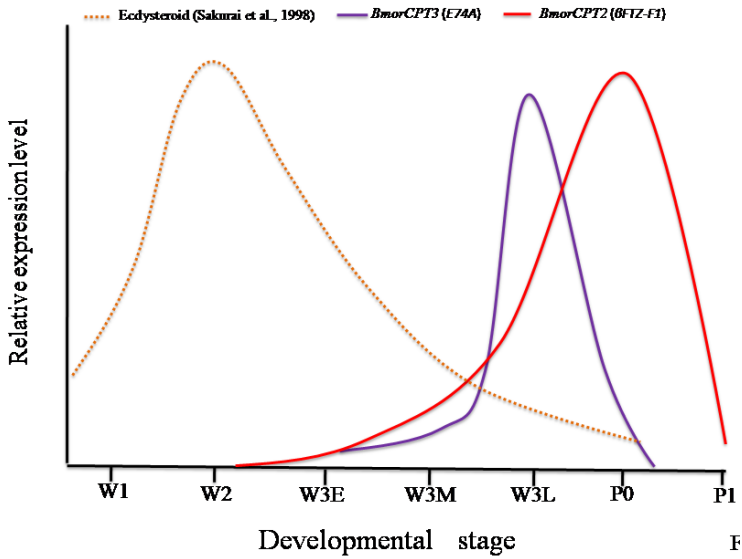

Figure 4. Schematic representation of cuticular protein genes and related ecdysone-responsive transcription factors in parentheses expressed in wing discs of *B. mori* in the late fifth larval instar. Ecdysteroid titer (Sakurai et al., 1998), E74A and βFTZ-F1 (Ali et al., 2013), *BmorCPT3* and *BmorCPT2* (this paper) are indicated.

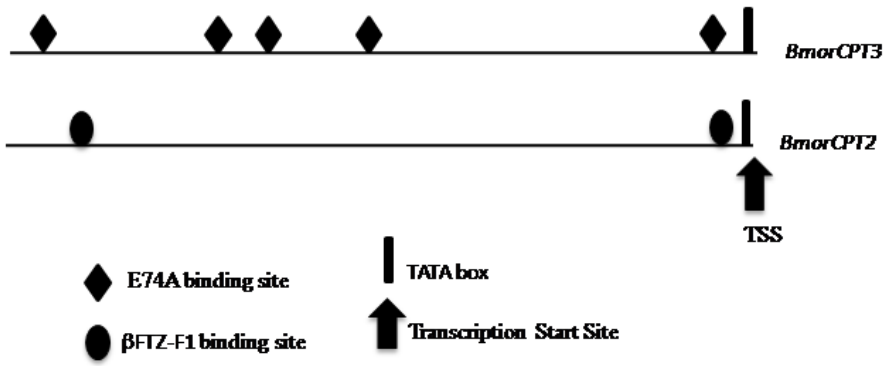

Figure 5. Schematic representation of the putative binding sites of the ecdysone-responsive transcriptional factor located on the upstream of indicated cuticular protein genes. The βFTZ-F1 and E74A binding sites are shown. Bars indicate 2kb upstream region from the transcription start site.