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Transcriptional Regulation of Cuticular Protein Glycine-Rich13 Gene Expression in Wing Disc of *Bombyx mori*, Lepidoptera

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ABSTRACT. Cuticular protein genes are good models to study the molecular mechanisms of signaling by ecdysteroids, which regulate molting and metamorphosis in insects. The present research demonstrates on hormonal regulation and analysis of the regulatory sequences and transcription factors important for *Bombyx mori* cuticular protein glycine-rich13 (*CPG13*) gene expression. Expression of *CPG13* was strong at prepupal stage in wing tissues of *B. mori*. *CPG13* expression was induced by the addition of 20E, which was inhibited by cycloheximide in the wing disc. The upstream region of the *CPG13* gene was analyzed using a transient reporter assay with a gene gun system and identified two BR-Z2 binding sites to be important cis-acting elements for the transcription activation of the luciferase reporter gene by an ecdysone pulse. Site-directed mutagenesis of these sites, followed by introduction into wing discs, significantly decreased the reporter activity. It was found that the regions carrying the binding sites for the ecdysone-responsive transcription factor BR-Z2 were responsible for the hormonal enhancement of the reporter gene activity in wing discs. Mutation of the BR-Z2 binding sites decreased the reporter activity suggesting that the BR-Z2 isoform can bind to the upstream region of the cuticle protein gene, *CPG13* and activates its expression.

Key Words: *CPG13,* BR-Z2, ecdysone, wing disc, gene gun

Recent whole-genome sequence of insect brought about a lot of findings. As for the cuticular protein genes, over 200 cuticular protein genes were identified in *Anopheles gambiae* (Cornman et al. 2008) and *Bombyx mori* (Futahashi et al. 2008), and several distinct families of cuticular protein were recognized (Willis 2010). Among them, CPRs have conserved chitin-binding sequences, and their names are derived from Rebers and Riddiford who first identified their sequences, and the original motif was G-(x8)-G-x(6)-Y-x(2)-A-x-E-x-G-F-x(7)-P-x-P (R&R residues; Rebers and Riddiford 1988) (where x represents any amino acid, the values in parenthesis indicate the number of residue). Three types of CPRs have been identified. RR1 is a soft cuticular protein, RR2 is a hard cuticular protein (Willis 2010), and RR3 is composed of few groups (Andersen 2000, Willis 2010). R&R consensus sequence was demonstrated to bind chitin (Rebers and Willis 2001, Togawa et al. 2004). Other types of cuticular proteins that have not R&R consensus sequence are suggested not to bind with chitin as observed in the case of CPF and CPFL (Togawa et al. 2007). Whether they are used to form epi-cuticle or pro-cuticle remains to be solved.

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,b, 2010; Ali et al. 2012a,b, 2013, 2014a,b, 2015). Cuticular protein genes have different expression profiles (Togawa et al. 2008) and regulatory systems by ecdysone-responsive transcription factors (Wang et al 2009b, 2010; 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.

Three different expression patterns of cuticular protein genes were observed in the wing discs of *B. mori* (Nita et al. 2009; Wang et al. 2009a,b). These expression patterns correspond with ecdysone responsiveness (Wang et al. 2009b, 2010). Expression of *BMWCP2, BMWCP5,* and *BMWCP9* was induced by ecdysone pulse through the transcription factor, βFTZ-F1, which bound to the upstream region of

them and enhanced their promoter activity (Nita et al. 2009, Wang et al. 2009b, Ali et al. 2012a). *BMWCP10* has an EcRE upstream of its transcription start site, and the binding of EcR and activation of its promoter were demonstrated (Wang et al. 2010). Thus, the expression of cuticular protein genes of *B. mori* appeared to be 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) enhancing the promoter activity of *BMWCP5* (Wang et al. 2009b) or *BMWCP10* (Wang et al. 2010). Therefore, BR-C is suggested to function together with other ecdysone responsive transcription factor and to induce the expression of pupal-specific genes (Wang et al. 2009b). BR-C is required for the pupal characterization of cuticular protein genes (Zhou and Riddiford 2002, Bayer et al.2003, Uhlirova et al. 2003). Although BR-C of insect consists of four isoforms and they 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), whose mechanism is not clarified.

Insect cuticle is made up by three cuticular layers: epi-cuticle, exo-cuticle and endo-cuticle. Exo- and endo-cuticle are thick and constructed by chitin and cuticle proteins having R&R residues, and other types of cuticular protein are suggested to construct or fill the space of three cuticle layers. However, the regulatory mechanism by which BR-C controls the expression of cuticle protein genes at a molecular level is not well understood. In this study, *CPG13* was selected for the examination of the promoter assay, since putative binding sites of BR-Z2 were found but lacks of βFTZ-F1 or E74A binding sites in the upstream region of *CPG13*. A detailed analysis of *CPG13* promoter region was performed and showed a strong transcriptional activity by ecdysone pulse. Furthermore, mutagenesis of these binding sites resulted in a reduction of the promoter activity. These results suggest that BR-Z2 positively regulate the promoter of the *CPG13* in a stage-specific manner.

Materials and Methods

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 after the sixth day of the fifth larval instar and pupation occurred 3 d 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 3 d 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 *CPG13* were used for BLAST search analysis. BLAST search was operated using 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).

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°C 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).

Quantitative RT-Polymerase Chain Reaction. Total RNA was isolated at distinct stages from wing discs using RNAiso (Takara, Japan) and first-strand cDNA was synthesized from 1 µg total RNA in a 10 µl reaction mixture using ReverTra Ace (Toyobo, Japan). Then, real-time polymerase chain reaction (PCR) was performed 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°C for 10 min followed by 40 cycles of treatment at 95°C for 10 s 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 using Primer3 software (http://frodo.wi.mit.edu/).The specificity of the primers was confirmed using NCBI BLAST (BLASTN) algorithms. The primers used were

C*PG13*: 5$^{\prime}$-AGCGCTCTGCTTAGTTTTCC-3$^{\prime}$
and 5$^{\prime}$-TTCAGCTTTGCTTTCTGTCG-3$^{\prime}$ and
R*pl*: 5$^{\prime}$-GATTCACAATCCACCGTATCACC-3$^{\prime}$ and
5$^{\prime}$-CCATCATGCGTTACCAAGTACG-3$^{\prime.}$
Gene Bank accession numbers of these genes are as follows:
C*PG13*: BR000434, *Rpl*: nm_001043792.

Plasmid Construction and Mutagenesis. The promoter region spanning from −2008 to +39 bp was amplified with the forward primer (5$^{\prime}$-CCCGGTACC AGGAGGCCATTTAGTATGTAATCG-3$^{\prime}$) and the *CPG13* reverse primer (5$^{\prime}$- CCCGCTAGC CCTCTTCAACGG TGGATGAAGTCGG-3$^{\prime}$), using *B. mori* P50 strain genomic DNA as the template. The amplified DNA fragment was digested with *KpnI* and *NheI* and then ligated into the *KpnI/NheI* sites of the luciferase reporter plasmid pGL3/basic to generate *CPG13-2008*. Deletion construct was obtained by PCR using the following forward primer:

C*PG13-428*: 5$^{\prime}$- CCCGGTACC GATTTATGTTTACGTTCTAGAA TG-3$^{\prime}$, combined with *CPG13* reverse primer. The restriction enzymes sites

are underlined. The PCR products were digested with *KpnI* and *NheI* and then subcloned into a pGL3-basic vector to generate *CPG13-428*.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).The Renilla luciferase activity was used to normalize the data.

Single mutant (−239/−236) BR-Z2mut and (−34/−31) BR-Z2mut for *CPG13* was performed with the Quick-Change TM Site-Directed Mutagenesis Kit (Stratagene) using the *CPG13-428* plasmid as the template. The double mutant (−239/−236) (−34/−31) BR-Z2mut was generated using (−239/−236) BR-Z2mut as a template. Twenty nanograms of plasmid DNA were amplified with *Pfu* DNA polymerase followed by digestion of the parental plasmid by *DpnI*. The mutated plasmid was introduced into XL1-Blue super-competent cells. The mutagenic reactions were performed according to the manufacturer's instructions. The introduction of each mutation was confirmed by sequencing. Oligonucleotide primers used to generate the mutations were shown as follows:

CPG13 (−239/−236) BR-Z2mut:
5$^{\prime}$-   CCAAAATTGAA<u>GCGC</u>AACGAAGCGTCTATAAAGAAT GGC-3$^{\prime}$
CPG13 (−34/−31) BR-Z2mut: 5$^{\prime}$-CGAAAGTAACAAATAAC<u>GG</u> A<u>T</u>TAT
AAAAACAGACGGCG-3$^{\prime}$
The underlined bases were mutated.

Luciferase Transient Expression Assays. Transient expression of the reporter constructs in wing discs was performed according to the procedure of Nita et al. (2009). According to the manufacturer's instructions, 12.5 mg of gold particles (diameter: 1.0 µm) was coated with plasmid DNA (50 µg each of pGL3-derived vectors and 5 µg phRG-hsp). The reporter constructs were introduced into wing discs using a particle gun (Bio-rad). Bombardment proceeded under helium pressure of 150 psi (pounds per square inch). After bombardment, wing discs were cultured for 48 h at 25°C in Grace's medium (Invitrogen) with or without 2 µg/ml of 20E (Sigma). The culture method was described previously (Kawasaki 1989). After 48 h of culture, wing discs were washed twice in phosphate-buffered saline. The tissues were suspended in a 25 µl 1x reporter Passive Lysis Buffer (Promega) and frozen/thawed for five cycles in liquid nitrogen before the supernatant was equilibrated at 4°C for 1 h. The supernatant was collected by centrifugation at $12,000 \times g$ for 2 min at 4°C. The luciferase reporter assay was carried out using a Dual-Luciferase reporter assay system (Promega) in a luminometer (Perkin Elmer) according to the manufacturer's protocol. The luciferase activity was normalized to the level of the Renilla luciferase activity. All experiments were performed at least five times. The results were expressed as the mean ± SEM, and significance was set at $P < 0.05$.

Results

To confirm the expression profile of *CPG13* at the transcription level, qRT-PCR analyses was performed using mRNA prepared from the wing discs during the prepupal stages. We looked at 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). *CPG13* transcripts increased gradually after the beginning of wandering and peaked at W3 late stage (Fig. 1), which was similar to the expression of BR-Z2 (Ali et. al. 2012b). A similar expression peak was induced by the ecdysone pulse in vitro (Fig. 2). *CPG13* transcripts increased 6 h after removal of 20E, peaked at 18 h, and then decreased. An increase of *CPG13* transcripts was not observed after 20E pulse treatment in the presence of cycloheximide (Fig. 2), which indicates that the induction of *CPG13* requires 20E-inducible factors. To determine whether the induction of *CPG13* 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 *CPG13* mRNA was assessed by
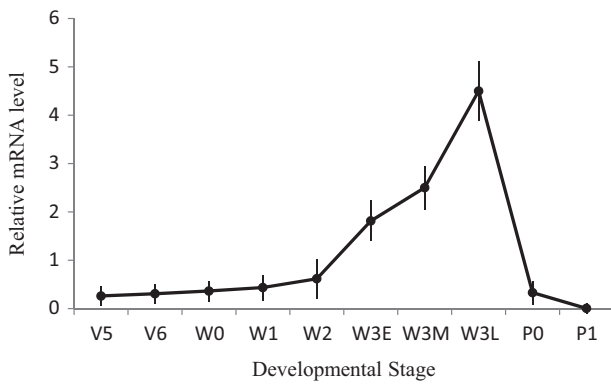
Fig. 1. Results of qRT-PCR of *CPG13*. Each datum was calculated from three independent experiments. RNA was extracted from wing discs and reverse transcribed to cDNA for use in qRT-PCR. Values were first normalized to rpl and then plotted relative to the highest value for each panel. Level of mRNA from V5 to pupation.
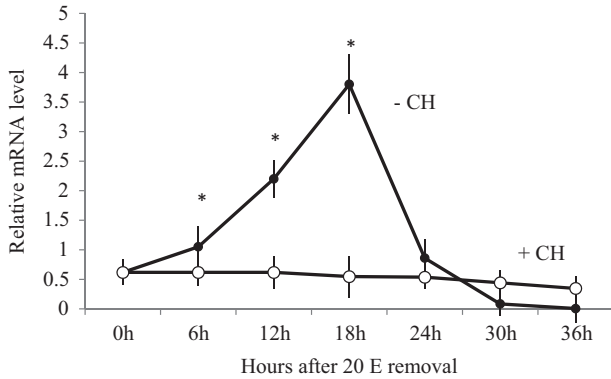

Fig. 2. Effect of ecdysone pulse treatment of *CPG13* gene. Wing discs of the W2 stage were incubated 12 h in a medium containing 2 μg/ml 20E and then transferred to a hormone-free medium with (open circle) or without cycloheximide (50 μg/ml) for the indicated time. RNA was extracted from wing discs and reverse transcribed to cDNA for use in qRT-PCR. Values were first normalized to rpl and then plotted relative to the highest value for each panel. Each datum was calculated from three independent experiments. The result is expressed as the mean ± SEM. Asterisks indicate $P < 0.05$ significance by the student's *t*-test. Level of mRNA of the ecdysone pulse treatment.

qRT-PCR. *CPG13* transcripts increased after 20E addition. Induction was not observed in the presence of cycloheximide. Thus, *CPG13* gene was upregulated by the 20E addition (Fig. 3).

We searched upstream region of cuticular protein genes derived from wing disc ESTs to compare the upstream regulatory sequences and found *CPG13* that has two putative BR-Z2 binding sites in the 2 kb upstream region (Fig. 4). To make clear the regulatory mechanism of cuticular protein genes by ecdysone responsive transcription factor, reporter assay was operated and clarified the relatedness of BR-Z2 and *CPG13*.

In vitro culture system of *Bombyx* wing discs were applied to examine the promoter activity of *CPG13*. According to Ali et al. (2013), W2 wing discs were used for in vivo reporter assay system. After the constructs were introduced into wing discs at W2, wing discs were cultured for 48 h with 20E (nonpulse treatment) or without 20E (pulse treatment). Since the ecdysteroid titer is high at W2 and wing discs are being exposed to ecdysone at the time of explantation, culturing the wings in hormone-free medium mimics a "Pulse" treatment. In contrast,
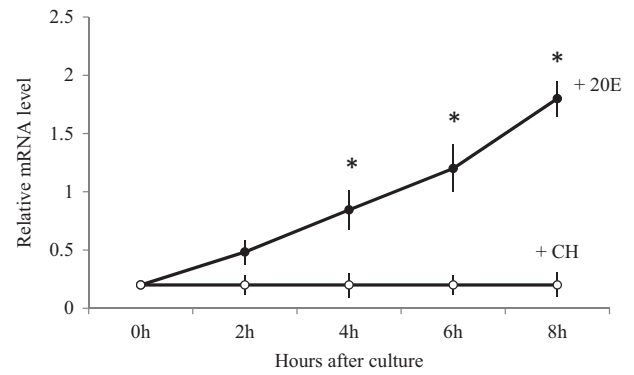

Fig. 3. Effect of *CPG13* after 20E addition. V4 wing discs were incubated for the indicated time in a medium containing 2 μg/ml 20E with (open circle) or without (closed circle) cycloheximide (50 μg/ml). RNA was extracted from wing discs and reverse-transcribed to cDNA for use in qRT-PCR. Values were first normalized to rpl and then plotted relative to the 0 h value for each panel. Each datum was calculated from three independent experiments. The result is expressed as the mean ± SEM. Asterisks indicate $P < 0.05$ significance by the student's *t*-test. Level of mRNA of the ecdysone treatment.

in the "Non-pulse," regimen continued to expose wing discs to high ecdysone.

The result indicated that the constructs showed much higher luciferase activity in the Pulse treatment than in the nonpulse treatment (Fig. 5). These results indicated that the presence of activators produced after the 20E pulse treatment promoted the luciferase activity.

To clarify the potential regulatory mechanisms directing the stage-specific usage of the *CPG13* promoter, a series of luciferase reporter plasmids containing various lengths of the promoter region was constructed and the promoter activity of each construct was analyzed using a luciferase transient expression assay. The −2008/+39 region of the *CPG13* promoter showed a substantially high level of luciferase activity in wing discs (Fig. 5). A substantial reduction in transcriptional activation was not resulted from the further deletion of the −2008 to −428 regions. These results indicated the existence of activators in the −428/+39 region in wing discs. Reduction of *CPG13* promoter activity by the deletion of 5′-flanking region between −2008 and −428 did not show significant decrease in luciferase activity. It was suggested that BR-Z2 binding sites exist in between −428 and +39 region (Fig. 4), which may explain of construct activities. In contrast, no significant difference was observed among nonpulse treatments. Moreover, the results suggested that the *CPG13* upstream region up to −428 was required for the in vivo *CPG13* expression.

We examined the functional contribution of these elements with site-directed mutagenesis of these sites (Fig. 6). Single mutation of putative BR-Z2 sites exhibited about 75% reduction in average in both the distal (−239/−236) and the proximal (−34/−31) cases. Double mutation of the two BR-Z2 sites showed no significant reporter activity compared with a single mutation. No clear difference was observed in the nonpulse treatment. These results indicated that the mutation affecting the binding of BR-Z2 resulted in the reduction of the luciferase activity. These results also suggested that both BR-Z2 sites were required for the promoter activity of *CPG13*-428, and BR-Z2 was an important positive regulator of the *CPG13* promoter.

Discussion

Recently, many 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 regulation of the transcriptional level are few. Therefore, in this

Fig. 4. Schematic representation of the putative binding sites of the ecdysone-responsive transcriptional factor located on the upstream of *CPG13*. The BR-Z2 binding sites are shown. Bars indicate 2 kb upstream region from the transcription start site.
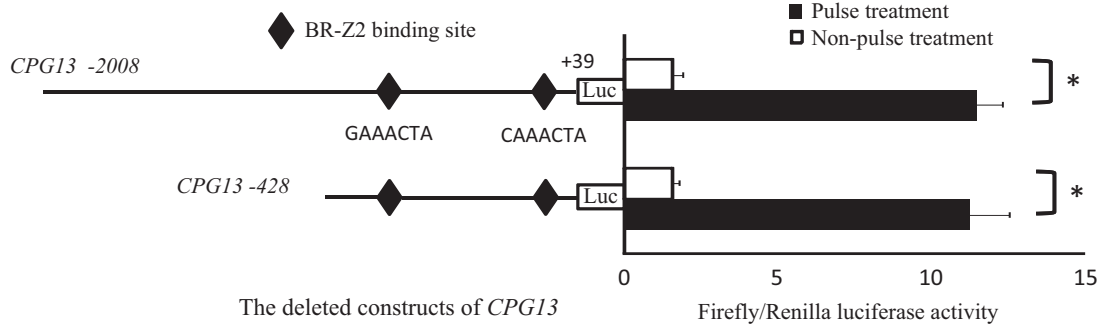

Fig. 5. Transient expression analysis of the *CPG13* promoter in wing discs at W2. The ecdysone responsiveness of the upstream sequences of *CPG13* was examined. Different lengths of constructs were introduced into wing discs (W2). The promoter activity was observed after bombarded wing discs were cultured for 48 h at 25°C with or without 2 μg/ml 20E. The left side of the graph shows different constructs and putative BR-Z2 binding sites existing in the upstream region of *CPG13*. All experiments were performed at least five times. The results are expressed as the mean ± SEM, and significance was $P < 0.01$ (*) by the student's *t*-test. The horizontal axis indicates the relative luciferase activity in arbitrary units. Filled boxes: wing discs were cultured without 20E (ecdysone pulse); empty boxes: wing discs were cultured with 20E (nonpulse treatment). The first panel indicates the promoter activity of construct *CPG13-2008*. The second panel shows the promoter activity of deleted construct *CPG13-428*.
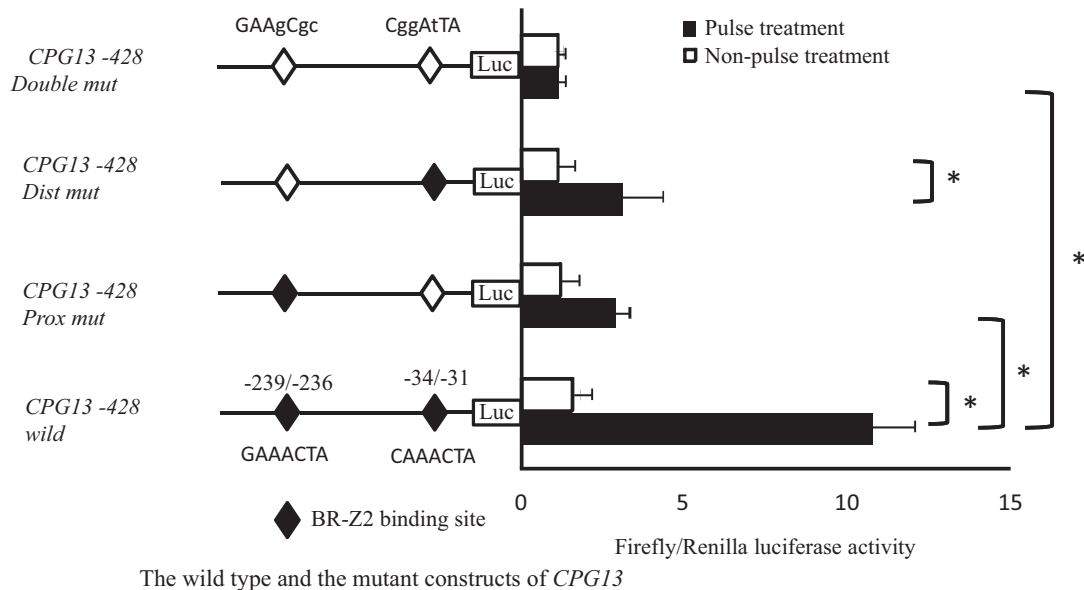

Fig. 6. Site-directed mutagenesis of the putative −239/−236 and −34/−31 BR-Z2 sites on *CPG13* promoter expression in wing discs at W2. Various mutant constructs in element BR-Z2 upstream of the *CPG13* promoter were created by site-directed mutagenesis. These constructs were introduced into wing discs (W2). The promoter activity was observed after bombarded wing discs were cultured for 48 h at 25°C with or without 2 μg/ml 20E. The left side of the graph shows different constructs and putative BR-Z2 binding sites existing in the upstream region of *CPG13*. The horizontal axis indicates the relative luciferase activity in arbitrary units. All experiments were performed at least five times. The results are expressed as the mean ± SEM, and significance was $P < 0.01$ (*) by the Student's *t*-test. Mutated nucleotides are indicated in small letters. The closed rhombs indicate the wild-type sequence, and the open rhombs indicate the mutated sequences. Filled boxes: wing discs were cultured without 20E (ecdysone pulse); empty boxes: wing discs were cultured with 20E (nonpulse treatment). The fourth panel shows the promoter activity of deleted construct *CPG13*-428. The first three panels indicate the promoter activity of the mutation at the putative −239/−236 and −34/−31 BR-Z2 binding sites of the construct *CPG13*-428.

study, we analyzed the expression and regulation of *CPG13* in wing disc using a transient assay system. Transcripts of *CPG13* and *BR-Z2* 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. As BR-C is inducible by a low concentration of 20E (Chen et al. 2004, Sekimoto et al. 2006, Muramatsu et al. 2008), it is suggested that BR-C expression is observed from around W0. The expression pattern of *CPG13* gene resembled that of *BR-Z2* (Ali et al.

2012b), and the promoter activity was closely related with the binding of BR-Z2. Henceforth, it is suggested that *CPG13* was induced by BR-Z2.

BR-Z2 transcription was induced by 20E in the existence of cyclo-heximide-suggested direct induction by 20E. The induction of BR-Z2 was also observed by ecdysone pulse treatment, which was inhibited by the addition of cycloheximide (Ali et al. 2012b). This result of pulse treatment indicated the existence of other factors affecting the expression of BR-Z2. Thus, BR-Z2 is induced by ecdysone directly and pulse treatment through other factors. This induction by different manner is similar to E74A (Karim and Thummel 1991, Stilwell et al. 2003). E74A is inducible by ecdysone (Karim and Thummel 1991) and ecdysone pulse (Stilwell et al. 2003). Transcripts of BR-Z2 showed peak at W3L stage, when the hemolymph ecdysteroid titer decreased after its peak (Ali et al. 2012b).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). *CPG13* expression showed similar profile to *BR-Z2,* except that *CPG13* was not induced by 20E in the medium containing cycloheximide. In correspondence with expression of *BR-Z2, CPG13* expression peaked at W3L stage. Together with the result of the expression in the wing disc, the strong relatedness of *CPG13* and BR-Z2 is suggested. Thus, the expression of cuticular protein gene *CPG13* indicates to be regulated by BR-Z2 transcription factor.

In this study, we used the wing disc culture system to compare the ecdysone pulse and nonpulse treatment on the promoter activity in different reporter constructs. The result indicated that all the constructs showed higher luciferase activity in the Pulse treatment than in the nonpulse treatment, confirming that *CPG13* was induced by an ecdysone pulse. Moreover, we showed by mutagenesis that BR-Z2 bound to the promoter of *CPG13* resulting in transcriptional activation. With the luciferase reporter gene assay, the mutation of BR-Z2 binding sites reduced the transcriptional activity about 75%, indicating the positive role of BR-Z2 through the both distal and proximal binding sites. The present result of the transcriptional activity by the BR-Z2 binding on the cuticle protein gene was similar with *BMWCP2* (Nita et al. 2009), which confirmed the system efficiency used in this study and transcription factor function on the ecdysone-pulse induced cuticle protein genes. We also found several potential BR-Z2 binding sites in the 5$^/$ flanking region of the *CPG12, 16, 17, 24*. Whether these genes are the targets of BR-Z2 remains to be elucidated.

Isoform-specific expressions of BR-C in the epidermis were reported in *B. mori* (Ijiro et al. 2004, Nishita and Takiya 2004, Reza et al. 2004, Nishita and Takiya 2006, Ali et al. 2012b) and *Manduca sexta* (Zhou and Riddiford 2001). BR-C isoforms bind to different DNA sequences, which would bring about the different target genes including cuticular protein genes and variety of cuticle layers in the different region. The binding sites of BR-Z2 and BR-Z4 were located in upstream region of *CPG13*. The transcripts of *BR-Z2* were observed in the cephalic region of the epidermis, wing discs, and silk glands (Ali et al. 2012b), whereas *BR-Z4* transcripts were not observed in the cephalic epidermis. Through RT-PCR, the transcripts of *CPG13* were observed in the cephalic region of the epidermis and wing discs (Supp Fig. 1 [online only]). From this, the relatedness between *CPG13* and *BR-Z2* was suggested as BR-Z2 was the only isoform expressed in both cephalic epidermis and wing discs. Mutagenesis of both −239/−236 and −34/−31 BR-Z2 binding sites significantly reduced the luciferase activity, suggesting the important positive regulatory role of *Bombyx* BR-Z2 in the *CPG13* promoter. Furthermore, the result of the mutagenesis of BR-Z2 indicated that BR-C actually bound and increased the promoter activity of *CPG13*. The results suggest that the BR-Z2 isoform can in fact binds to the cuticle protein *CPG13* gene promoter and activate its expression in a transient expression assay. The findings suggest that BR-Z2 both enhances the promoter activity and directs pupal-type cuticle formation. These data agree with a previous report of the

metamorphic defects resulting from BR-Z4 RNAi in *B. mori* (Uhlirova et al. 2003). The data presented in this article indicate that the BR-Z2 isoform is an important regulator of *CPG13* transcription, which suggests that BR-C affects the expression of the pupal-specific genes. Future experiments will focus on the role of different isoforms in cuticle protein gene transcription.

Thus, expression of *CPG13* was regulated by the fluctuation of ecdysteroid titer through BR-Z2 as illustrated in Supp Fig. 2 [online only]. The results of promoter activity in this study coincide well with this schema. Expression of *BR-Z2* is induced by the ecdysone pulse, which results in the induction of *CPG13*.These studies provide a basis for investigating temporal regulation of cuticle protein gene by ecdysone. An RNAi-mediated knockdown of BR-Z2 using the luciferase reporter constructs would provide more definitive proof that the BR-Z2 isoform is regulating *CPG13*. We hope to consider about RNAi in future experiment. In conclusion, the results of this study have demonstrated that the upstream region (−428/+39) of *CPG13* contains essential elements for ecdysone-mediated transcription and regulatory elements for temporal regulation in the wing disc.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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