Transgenic Ectopic Overexpression of Broad Complex (BrC-Z2) in the Silk Gland Inhibits the Expression of Silk Fibroin Genes of Bombyx mori

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Abstract: Bombyx mori silk protein genes are strictly turned on and off in different developmental stages under the hormone periodically change. The broad complex (BrC) is a transcription factor mediating 20-hydroxyecdysone action, which plays important roles during metamorphosis. Here, we observed that two isoforms of BmBrC (BmBrC-Z2 and BmBrC-Z4) exhibited contrasting expression patterns with fibroin genes (FibH, FibL and P25) in the posterior silk gland (PSG), suggesting that BmBrC may negatively regulate fibroin genes. Transgenic lines were constructed to ectopically overexpress BmBrC-Z2 in the PSG. The silk protein genes in the transgenic line were decreased to almost half of that in the wild type. The silk yield was decreased significantly. In addition, the expression levels of regulatory factors (BmKr-h1 and BmDimm) response to juvenile hormone (JH) signal were inhibited significantly. Then exogenous JH in the BmBrC-Z2 overexpressed lines can inhibit the expression of BmBrC-Z2 and activate the expression of silk protein genes and restore the silk yield to the level of the wild type. These results indicated that BmBrC may inhibit fibroin genes by repressing the JH signal pathway, which would assist in deciphering the comprehensive regulation mechanism of silk protein genes.

Keywords: broad complex; fibroin genes; gene regulation; silk proteins; Bombyx mori

1. Introduction

Insects undergo developmental transitions during their growth, which are aided by two major hormones: 20-hydroxyecdysone (20E) [1] and juvenile hormone (JH) [2]. The hormone 20E is necessary for the transformation during larva–larva and larva–adult, while JH maintains the larval growth process. Both 20E and JH change periodically that resulted in the repression or activation of the target genes in insects [3]. The topic of the relationship of hormones and gene regulation has always been a hotspot research in insect development. The silkworm, Bombyx mori, is an important economic insect and a lepidopteran model with applications to biologic science, modern agriculture and biotechnological industry [4]. Their silk-producing organ, the silk gland is considered an ideal system to elucidate the relationship between gene expression and hormone because the silk protein genes are turned on and off during the intermolt to molt stage under JH and 20E control with temporal specific manner [4].

The silk gland of the silkworm is divided into three compartments: the anterior silk gland (ASG), the middle silk gland (MSG) and the posterior silk gland (PSG). As the spatio-specific protein data, the MSG synthesizes sericin proteins, including Ser1, Ser2, Ser3 and Ser4 [5–8]. The PSG synthesizes fibroin proteins, including fibroin heavy chain (FibH), fibroin light chain (FibL) and fibrohexamerin/P25 [9]. According to the temporal-specific gene expression data, the sericin and fibroin genes are highly
expressed during the inter-molting stage, especially in the last instar, but are hardly expressed during the molting stage [10,11]. Currently, the general opinion is that the spatio- and temporal-specificities of silk protein genes are controlled by the signaling pathways of segmental differentiation factors and hormones at the transcriptional level [4].

Silk protein genes are repetitively turned on and off in the inter-molting and molting processes during the whole larval stage [10]. In order to reveal the molecular mechanisms of transcriptional regulation, especially of the fibroin genes, regulatory elements or regions upstream of the promoters of these genes were identified [12–20]. Correspondingly, many factors were identified to regulate the transcription of silk protein genes, including BmFkh/SGF-1 [21], SGF-2 [22], POU-M1/SGF-3 [23], SGF-4 [13], FBF-A1 [13], FMBP-1 [17,24], BmFTZ-F1 [25], BmSage [26] and BmDimm [27]. Most of them function as activators of silk protein genes. For example, BmSage and BmDimm are mainly expressed at the inter-molting stage [26,27]. BmSage can interact with the silk gland factor-1 (SGF-1), form a complex after binding to A and B elements on the FibH promoter and activate the fibroin synthesis [26]. BmDimm can interact with BmSage to regulate the transcription of FibH by binding to the E-box element and be upregulated by Krüppel homolog 1 (BmKr-h1) by the JH-signaling pathway [27]. Both FMBP-1 and BmFTZ-F1 could repress the activity of FibH promoter by binding to the upstream elements at the cellular level, suggesting that they may be repressors of the FibH gene [24,25].

Broad complex (BrC) is one of the early responding transcription factors of 20E, which has been widely studied as a medium of the 20E and JH-signaling pathways [28–31]. In B. mori, BrC gene can produce four isoforms (Z1–Z4) with an evolutionarily conserved bric-a-brac–tramtrack–broad (BTB) domain and a zinc–finger DNA-binding domain and four another isoforms (NZ1–NZ4) without a zinc finger domain [2,32,33]. Among them, BmBrC-Z2 and BmBrC-Z4 perform multiple biologic functions in different tissues and developmental stages. For example, female moths treated with BmBrC-Z2 dsRNA laid fewer and whiter eggs [34]. BmPOUM2 binds only to BmBrC-Z2 to collaboratively regulate BmVg expression by 20E induction to control vitellogenesis and egg formation [35]. BmBrC-Z4 enhances the expression of chitinase 5 during molting and metamorphosis [36]. During the development of wing disc, BmBrC-Z2 regulates the cuticle protein gene BmWCP10 induction by 20E-signaling pathway [37], and BmBrC-Z4 regulates the pupal-specific expression of the wing disc cuticle protein gene BmWCP4 through BmPOUM2 [38]. In the innate immune response, BmBrC-Z2 and BmBrC-Z4 also play an important biologic role in regulating the expression of immune-related genes, including lysozyme and lebocin [39,40].

According to our previous transcriptomic analysis of the silk glands from molting to inter-molting stages, BmBrC-Zs were significantly overexpressed, but the expression of the fibroin genes at the molting stage was almost absent [11]. Synthetic oligopyrrole carboxamides can downregulate the expression levels of various isoforms of BmBrC gene in the silk gland, which resulted in statistically enhanced cocoon weight, shell weight and silk yield [41]. Therefore, we hypothesized that BmBrC may be involved in the negative regulation of fibroin genes. To test the hypothesis, in this study, we further investigated their expression patterns from molting to inter-molting stages by RT-PCR. Transgenic lines with ectopic overexpression of BmBrC-Z2 in the PSG was obtained to investigate the phenotype and gene expression levels. These results may provide an insight into the regulatory mechanism of silk proteins in the silkworm.

2. Materials and Methods

2.1. Experimental Insects

The silkworm strain Dazao was used for transgenic microinjection was obtained from the gene resource library of domesticated silkworm, Southwest University, China. Silkworm larvae were fed with fresh mulberry leaves at 25 °C with a photoperiod of 12 h light/12 h dark.
2.2. Semi-RT-PCR and qRT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the protocol provided by the manufacturer. For reverse transcription, we used the PrimeScript RT reagent Kit (Takara, Kyoto, Japan) according to the manufacturer’s instructions. The primers used for semi-RT-PCR are given in Table 1. The following conditions: 94 °C for 30 s, followed by 30 cycles at 94 °C for 10 s, 60 °C for 15 s and 72 °C for 30 s, then elongation at 72 °C for 7 min and held at 16 °C. The ribosomal protein L3 (RPL3) gene of the silkworm was used as the reference gene.

Table 1. Primers used in the semi-RT-PCR and quantitative RT-PCR.

| Purpose | Gene     | Forward (5′–3′)         | Reverse (5′–3′)         |
|---------|----------|-------------------------|-------------------------|
| RT-PCR  | BmBrC-Z1 | CCCAAGAAAGATTACAGATGCG  | AGGTGCGCTGGTTAGGGTG     |
| RT-PCR  | BmBrC-Z2 | TCGCTGACAAACACGCTG      | ATGGTAAGAACCGGAGAC      |
| RT-PCR  | BmBrC-Z4 | GCCACAAGGTCTTCCGCA      | AAGGACGACGGGAAAGGAT     |
| RT-PCR  | FibH     | CAGCATCAGTTCGGTTCC      | GACTCGTACCCTCGGAATTC    |
| RT-PCR  | FibL     | ATACCGATTGGTCACATAACAG  | GCAGATAGATGGCGCATAAA    |
| RT-PCR  | P25      | AGGCGGCTGTCGGCAGTTTTG   | TAGGTCGGCGTTGAAGTATGG   |
| RT-PCR  | RPL3     | TCGTCACTGTCGTAAGGTCAA   | TTTGATTCTTTCGCTTTGCTTG |
| qRT-PCR | BmBrC-Z2 | TCGTCAACAAACACGCTG      | ATGGTAAAGACCGGGAAC      |
| qRT-PCR | BmBrC-Z4 | GCCACAAGGTCTTCCGCA      | AAGGACGACGGGAAAGGAT     |
| qRT-PCR | FibH     | TCTGTGTCATCTGCTCATCTCG  | TATCCAGGAGCAAGAAAGAACA |
| qRT-PCR | FibL     | ATACCGATTGGTCACATAACAG  | GCAGATAGATGGCGCATAAA    |
| qRT-PCR | P25      | AGGCGGCTGTCGGCAGTTTTG   | TAGGTCGGCGTTGAAGTATGG   |
| qRT-PCR | BmKr-h1  | CATCGTTTTCAACATTTTGCGAG| CACATCACTTACATCGCGAC    |
| qRT-PCR | BmDimm   | CCGGAACACGGCACTTTGTA    | AACCTCGGCAATCCAGCTCG    |
| qRT-PCR | RPL3     | TCGTACTGCGCTCTTCTCGT    | CAAAGTTGATAGCAATTTCCC  |

BrC—broad complex; FibH—fibroin heavy chain, FibL—fibroin light chain; Kr-h1—Krüppel homolog1; RPL3—ribosomal protein L3; Bm—Bombyx mori.

Quantitative RT-PCR (qRT-PCR) was conducted with the ABI7500 real-time PCR machine (Applied Biosystems, Foster, CA, USA) using FastStar Universal SYBR Green Master (Roche, Switzerland). Each qRT-PCR reaction was performed under the following conditions: denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 30 s and 72 °C for 35 s. RPL3 gene was used as the reference. Three repeat experiments were set up. The expression of the target gene was calculated by Ct value and graphics were created based on this data. The primers used are shown in Table 1.

2.3. Transgenic Overexpression of BmBrC-Z2 in the PSG

A transgenic ectopic overexpression vector was constructed. The sequence encoding BmBrC-Z2 and a MYC-tag were cloned into the PMID-19 vector between the BamHI and NotI, then sub-cloned into the mid-vector containing a modified FibH promoter, which was deleted the predicted Br–C binding sites. Finally, the unit [MFibH-BmBrC-Z2-myc-Sv40] was sub-cloned into the piggyBac vector [3xP3-Red-Sv40] with the red fluorescence marker by Ascl digestion site. The recombinant vector [3xP3-Red-Sv40-MFibH-BmBrC-Z2-myc-Sv40] was transformed into the E. coli strain Trans1-T1 cells (TransGen, Beijing, China), screened for positive clones and the plasmid was extracted. The piggyBac transposase-expressing plasmid pHA3PIG helper was obtained from a previous study [42]. Transgenic plasmid and helper plasmid were mixed both to 500 µg/µL and injected into preblastoderm embryos at 2 h after oviposition. Positive individuals were mixed in the pupal stage by red fluorescence using a fluorescence stereomicroscope (Olympus, Tokyo, Japan). The phenotypic characters were investigated between the transgenic overexpressing BmBrC-Z2 lines and the wild type as per the protocols given in our previous study [43].
2.4. Juvenile Hormone Treatment

Overexpressing BmBrC-Z2 lines on the 1st day of the 5th instar larvae (V1stD) were treated with 1 µL JH analogs methoprene (Sigma, St. Louis, Missouri, USA) on the back by the pipette with the concentration of 2 µg/µL. For the controls, equal volumes of DMSO were applied. A quarter of the treatment samples were collected the 2nd day of the 5th instar larvae (V1stD) and stored at -80 °C for subsequent investigation of gene expression patterns. The remaining larvae were raised to the pupal stage and were used to investigate the phenotypic data, including total weight of cocoons and weight of cocoon layer.

2.5. Statistical Analysis

All data were statistically analyzed by t-test. Each set of data were repeated three times. Asterisks were used to indicate significant differences (* p < 0.05; ** p < 0.01; *** p < 0.001).

3. Results

3.1. Expression Patterns of BmBrC-Zs and Fibroins in the Silk Gland

Previous studies showed that BmBrC was highly expressed in the PSG during the 4th molt (IVM) stage, while fibroin genes were almost undetectable by northern blot and transcriptomic analysis [11,32]. Here, we investigated the expression patterns of three isoforms (BmBrC-Z1, Z2 and Z4) and fibroin genes (FibH, FibL and P25) using semi-RT-PCR (Figure 1A). The results indicated that BmBrC-Zs were expressed from the beginning of IVM to 1st day of the 5th instar stage (V1stD). By qRT-PCR analysis, the relative expression level of BmBrC-Z2 was higher than that of BmBrC-Z4 (Figure 1B). The expression levels of fibroin genes were gradually inhibited at the IVM and the expression levels of fibroin genes started to recover at V1stD when the expression of BmBrC-Zs decreased. Thus, we suspected that BmBrC may negatively regulate the expression of fibroin genes.

Figure 1. Expression patterns of BmBrC-Zs and fibroin genes in Bombyx mori. (A). Expression levels of BmBrC-Zs and fibroins in the PSG from 3rd day of the 4th instar to the 2nd day of the wandering stage by semi-RT-PCR analysis; (B) relative expression level of BmBrC-Zs in PSG at 3rd day of the 4th instar (IV3) to the 3rd day of the 5th instar (V3) by qRT-PCR analysis. RPL3 shown as a control. d—day; h—hour; E—ecdysis; IV—4th instar; M—molting; V—5th instar.
3.2. Transgenic Ectopic Overexpression of BmBrC-Z2 in the PSG

Previous studies on transcription factors regulating the expression of silk protein genes confirmed it in the cellular level [24–27]. To confirm the biologic function of BmBrC-Z2 in vivo, a transgenic vector with ectopic overexpression of BmBrC-Z2 was constructed with a modified FibH promotor, in which the predicted Br–C binding site was deleted (Figure 2A). After embryo injection and screening from 366 eggs, fourteen individuals positive for overexpression of BmBrC-Z2 were obtained. The investigation showed that in the silk glands, there were no obvious phenotypic differences between the transgenic lines and the wild types in the V5thD larvae (Figure 2B). Interestingly, the cocoons of transgenic lines became thinner and lighter color compared with those of the wild types (Figure 2C). The cocoon layer ratios decreased significantly in both the male and female individuals (Figure 2D, \( n = 50 \)). The results indicated that ectopic overexpression of BmBrC-Z2 in the PSG can lead to a decline in the silk protein synthesis.

![Figure 2](image-url)

**Figure 2.** Transgenic overexpression of BmBrC-Z2 in the PSG. (A) Transgenic overexpression vector was constructed to overexpress BmBrC-Z2 in B. mori PSG. The positive transgenic phenotype was observed in the pupae; (B) silk gland phenotype exhibited no obvious changes at the V5thD; (C) cocoons became thinner and lighter and (D) cocoon layer ratio (weight of cocoon layer/total weight of cocoon) was obtained by statistical analysis (\( n = 50 \)). Wild type was used as the control. WT—wild type; OE—overexpression; ***—\( p < 0.001 \).
3.3. Transgenic Overexpression of BmBrC-Z2 Downregulates Gene Expression

To investigate the effect of overexpression of BmBrC-Z2 in the PSG, genes encoding silk proteins and transcription factors were detected by qRT-PCR at the V5thD (Figure 3). The results showed that BmBrC-Z2 was increased significantly in the transgenic line than those of the wild type (Figure 3A). All of the silk protein coding genes, including FibH, FibL and P25, were inhibited in the transgenic line (Figure 3B). Interestingly, the expression of two transcription factors, BmKr-h1 and BmDimm, was also significantly inhibited (Figure 3A). We also tested the endogenous BmBrC-Z4 and found that it was significantly upregulated in the transgenic line (Figure 3A). Our previous study had confirmed that these two transcription factors were important regulators to activate the expression of FibH gene by the JH-signaling pathway [27]. These results not only indicated that the ectopic overexpression of BmBrC-Z2 may inhibit the expression of silk protein genes in the PSG of the V5thD larvae, but also showed that the overexpression of BmBrC-Z2 affected the positive regulatory factors of fibroin genes.

3.4. Exogenous JH Analog Can Rescue the Phenotype of the Transgenic Line Overexpressing BmBrC-Z2

To further determine whether the overexpression of BmBrC-Z2 repressed the JH-signaling pathway, we treated the transgenic line and wild type larvae at V1stD with exogenous JH analog methoprene (JHA), where dimethyl sulfoxide (DMSO) was used as the control (Figure 4). The results showed that the coconum phenotypes of the transgenic line overexpressing BmBrC-Z2 were rescued similar to those of the wild type with the application of JH analog (Figure 4A). Further, the cocoon layer ratios reached 8.9% and 11.9% in the female and male individuals, respectively, which were close to that of the wild type (Figure 4B). The expression levels were tested by qRT-PCR in the transgenic line after JH analog treatment. Interestingly, the expression levels of BmBrC-Z2 and BmBrC-Z4 was inhibited (Figure 4C). The expression levels of BmKr-h1 and BmDimm were significantly increased. In addition, the expression levels of all the three fibroin genes were significantly increased (Figure 4D). These results indicated that the exogenous JH analog can reverse the effect of the ectopic overexpression of BmBrC-Z2 in the PSG to increase the expression levels of silk protein genes.
were obtained by the ectopic overexpression of BmBrC-Z2 using a modified FibH promoter region [24]. However, evidence on their in vivo regulatory functions is still mostly absent. Among most of the previous studies, the activity of FibH gene, such as SGF1, Sage and Dimm, these results may be credible [26,27], but for inhibitors, the cellular-level results may be insufficient to explain the molecular regulatory mechanisms in vivo, as the silk gland is a specialized organ. In this study, we constructed a transgenic line to ectopically overexpress BmBrC-Z2 using the FibH promoter in the PSG at the inter-molting stage. The phenotype and molecular investigation demonstrated that the ectopic overexpression of BmBrC-Z2 not only inhibited the expression of silk protein genes and silk protein synthesis, but also repressed the expression of positive regulatory factors BmKr-h1 and BmDimm, which respond to the JH signal to activate the expression of silk protein genes [27].

Several transcription factors have been reported to inhibit the expression of silk protein genes at the molting stage. Both of FTZ-F1 and FMBP-1 could downregulate the expression of FibH at the cellular level by binding to the regulatory element on its promoter [24,25]. An element −397 to −389 upstream of the FibH promoter was bound by FTZ-F1 to inhibit the promoter activity [25]. FMBP-1 repressed FibH promoter activity by directly binding to the −152 to −125 element in the FibH promoter region [24]. However, evidence on their in vivo regulatory functions is still mostly absent. Among most of the previous studies, the activity of FibH promoter was very weak in the silkworm cell lines. For the activators of FibH gene, such as SGF1, Sage and Dimm, these results may be credible [26,27], but for inhibitors, the cellular-level results may be insufficient to explain the molecular regulatory mechanisms in vivo, as the silk gland is a specialized organ. In this study, to explore whether BmBrC-Z2 could inhibit the expression of silk protein genes in vivo, transgenic lines were obtained by the ectopic overexpression of BmBrC-Z2 using a modified FibH promoter in the

4. Discussion

The transcriptional regulation of silk protein genes has always been a research hotspot in research on silkworms. Silk protein genes are repeatedly turned on and off from the inter-molting to the molting stages and are controlled by a large number of positive and negative transcriptional factors. In this study, we constructed a transgenic line to ectopically overexpress BmBrC-Z2 using the FibH promoter in the PSG at the inter-molting stage. The phenotype and molecular investigation demonstrated that the ectopic overexpression of BmBrC-Z2 not only inhibited the expression of silk protein genes and silk protein synthesis, but also repressed the expression of positive regulatory factors BmKr-h1 and BmDimm, which respond to the JH signal to activate the expression of silk protein genes [27].

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Figure 4. Juvenile hormone (JH) analog rescues the cocoon phenotype of the transgenic overexpression line. (A,B) Phenotype observation and cocoon layer ratio statistics (n = 10). Treatments of DMSO was used as the control; (C,D) relative mRNA levels of genes were investigated by qRT-PCR after treatment of JH analog in the transgenic overexpressing BmBrC-Z2 line. Including: BmBrC-Z2, BmBrC-Z4, BmDimm, BmKr-h1, FibH, FibL, P25. RPL3 was used as the control. *—p < 0.05, **—p < 0.01, ***—p < 0.001; WT—wild type; OE—overexpression.
In insects (Figure 2). In the overexpression--transgenic line, the relative mRNA level of BmBrC-Z2 was significantly increased in the PSG at the fifth instar stage (Figure 3), the expression levels of silk protein genes were also significantly inhibited to almost half of the wild type, thus resulting in a significant reduction in the cocoon weight and cocoon layer ratio. We believed that BmBrC was a strong inhibitor of silk protein genes, but the high efficiency of inhibition is still unknown which need further explore in our next step.

FibH promoter was very specific and strong to promote foreign gene expression in the PSG [42]. Although the promoter was modified by deleting the BmBrC-Z2 binding site to eliminate its influence, the expression level of BmBrC-Z2 in the transgenic line was not as high as we expected. It should be noted that the overexpression of BmBrC-Z2 significantly downregulated the expression of BmKr-h1 and BmDimm in the PSG at the inter-molting stage (Figure 3). Thus, we assumed that the reduction of BmKr-h1 and BmDimm may be the reason behind the low activity of the modified FibH promoter in the overexpression of BmBrC-Z2 in the transgenic line. JH-mediated BmKr-h1 repressed the expression of BmBrC by binding to the BmBrC promoter during the larval-adult metamorphosis [31,44]. Our previous research reported that BmKr-h1–BmDimm cascade response to the JH signal could activate the FibH gene expression [27]. After JH analog treatment of the BmBrC-Z2 overexpression--transgenic line, the expression levels of BmKr-h1 and BmDimm were significantly increased, and then the expression levels of BmBrC-Z2 were inhibited significantly. Moreover, the expression levels of all the three fibroin genes were significantly increased (Figure 4). It is suggested that JH-signaling was inhibited when ectopically overexpressing BmBrC-Z2 in the PSG at the inter-molting stage. However, how BmBrC-Z2 repressed the expression of BmKr-h1 and BmDimm needs to be explored.

In addition, the endogenous BmBrC-Z4 was significantly upregulated in the inter-molting stage and was also downregulated when treatment of JH in the transgenic line (Figures 3A and 4C), suggesting that overexpression of BmBrC-Z2 may function as a feedback regulation on ecdysone-signaling pathway. This present study is the inadequacy of the lack of the in vitro activity analysis of promoters of silk protein genes and genetic loss-of-function of BrC in the silk gland in vivo. The promoters of silk protein genes are almost hardly active in silkworm cell lines, which is not applicable to the negative regulation of BrC to the silk protein genes in vitro. In addition, knocking-out BrC may not be suitable because BrC is very crucial in the hormone-signaling pathway for the development and physiology in the silkworm life cycle [28–30]. Based on the present and previous studies [11,27,31], here, we propose a hypothesis for the regulatory mechanism for the inhibition of the expression of silk protein genes by BmBrC at the molting stage (Figure 5). BmBrC could inhibit the expression of fibroin genes directly by binding to the upstream regulatory elements or indirectly by BmKr-h1 and BmDimm cascade in the JH signal pathway. At the inter-molting stage, JH binds to a heterodimer of BmMet2/SRC, which directly activates the expression of BmKr-h1. BmKr-h1 inhibits the expression of BmBrC-Z2 and meanwhile activates the expression of BmDimm and then activates the expression of the fibroin genes in the silkworm. Therefore, our findings will assist in deciphering a comprehensive regulation mechanism of silk proteins in the silkworm.
Figure 5. Schematic representation of the hypothesis for molecular regulatory mechanism of BmBrC inhibiting the expression of silk protein genes. At the molting stage, 20-hydroxyecdysone (20E) activates the expression of BmBrC. BmBrC-Z2 inhibits the expression of fibroin genes directly by binding to the upstream regulatory elements or indirectly by BmKr-h1 and BmDimm in the JH signal pathway. At the inter-molting stage, JH activates the expression of BmKr-h1. BmKr-h1 inhibits the expression of BmBrC-Z2 and meanwhile activates the expression of BmDimm, then activates the transcription of fibroin genes in the silkworm.

5. Conclusions

The expression regulation of silk protein genes are comprehensive, which repetitively turned on and off in the inter-molting and molting processes. In this study, we found that BmBrC could significantly repress the expression of silk protein genes and several transcriptional regulatory factors by a transgenic ectopic overexpression of BmBrC-Z2 in the inter-molting PSG. It is suggested that at the molting stage BmBrC could inhibit the expression of silk protein genes directly by binding to the upstream regulatory elements or indirectly by BmKr-h1 and BmDimm in the JH signal pathway, however, at the inter-molting stage, BmKr-h1 could inhibit the expression of BmBrC-Z2 and then activate the expression of silk protein genes. Our findings will assist in deciphering a comprehensive regulatory mechanism of silk protein genes by the alternate signals of 20E and JH in the silkworm.

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