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Full Length Research Paper

Effect of inhibiting the expression of insulin-like peptide gene *BBX-B*8 on development and reproduction of silkworm, *Bombyx mori*

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Bombyxin (BBX) of the silkworm, *Bombyx mori*, is an insulin-like peptide (ILP). To further study the function of the *BBX-B*8 gene, the effects of inhibiting the expression of *BBX-B*8 gene on organ development, reproduction and trehalose metabolization of the silkworm were investigated. 48 h after the injection of *BBX-B*8 dsRNA into the 3-day larvae of fifth-instar (10 µg per larvae; 10 µl; the average weight: 3 g/larvae), the mRNA level of *BBX-B*8 in the brain of silkworm was declined strikingly by 37.33%; 72 h after injection, the mRNA level of BBX-B8 was decreased by 30%. After the injection of *BBX-B*8 dsRNA into the 3-day larvae of fifth-instar (1 or 5 µg per larvae; 10 µl), development of pupal wing imaginal discs was suppressed, more deformed adult wings were found. The number of mature eggs in the oviduct respectively increased by 7.86 and 12.62% when pupa were injected with *BBX-B*8 dsRNA (10 or 15 µg per larvae; 10 µl) on the first day of pupation. 72 h after the injection of *BBX-B*8 dsRNA (10 µg per larvae; 10 µl) into the 3-day larvae of fifth-instar, the trehalose level of hemolymph was elevated by 12.68% in silkworm. These results suggest that BBX-B8 plays an important role in organ development, reproduction and the metabolization of trehalose of silkworm.

Key words: Silkworm, insulin-like, RNAi, organ growth, reproduction.

INTRODUCTION

The insulin-like growth factor 1 (insulin/IGF) like signaling pathway plays an important role in growth (Rulifson et al., 2002), development (Kimura et al., 1997; Arquier et al., 2008), fecundity (Tatar et al., 2003), stress resistance (Clancy et al., 2001; Holzenberger et al., 2003), metabolism (Saltiel et al., 2001) and lifespan (Kenyon., 2005; Selman et al., 2008) in most multi-cellular organisms. Insect insulin-like peptides (ILPs) are a structurally diverse group encoded by large multigene families that mainly expressed in brains and are similar in structure to vertebrate insulin but serve different functions (Imami et al., 1998; Sebastian and Linda, 2010).

Bombyxin (BBX) is the first insect insulin-like peptide to be discovered (Nagasawa et al., 1996). And there are also many detailed reports on the gene structure, transcription modality, tissue-specific expression and the evolutionary features of BBX (Kondo et al., 1996; Iwami, 2000; Ishizaki, 2004). Compared with *Drosophila* and other model organisms, we have known little about the functions of BBX of silkworms (*Bombyx mori*). BBX can stimulate the secretory activity of prothoracic glands of pupae (*Samia cynthia ricini Boisduval*) whose brain had been removed (Ichikawa and Ishizaki, 1961), and participate in glucose metabolism (Satake et al., 1997) and hematopoiesis (Nakahara et al., 2006) of silkworm. Moreover, BBX may play an important role in the ovarian development of silkworms (Luc and Kostas, 2003).

As the Lepidoptera model organism (Xia et al., 2004; International Silkworm Genome Consortium 2008), it is undoubtedly that *Bombyx mori* is a suitable subject for the study of the function of ILPs in insects. Compared

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with many vertebrates and other multi-cellular animals whose genotype is single, to date, 7 super-gene families (A, B, C, D, E, F, G) comprising almost 38 types of BBX have been described (Kondo et al., 1996; Ishizaki, 2004). The *B*8 (GenBank Accession No. D00784) of *BBX-B* family which encodes 88 amino acids is highly expressed in the neurosecretory cells of silkworm brain (Iwami et al., 1996, 1998). In this study, to further approach the function of *BBX-B*8, *BBX-B*8 dsRNA synthesized by transcription *in vitro* was injected into fifth-instar larvae or pupae to silence the expression of *BBX-B*8. The results showed that the development of brains and the wing imaginal discs of silkworms were inhibited, the level of trehalose in the hemolymph was elevated and the fecundity was increased by down-regulating the expression of *BBX-B*8.

MATERIALS AND METHODS

Silkworms and the artificial diets

The Kinshu × Showa and Haoyue strains of *B. mori* were used as the experimental animals. The larvae were reared on the artificial diet Silkmate II (Nihon Nosan Kogyo, Yokohama, Japan) or common artificial diet (Sericultural institute of Shangdong province, Yantai) at 25 ± 1°C, under alternating 12 h light and 12 h dark (Sakurai et al., 1984), respectively.

Preparation of double-stranded RNA (dsRNA)

The total RNA was extracted from brains dissected from the 2-day larvae of fifth-instar using the GTPC method (Chomczynski et al., 1987). The cDNA were synthesized with 1 µg of the total RNA by RT-PCR (Iwami et al., 1996), subsequently, the *BBX-B*8 gene was amplified with a pair of specific primers P1 (5'-GGC ACT CGA ATA ACT ACT TTC-3') and P2 (5'- GTA CAG TTT GCA GGT CAC G-3'). The PCR product contains the complete ORF (267 bp) including 65 bp upstream sequence and 29 bp downstream sequence of *BBX-B*8. The plasmids pGEM-B8 was linearized with *Nco*I and *Pst*I, respectively. Sense and antisense RNAs were synthesized *in vitro* using T7 RNA polymerase (Takara, Otsu, Japan) and SP6 RNA polymerase (Takara), respectively. To generate dsRNA, equal amounts of sense and antisense ssRNA were mixed, heated at 95°C (5 min), and gradually cooled to 25°C. After the treatment with RNaseA (Nacalai Tesque, Kyoto, Japan) and DNase RQ1 (Promega) for 45 min at 37°C, the dsRNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) to remove protein and precipitated by ethanol. The RNA precipitate was dissolved in DEPC water and then stored at -20°C. The concentration of the dsRNA was estimated with an ultraviolet spectrophotometer (Shimadzu Bio Spec-1600, Japan). pGEM-7Zf (+) - egfp (Monwar et al. 2008), a gift from Prof. Iwami Masafumi of Kanazawa University, and were linearized with *Xho*I or *Hin*dIII, respectively. The 0.72 kb of *egfp* dsRNA was synthesized according to the method previously metioned.

RNA interference

After being starved for 2 h, the 3-day larvae of fifth-instar was injected with 5 µg (10 µl; the average weight: 3 g/ larvae) or 10 µg (10 µl; the average weight: 3 g/larvae) of *BBX-B*8 dsRNA. A negative control and a blank control of larvae were injected with 10 µg of egfp dsRNA and 10 µl of Insect Ringer buffer (130 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$), respectively. The injected larvae were reared under the standard conditions.

Semi-quantitative reverse transcription-polymerase chain reaction (sqRT-PCR)

The total RNA was extracted from the larva brains at the second day of post-injection. Then the cDNA were synthesized by RT-PCR. Using 0.20 µg of the cDNA as template, semi-quantitative reverse transcription-polymerase chain reaction (sqRT-PCR) was carried out with primers P1 and P2 (mentioned previously). The sq RT-PCR protocol consisted of an initial denaturation at 94°C for 1 min and then 20, 25, or 30 cycles: 94°C for 30 s, 57°C for 60 s, and 72°C for 45 s, respectively. After then the PCR products were separated on agarose gel and a scion image analysis was carried out using a gel imaging system (AE-6932 GXCF, ATTO Japan).The *RpL*3 (GenBank Accession No. AB024901) gene was used as an endogenous reference and sqRT-PCR was performed with the primers P3 (5'-AGC ACC CCG TCA TGG GTC TA-3') and P4 (5'-TGC GTC CAA GCT CAT CCT GC-3').

Western blot

Three days after the injection of dsRNA, the brains and wing imaginal discs (negative control group) of larvae were homogenized on ice and their protein levels determined by Dc Protein Assay-kit (Bio-Rad Dc, USA) according to the manufacturer's instructions, respectively. Brain homogenates were mixed with 4 × Tricine SDS sample buffer (0.05M Tris/Cl, 4% SDS, 12% glycerol, 2% mercaptoethanol, 0.01% Commasie G250). After centrifugation, the supernatant (60 µg of protein, equal to 10 silkworm brains) was used as the sample for Tricine-SDS-PAGE and Western blot analysis (Schägger et al., 1987; Saegusa et al., 1992). A mouse anti-Bombyxin II monoclonal antibody (a gift from Prof. Iwami Masafumi of Kanazawa University) was diluted at 1:10000 (Mizoguchi et al., 1987). Goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) was used at a dilution of 1:20000. The hybrid band was visualized by the Western Blotting Detection System (ECL Advance™ Western Blotting Detection kit). Quantitative analysis was performed with a fluorescence and chemiluminescence image analyzer (FUJIFILM Las-3000, Japan). The experiments were in triplicate.

Effects of down-regulating the expression of *BBX-B*8 gene on Morphology

*BBX-B*8 dsRNA (1 µg or 5 µg per larvae; 10 µl; the average weight: 3 g/ larvae) was injected into the 3-day larvae of fifth-instar, respectively. The pupal wing imaginal discs, the wing shape of moths were observed at different developmental stages. Meanwhile, the sizes of the wing were also measured. The control larvae injected with *egfp* dsRNA (5 µg per larvae; 10 µl; the average weight: 3 g/ larvae) or the Ringer buffer (10 µl) were also investigated.

Effects of down-regulating the expression of *BBX-B*8 gene on fecundity

One day (24 ± 3 h) after pupation, each pupae was injected with 10 or 15 µg (10 µl) of *BBX-B*8 dsRNA (11 female and 11 male involved in each treatment group), respectively. The injected pupae were kept at 25°C under alternating periods of 12 h light and 12 h darkness. After adult emergence, the resulting moths were allowed

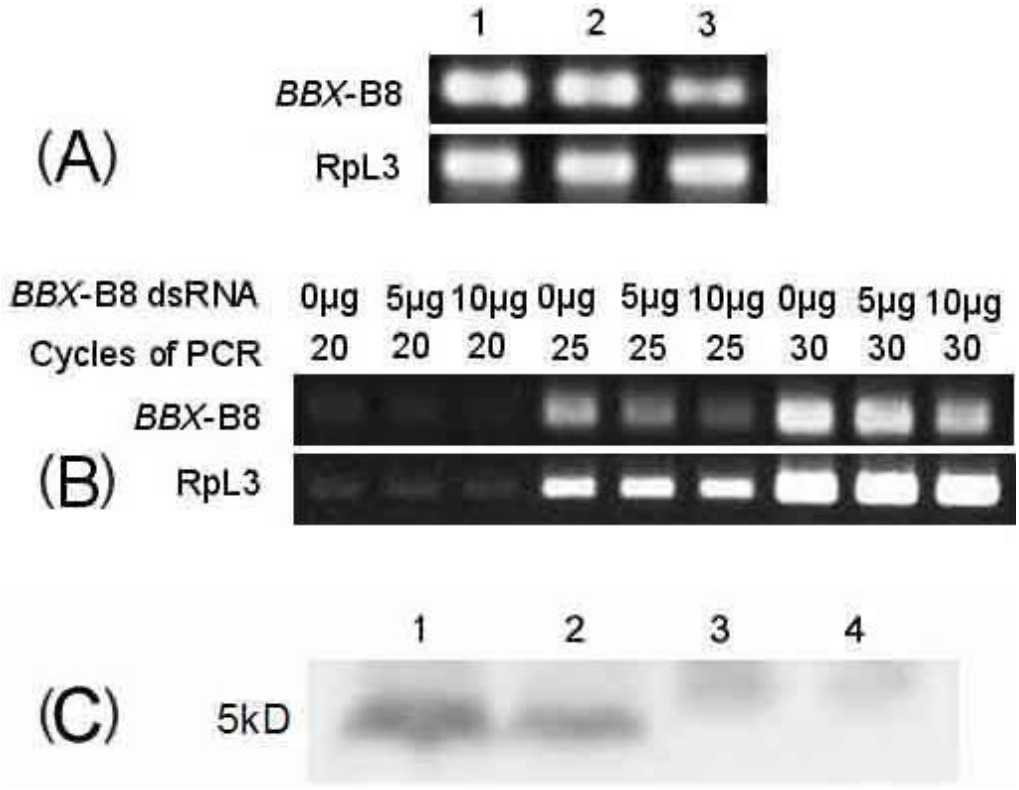

Figure 1. Effects of injected dsRNA into silkworm on *BBX–B*8 expression in the fifth instar larval brain and wing imaginal disks. *RpL*3 was used as an internal standard. (A) Lanes 1, 2, 3 indicated the worm was injected with *egfp* dsRNA (10 µg per larvae), Insect Ringer Buffer (10 µl per larvae) and *BBX-B*8 dsRNA (10 µg per larvae), respectively. (B) Analysis of semi-quantitation RT-PCR for *BBX-B*8 mRNA. (C) Western blot for silkworm brain and wing imaginal discs. Lanes 1 and 2 are western blot for the brains of silkworms injected with *egfp* dsRNA (10 µg per larvae) and *BBX-B*8 dsRNA (10 µg per larvae), respectively; lanes 3 and 4 are western blot for wing imaginal disks of silkworm injected with *egfp* dsRNA (10 µg per larvae) and *BBX-B*8 dsRNA(10 µg per larvae), respectively; The concentrations of the stacking gel, spacer gel, and separating gel was 4, 10 and 16.5%, respectively. Anti-Bombyxin II Mouse monoclonal antibody (1:10000) was used as primary antibody and goat anti-mouse IgG conjugated with HRP (1:20000) was used as the secondary antibody. Total proteins prepared from ten brains or ten wing imaginal disks were added to each lane; *B. mori* varieties: Kinshu × Showa.

to mate and spawn normally and the daily oviposition number was counted for 4 consecutive days. The reproductive tracts of the moths were dissected to record the eggs remaining in the ovarioles on the fifth day. The total produced egg numbers was consistent of the daily oviposition number and the eggs remaining in the oviduct. The total produced egg numbers for control larvae injected with *egfp* dsRNA (10 µg per larvae; 10 µg; the average weight: 3 g/ larvae) were also investigated.

Detection of trehalose activity

Three days old fifth-instar larvae was injected with 10 µg (the average weight: 3 g/ larvae) of *BBX-B*8 dsRNA. At 48 h post-injection, the hemolymph was collected, mixed with phenylthiourea (the final concentration was 1%) and then centrifuged at 5000 rpm for 5 min. The supernatant was used for detection. Trehalose level was determined by the Anthrone method (Pons et al., 1981). The detection of trehalose activity for control injected with *egfp* dsRNA (10 µg per larvae; 10 µl; the average weight: 3 g/ larvae) was also investigated.

RESULTS

Effect of *BBX-B*8 dsRNA on inhibiting the expression of *BBX-B*8 gene

In order to determine the effect of *BBX-B*8 dsRNA on silencing the *BBX-B*8 gene, the level of *BBX-B*8 mRNA was estimated using sqRT-PCR following injection of the larvae with dsRNA. The result is shown in Figure 1A. The amount of *BBX-B*8 mRNA showed no obvious change in the control groups injected either with *egfp* dsRNA or Insect Ringer buffer. In contrast, the amount of *BBX-B*8 mRNA decreased by 37.33% in the experimental group injected with *BBX-B*8 dsRNA (10 µg for each larva). After the PCR products of 20, 25 and 30 cycles were subjected to gel electrophoresis, the results of the NIH Scion Image showed a dose-response effect of *BBX-B*8 dsRNA in silencing the *BBX-B*8 gene (Figure 1B). For instance, the

sample injected with 10 µg dsRNA had a better silencing effect on the *BBX-B*8 gene than that injected with 5 µg dsRNA.

To further verify the effect of *BBX-B*8 dsRNA on silencing *BBX-B*8 gene expression, Western blot and immunofluorescence were performed with anti-Bombyxin mouse monoclonal antibodies. The results showed that a specific band with the molecular weight of 5 kDa representing *BBX-B*8 (GenPept accession No. P26742) could be detected in the extracts of silkworm brain, whereas no specific band was found in the extracts from wing imaginal discs (Figure 1C). The quantitative analysis showed that the level of BBX-B8 dramatically decreased by 30% in the brains after the injection of *BBX-B*8 dsRNA (10 µg per larvae).

Effects of suppression of *BBX-B*8 gene expression on tissue development and morphology

After the injection of *BBX*-B*8* dsRNA (1 µg or 5 µg per larvae; 10 µl) into the larvae, the wing imaginal discs with different degrees of deformity could be observed during the pupal stage, and the deformity rate reached about 10 to 15%. Likewise, many moths with small wings or scrolling wings could be found during the moth stage (Figure 2A). The ratios of moths with small wings were 15.38% (for 1 µg *BBX-B*8 dsRNA per larvae) and 17.50% (for 5 µg *BBX-B*8 dsRNA per larvae), while the ratios of moths with scrolling wings were 25.64% (for 1 µg *BBX*-B8 dsRNA per larvae) and 27.52% (for 5 µg *BBX-B*8 dsRNA per larvae), respectively (Figure 2B). However, there were no effects on the development or shape of the wing imaginal discs in the pupal-instar or the wings of moths in the control groups injected with *egfp* dsRNA.

Effects of suppression of *BBX-B*8 gene expression on fertility

It has been shown that removing insulin-like ligand cells could lead to lower fertility (Susan et al., 2005). Our research indicated that silencing *BBX-B*8 gene expression caused varied degrees of change in the number of mature eggs in the oviduct (Figure 3). The total produced egg numbers for a moth injected with 10 and 15 µg of *BBX-B*8 dsRNA was increased by 7.86 and 12.62%, respectively.

Effects of silencing *BBX-B*8 gene on the level of trehalose

In order to investigate the effects of silencing the *BBX-B*8 gene on glycometabolism in silkworm blood, the levels of trehalose in the hemolymph of silkworms were determined 2 days after injecting *BBX-B*8 dsRNA (10 µg per larvae). The level of trehalose was 12.38 mmol/ml (4♀+4♂, p=0.0001) in the control group injected with *egfp* dsRNA (10 µg per larvae) while it was 13.95 mmol/ml (4♀+4♂, p=0.00001) in the RNAi group injected with *BBX-B*8 dsRNA (10 µg per larvae). Compared with the control group, the level of trehalose in the RNAi group was elevated by about 12.68%.

DISCUSSION

BBX, the insulin-like peptides of silkworms, were mainly synthesized by four pairs of large neurosecretory cells in the pars intercerebralis and finally released into the blood. All the genes of the 7 (A~G) super-gene families could be expressed in the four pairs of large neurosecretory cells (Iwami et al., 1998). In this study, the chosen BBX-B8 was highly expressed in the BBX-B family. The results showed that the levels of the mRNA and the protein of the *BBX-B*8 had a dramatic decline in the silkworms after being injected with *BBX-B*8 dsRNA. There is a high homology among the families of BBX, especially between the family A and B (Nagasawa et al., 1984; Ishizaki, 2004). Therefore, it was assumed that the expression of other *BBX* genes of the super-gene families could also be silenced synchronously by injection of *BBX-B*8 dsRNA into silkworm as well as the expression of *BBX-B*8 gene.

In our research, many seriously defective wing imaginal disks were observed in the newly pupae after injection of fifth instar 3-day larvae with *BBX-B*8 dsRNA to depress the expression of *BBX-B*8 gene (Figure 2Ac). The elevation of Bombyxin level begins at the middle stage of the fifth-instar larvae and sharply gone up after pupation (Iwami et al., 1998). It was known that the rapid growth and the vigorous development of wing imaginal disks last from middle stage of the fifth-instar to the wandering stage before spinning (Nijhout et al., 2007). It therefore appears that *BBX-B*8 is a growth factor for wing imaginal disks in silkworm, because silencing the *BBX-B*8 gene severely depresses their development. In addition, 3-day larvae of fifth-instar were injected with *BBX-B*8 dsRNA; abnormalities were observed in the resulting moth's wings. There were many moths with small and scrolled wings, indicating that decreasing the expression of *BBX-B*8 gene continually suppresses the growth and development of wing imaginal disks. These results are consistent with the studies on the differentiation and development of wing imaginal disks in *Precis coenia* and *Manduca sexta* (Nijhout and Grunert, 2002; Nijhout et al., 2007). The development of wing imaginal discs is regulated by 20E and JH (Koyama et al., 2004). Bombyxin and 20E (20-hydroxyecdysone, 20E) play an important role in the development of wing imaginal discs by acting alone or in combination in *Manduca sexta* (Nijhout and Grunert, 2002; Nijhout et al., 2007). Maybe this hypothesis is also suitable to silkworm.

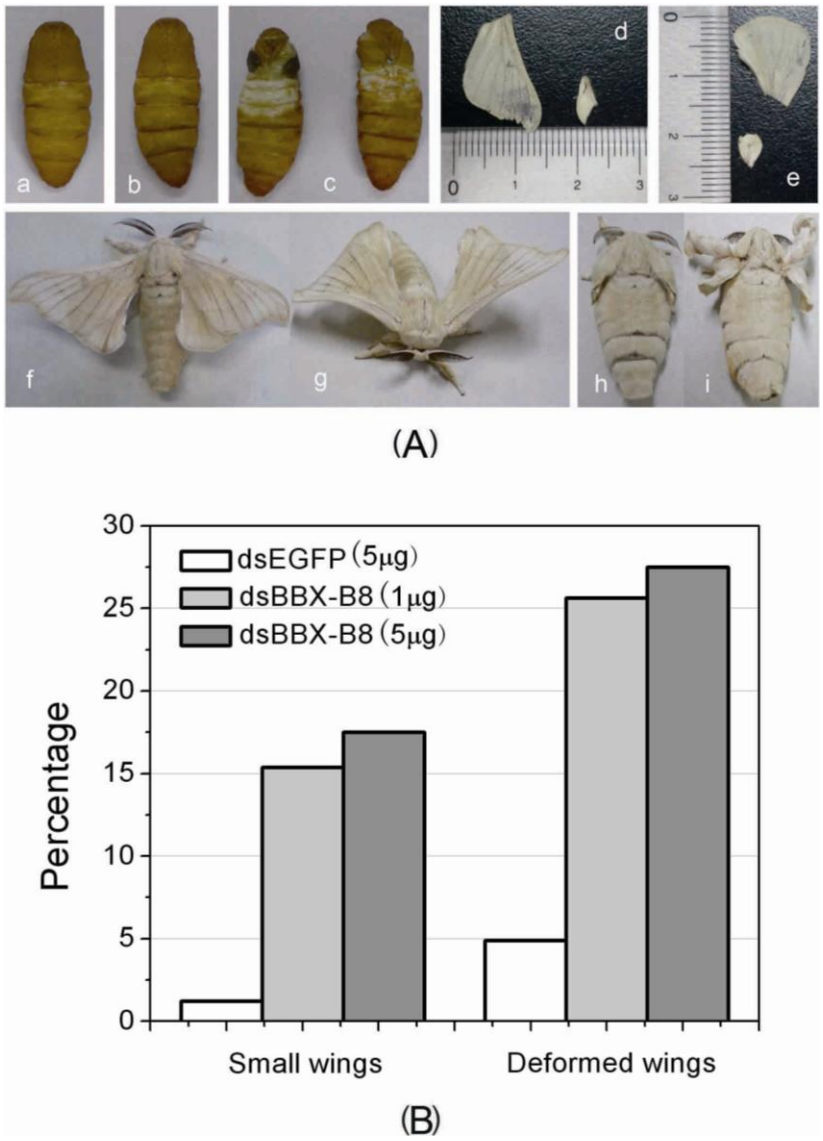

Figure 2. Effect of silencing *BBX-B*8 gene in fifth instar larva on the development of moth wings. (A) Wing imaginal discs and moth wings; (a) injected Ringer buffer; (b) injected *egfp* dsRNA; (c) injected *BBX-B*8 dsRNA; (d) moth anterior wings (left for *egfp* dsRNA), right for injected the *BBX-B*8 dsRNA); (e) posterior wings (upper for injected *egfp* dsRNA, down for injected the *BBX-B*8 dsRNA); (f) wings for injected Ringer buffer; (g) wings for injected *egfp* dsRNA; (h) small wings moth for injected *BBX-B*8 dsRNA; (I) scrolling wings moth for injected *BBX-B*8 dsRNA. (B) Effect of silencing *BBX-B*8 gene in fifth instar larva on proportion of deformed wings. That wings area was 1/5 of the normal wing area is define as small wings. The number of the silkworm injected *egfp* dsRNA (5 μg), *BBX-B*8 dsRNA (1 μg each larvae), *BBX-B*8 dsRNA (5 μg each larvae) was 81, 80 and 79, respectively. Varieties: Kinshu × Showa.

It had been reported that the concentration of trehalose decreased in hemolymph when Bombyxin II was injected into the larva of silkworm. So it was assumed that Bpmbyxin II could promote the depletion of carbohydrate in silkworm (Satake et al., 1997). Huang et al. (2007) also considered that the metabolism of trehalose might be related to the soaring of silkworm. Our research indicated that the concentration of the trehalose in the hemolymph could be elevated by down-regulating the expression level of the BBX-B8 suggesting that reducing the level of the BBX-B8 in the hemolymph of silkworm larvae could promote the accumulation of carbohydrate. BBX may take part in regulating the metabolization of the carbohydrate, down-regulating the expression level of

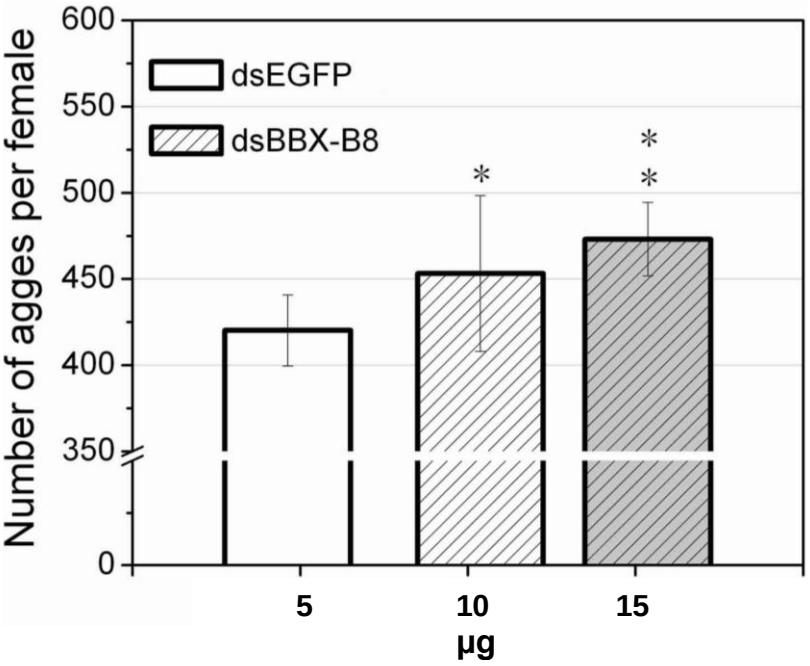

Figure 3. Effect of *BBX-B*8 gene silencing on the total produced egg numbers for each moth. Effect of *BBX-B*8 gene silencing on the number of laid eggs of one moth, the number of samples were 11 female and 11 male moths involved in each treatment group. *, $0.05 > p > 0.01$; **, $p < 0.01$ (paired t test). Varieties: Haoyue.

BBX-B8 during the larva stage, changing the concentration of hemolymph trehalose and affecting the development of the wings and soaring of silkworm.

Using *BBX-B*8 dsRNA to inhibit the expression of BBX-B8, it may reduce the function of one or more BBX from BBX family and cause blood and other tissue BBX decline in titer leading to lowered systemic insulin signaling. The lowered insulin signaling in pupal stage could also make changes in the amount of eggs that may be related to the changes in metabolic activity of carbohydrate. It has been shown that treatment with synthetic ILP3 leads to promotion of ecdysteroid production by the ovaries, while at the same time the metabolic levels of carbohydrate and lipid storage rise in *Aedes aegypti* (Mark et al., 2008). Endogenous IPLs are also involved in regulating the production and activity of ovarian hormone in *Phormia regina* (Manière et al., 2004). Our results indicated that inhibiting the expression of endogenous BBX-B8, a IPL, elevated trehalose levels in hemolymph which resulted in enhancing fecundity, suggesting that *BBX-B*8 is involved in the regulation of carbohydrate metabolism and is a critical regulator of the number of eggs laid by silkworms.

There are tissues or stage-specifics of the expression of insulin-like Bombyxin of silkworm (Iwami et al., 1998; Sebastian and Linda Clemmons et al., 2010; Mizoguchi et al., 1990). Bombyxin may have varied functions in different tissues or in different developmental stages of silkworms. Our research showed that down-regulating the expression of *BBX-B*8 gene can exert an influence on the organ development and the fecundity. So it could be assumed that silkworm insulin-like peptides have different functions in the different developmental stages and tissues, although these phenotypes and the other traits regulated by insulin signaling pathway are not understood.

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