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A novel GATA transcription factor GATAβ4 promotes vitellogenin transcription and egg formation in the silkworm *Bombyx mori*

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ABSTRACT

GATA transcription factors (GATAs) are widely expressed among various organisms and belong to the zinc finger protein family. GATA transcription factors play important roles in the proliferation, differentiation, and development of eukaryotes. Previous studies have shown that GATA participates in oogenesis by selective splicing in silkworms. In this study, we investigated the function of GATAs during vitellogenesis using female silkworms (*Bombyx mori*). Six types of GATA transcription factors were successfully cloned in the fat body of silkworms during the wandering stage and only BmGATAβ4 induced the activity of the *Bombyx mori* vitellogenin (*BmVg*) promoter. Furthermore, *BmVg* and *BmGATAβ4* exhibited similar expression patterns in the fat body of female silkworms during the wandering stage. Electrophoretic mobility shift assays, cell transfection assays, and chromatin immunoprecipitation showed that BmGATAβ4 was involved in regulating the transcription of *BmVg* by directly binding to the GATA *cis*-response element 1 (CRE1) and GATA *cis*-response element 2 (CRE2) in the promoter of the *BmVg* gene. RNA interference of *BmGATAβ4* in female silkworms downregulated *BmVg* transcription, resulting in a decrease in egg size and shortening of the length of egg tubes relative to the control. In summary, our results indicated that BmGATAβ4 bound to the GATA CRE1 and CRE2 motifs in the *BmVg* promoter to upregulate *BmVg* expression in the fat body of female silkworms.

1. Introduction

Vitellogenin (Vg), which is the precursor of the egg yolk protein vitellin, is an essential nutrient for embryo development and egg formation. The amino acid sequence and structure of Vgs are highly conserved among insects and other oviparous species (Sappington et al., 2002). In insects, Vgs are mainly synthesized in the female fat body and then transferred to oocytes (Raikhel and Dhadialla, 1992). Female-specific expression is mainly regulated by the sex-specific transcriptional regulator doublesex (Suzuki et al., 2003). However, the synthesis periods of Vgs differ dramatically, and their regulation models are highly complex; thus, the mechanisms regulating *Vg* expression have been studied extensively.

In fruit fly *Drosophila melanogaster*, there are three yolk protein (*yp*) genes; two of these *yp* genes are specifically expressed in fat body, and the other is specifically expressed in the ovaries in adults. This tissue and stage specificity are regulated by the tissue-specific enhancers and hormones including 20-hydroxyecdysone (20E) and juvenile hormone (JH) (Garabedian et al., 1985; Hutson and Bownes, 2003; Raikhel, 2005). In mosquito *Aedes aegypti*, *Vg* is expressed beginning at 8 h, and expression peaks are at 24 h after blood fed; this process is mainly regulated by nutrition and 20E (Attardo et al., 2005; Raikhel et al., 2002). In German cockroach *Blattella germanica*, *Vg* mRNA begins to appear on day 1 of the adult stage and peaks on day 5; the process is regulated by nutrition and JH (Abrisqueta et al., 2014; Cruz et al., 2003; Suren-Castillo et al., 2012). Additionally, in bean bug *Riptortus clavatus*, *Vg* begins to appear on day 2 of the adult stage in non-diapausing strains. In contrast, in diapause strains, *Vg* expression is observed beginning on day 8 after dissolving diapause, although *Vg* can be detected on day 2 following JH treatment (Hirai et al., 1998). In *Bombyx mori*, *Vg* is expressed on day 2 of the wandering stage, and the expression level reaches a maximum on day 3 of the wandering stage (Yang et al., 2014a, 2014b). However, the mechanisms mediating this specific expression pattern are still unclear.

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Bombyx mori is a model insect from the order Lepidoptera. Previous studies have shown that *BmVg* is regulated by ecdysone; however, there is no ecdysone receptor response element predicted in the regulatory region of the *BmVg* promoter (Yang et al., 2014a). Ecdysone can induce the expression of its early response gene broad complex isoform 2 (BRC-Z2). BRC-Z2 and a member of the HOX family, POU homeodomain transcription factor 2 (POUM2) bind to the adjacent BRC-Z2 *cis*-response element (CRE) and POUM2 CRE in the *BmVg* promoter to induce the upregulation of *BmVg* (Lin et al., 2017; Yang et al., 2014a). Additionally, *Bombyx mori* estrogen-related receptor (BmERR) participates in the regulation of *BmVg* by engaging in crosstalk with the ecdysone receptor pathway in silkworms (Shen et al., 2018). No recent studies have examined the regulation of *BmVg* in silkworms. However, in our previous study, we found that there were 10 GATA-like CREs on the promoter of *BmVg* in *Bombyx mori*. It is still unclear whether these GATA-like CREs can regulate the expression of *BmVg*.

GATA transcription factors (GATAs) belong to the zinc finger protein family and bind to a conserved (T/A) GATA (A/G) motif on the promoter sequence (Orkin, 1992; Patient and McGhee, 2002; Vonderfecht et al., 2008). GATA transcription factors are highly conserved. Six GATA transcription factors are found in mammals, and five are found in fruit flies. GATAs play important roles in the proliferation, differentiation, and development of eukaryotes (Patient and McGhee, 2002). Additionally, in mosquitos, amino acid signaling can induce the synthesis of GATA after a blood meal, which in turn upregulates the expression of *Vg* (Attardo et al., 2005; Park et al., 2006). In hard tick *Haemaphysalis longicornis*, GATA is synthesized after a blood meal and activates the expression of *Vg* (Boldbaatar et al., 2010). In brown planthopper *Nilaparvata lugens*, high- and low-fecundity populations have been identified based on a single nucleotide polymorphism in the GATA-1 CRE on the promoter of *Vg* (Sun et al., 2018). Notably, the activity of GATA-1 CRE in the high-fecundity population is higher than that in the low-fecundity population, leading to high expression of *Vg* and high-fecundity ability.

However, some studies have evaluated GATA function in silkworms. For example, in 1994, Drevet et al. identified two GATA transcription factors (GATAα and GATAβ) in genome of the silkworm (Drevet et al., 1994); they confirmed that GATAβ encoded the chorion transcription factor BCFI through a series of experiments. Subsequent research showed that BmGATAβ had various alternative splicing types during different developmental stages; BmGATAβ1 is specifically expressed in the gonads, BmGATAβ2 is expressed in various tissues during larvae and pupa stages, and BmGATAβ3 is only expressed in follicular cells (Drevet et al., 1995). Subsequently, researchers showed that GATA plays an important role during choriogenesis (Lecanidou and Papantonis, 2010; Papantonis et al., 2008; Sourmeli et al., 2003). However, the roles of the GATA transcription factors in vitellogenesis are still unknown in silkworms.

In this study, we explored whether GATA was involved in the transcriptional regulation of *BmVg* and the formation of eggs in silkworm. Our results provide important insights into the roles of GATA transcription factors in silkworms and are expected to facilitate further studies of the regulatory networks of *BmVg* in silkworms.

2. Materials and methods

2.1. Animal strain

The silkworm strain dazao was obtained from the silkworm gene bank of the Southwest University of China. The larvae were reared on fresh mulberry leaves (*Morus* sp.) and began wandering on day 7–8 of the last instar stage. We collected fat body samples from wandering to day 3 after pupation for total RNA isolation to analyze the expression of *BmGATAβ4*.

2.2. RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, WI, USA) was used to synthesize the corresponding cDNA.

A SYBR Green Kit (TaKaRa Biotech, Osaka, Japan) was used for qPCR according to the manufacturer's instructions. The relative mRNA levels of the target genes were normalized to the internal marker gene translation initiation factor 4A, and expression levels were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Three independent replicates were performed for each experiment. The primers for qPCR are shown in Table S1.

2.3. Cloning and sequence analysis of GATAs

The sequences of *GATA6*, *GATA-A-X3*, and *GATAβ* were obtained from the National Center for Biology Information (NCBI) with accession numbers XM_004924410.3, XM_012688907.2, and NM_001043981.1, respectively. Using sequences in SILKDB (http://silkworm.genomics.org.cn/) and transcriptome data of the fat body in pupae (transcriptome data provided by our research team), we designed primers to clone GATAs in the fat body. The primers are given in Table S1. The protein sequences of GATAs were translated using the Expasy-translate tool (https://web.expasy.org/translate/). Other amino acid sequences of GATAs were obtained from NCBI to generate a phylogenetic tree using MEGA7 software. The accession numbers of GATAs are shown in Table S2.

2.4. Vector construction

The upstream regulatory sequence of the *BmVg* gene promoter was amplified from the silkworm genome and truncated to different fragment lengths according to the position of the GATA CREs. Promoters with different lengths were digested with the restriction enzymes *Xho*I and *Hind*III and then ligated to the pGL3-basic vector (Promega). The constructed vectors were designated pGL3-VgP1642 Luc, pGL3-VgP1573 Luc, pGL3-VgP1342 Luc, pGL3-VgP1281 Luc, pGL3-VgP1112 Luc, pGL3-VgP1084 Luc, pGL3-VgP499 Luc, pGL3-VgP486 Luc, pGL3-VgP89 Luc, and pGL3-VgP64 Luc. To analyze the effects of the first two GATA CRE groups, vectors were ligated to the base promoter 78 ML (a basic promoter that does not contain any cis-regulatory element except TATA box, stored in our laboratory) to generate pGL3-78 ML Luc, pGL3-78 ML + CRE1 Luc, and pGL3-78 ML + CRE2 Luc.

Mutation vectors of pGL3-VgP499 Luc with disruption of the GATA CREs were constructed by site-specific mutagenesis. The mutation sequences were amplified using a unique primer containing the mutated GATA CRE. The core sites of GATA CRE1, i.e., “TTATCT” and “ACTA TCT”, were changed to “CCGCTC” and “GTCGCTC”, respectively. The GATA CRE2 sequence “CTATCA” was changed to “TCGCTG”. The mutation reporter vectors were designated pGL3-VgP499-M1 Luc, pGL3-VgP499-M2 Luc, and pGL3-VgP499-M Luc.

The open reading frames of GATA6, GATA-A, and GATAβ were amplified from the cDNA of the fat body on day 3 after wandering and cloned into the psl1180-Hr3-A4 SV40 expression vector (stored in our laboratory) with *Bam*HI and *Not*I or *Sph*I. The primers for vector construction are given in Table S1.

2.5. Cell culture and transfection assay

The *B. mori* embryo cell line BmE-SWU1 (stored in our laboratory) was maintained at 27 °C in Grace's insect medium (GIBCO, Invitrogen, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; HyClone, Thermo Fisher, Waltham, MA, USA).

Cell cotransfection assays were carried out using X-tremeGENE HP

DNA Transfection Reagent (Roche, Basel, Switzerland), as previously described (Liu et al., 2018). The PRL-VgP78M vector was transferred as an inner control (Liu et al., 2018). The ratio (w/w) of reporter vector with *BmVg* promoter to control reporter vector PRL-VgP78M was 1:0.1. The ratio (w/w) of reporter vector with *BmVg* promoter to psl1180-Hr3-A4 BmGATAβ4 SV40 was 1:1.

After culturing for 7 h, the transfection mixture was replaced with 500 μL fresh medium with 10% FBS. The cells were cultured for an additional 48 h and then harvested for luciferase activity assays. Luciferase activity was measured using commercially available kits (Promega) according to the manufacturer's instructions. Transfection was repeated three times independently, and the average luciferase activity was presented as the mean ± standard error. The significance of differences in regulatory activity was analyzed using Student's *t*-tests.

2.6. Immunofluorescence histochemistry

BmE-SWU1 cells transfected with BmGATAβ4 fused with the Flag tag were fixed in 4% (v/v) formaldehyde for 10 min at room temperature (25 °C) and blocked with 5% bovine serum albumin in phosphate-buffered saline (PBS) containing 0.5% TritonX-100 (Sigma, Santa Clara, USA) for 1 h at room temperature. Cells were then incubated with primary anti-Flag tag antibodies, followed by secondary Alexa FluorVR 594 Donkey Anti-Mouse IgG (H + L) antibodies (Invitrogen) for 1 h each. The primary and secondary antibodies were both diluted 1:500 in PBS with 0.2% Tween20 (Sigma) and washed three times after each step. 4′,6-Diamidino-2-phenylindole (DAPI) was used to stain cell nuclei. Labeled cells were visualized with a fluorescence microscope (EVOS FL Auto; Life, USA).

2.7. Electrophoretic mobility-shift assay (EMSA)

To analyze whether BmGATAβ4 could bind to the putative GATA CRE in the *BmVg* promoter, EMSAs were used, as previously described (Hellman and Fried, 2007). The probe was labeled with biotin at the 5′ terminus (synthesized by BGI, Beijing, China). On day 1 after pupation, nuclear proteins were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, China). Recombinant BmGATAβ4 was expressed with a sumo tag in *Escherichia coli* and purified with a nickel affinity column. EMSAs were carried out according to the manufacturer's instructions (EMSA/Gel-Shift Kit; Beyotime Biotechnology). The DNA-protein binding reaction mixtures were loaded onto 5% (w/v) native polyacrylamide gels and electrophoresed in TBE buffer (45 mM Tris-borate, 1 mM ethylenediaminetetraacetic acid, pH 8.3). After electrophoresis, the protein/nucleic acid complexes were transferred electrophoretically to a nylon membrane (Roche, Indianapolis, IN, USA). Horseradish peroxidase-labeled anti-biotin antibodies were detected using an enhanced chemiluminescence system (Thermo, USA) with a Clinx ChemiScope 3400 Mini instrument (Science Instrument, China).

2.8. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed with an EZ-ChIP kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Cells overexpressing Flag-BmGATAβ4 were fixed in 4% (v/v) formaldehyde for 10 min at room temperature, and DNA fragments were disrupted by sonication. The lysates were incubated with immunoglobulin G isotype antibodies and anti-Flag antibodies to precipitate protein/nucleic acid complexes. The DNA fragments were purified on a column using a gel extraction kit protocol (Imega, Norwalk, CT, USA). The DNA fragments were then analyzed by PCR and confirmed by sequencing. The primers used for ChIP are shown in Table S1.

2.9. RNA interference (RNAi) assays

Based on the many splicing forms of BmGATAβ, we select a unique nucleic acid sequence of BmGATAβ4 (amino acid positions 236–289) to synthesize double-stranded RNAi fragments using the T7 RiboMAXTM Express RNAi System (Promega). dsRNA fragments of the enhanced green fluorescent protein (*EGFP*) gene were used as a negative control. The primers used for this assay are described in Table S1.

For RNAi in cells, due to the low expression of *BmVg* in cells, 1180-Hr3-A4-BmGATAβ4 SV40 were first transfected to BmE-SWU1 cells to increase the expression of *BmVg*. Subsequently 1 μg interference fragments were added to each well of cells. In order to analyze the effects of *BmGATAβ4* interference on the activity of promoter containing GATA-like CRE activity, pGL3-78 ML + CRE1 Luc was also transferred into cells. The cells were then collected after 48 h, and gene expression and luciferase activity were evaluated.

For RNAi in individuals, we injected 10 μg/head dsRNA on day 2 of the wandering stage and day 2 of pupae. After eclosion, eggs in the abdomen were harvested for detection of BmVg protein concent.

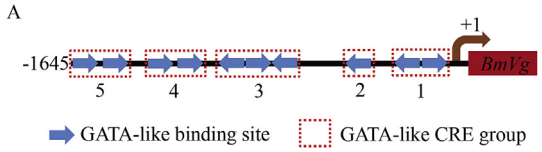
3. Results

3.1. GATA-like CREs on the BmVg promoter

To analyze the effects of GATAs on transcriptional regulation of the *BmVg* gene, we evaluated the 1682 bp (−1642 to +40) upstream from the translation initiation site of *BmVg* and then predicted the GATA binding sites in this sequence. In total, 10 GATA-like CREs were found in the promoter of *BmVg* (Fig. 1A). In order to further analyze the effects of these GATA-like CREs on the activity of the *BmVg* promoter, we grouped the GATA-like CREs according to the positions of these CREs into five groups (Fig. 1A). The specific GATA binding motifs (T/A)GATA(A/G) of each group were identified according to the GATA conserved binding motif (Fig. 1B).

3.2. Cloning and structural analysis of BmGATAs

In order to identify GATAs associated with the expression of *BmVg*, the cDNA of the fat body on days 1 and 3 after wandering were used as templates to clone *GATA* genes in the absence or presence of *Vg* expression. Based on these experiments, we successfully cloned six GATA transcription factors, i.e., *GATA6*, *GATA-A-X3*, *GATA-A-X5*, *GATAβ4*,



B

	sequence (5′–3′)
1	ATTTTATCTGTGTTTACTATCTAGG
2	AGGCTATCAGCA
3	TCTAATAGAAAAGATGATAAATAGATCA
4	GACAGATAGGTT……TTCAGATAGTAA
5	AGAAGATAAAAA……GACACTATCAGAA

Fig. 1. The putative GATA-like CREs in regulatory region of the *BmVg* gene. A. Schematic illustration of GATA-like CREs in the regulatory region of the *BmVg* gene. "+1" represents the transcription start site, the box indicates a GATA group, and the numbers below the boxes refer to the group number. B. The nucleotide sequences of GATA-like CREs in the regulatory region of the *BmVg* gene. The core nucleotide sequences of GATA-like CREs are underlined. The ellipses in groups 3 and 4 indicate that many nucleotides were omitted in the middle.

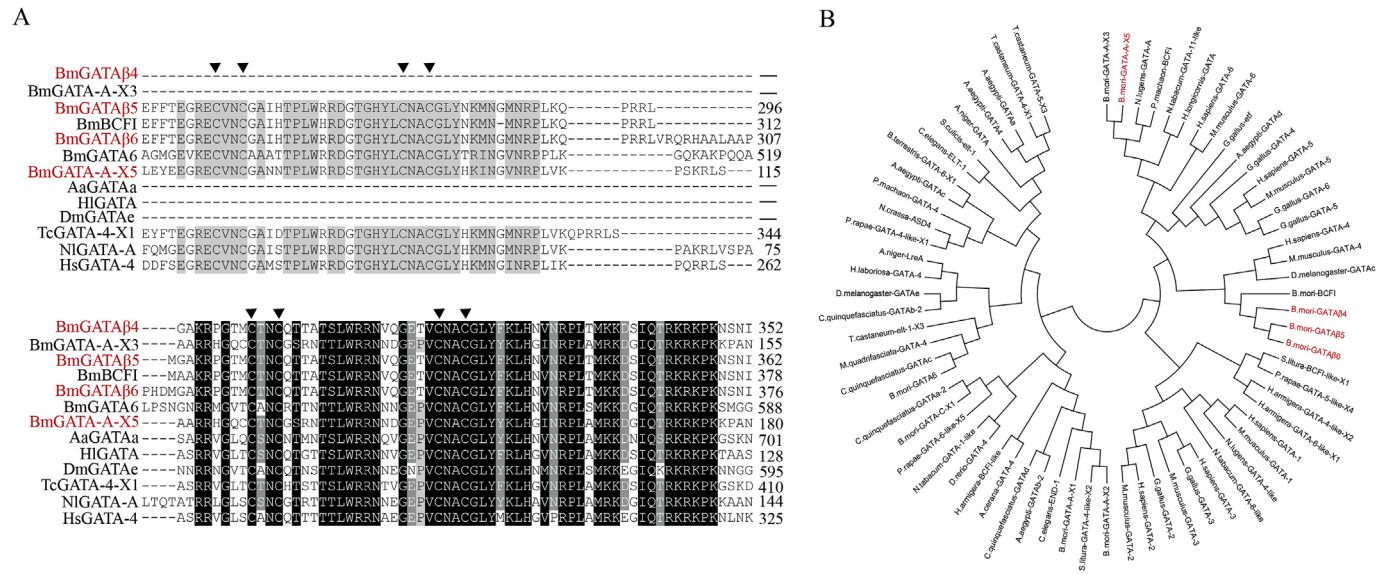


Fig. 2. Analysis of the cloned GATA transcription factors in silkworms. A. Sequence alignment analysis of the GATA zinc finger domains. B. Phylogenetic tree analysis of the cloned GATA transcription factors. The red font were newly cloned GATA transcription factors in silkworm, and their accession numbers were MK033958, MH378168, MK033956 and MK033957 respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

GATAβ5, and *GATAβ6* in the fat body. *GATA-A-X5*, *GATAβ4*, *GATAβ5,* and *GATAβ6* were new cloned, and their accession numbers in NCBI were MK033958, MH378168, MK033956 and MK033957 respectively. After predicting the GATA domains of these GATA transcription factors, we found that the GATA domains were highly similar to GATAs in other species (Fig. 2A), indicating that the GATA transcription factors were highly conserved in silkworms. At the same time, we performed phylogenetic tree analysis of the cloned GATAs and GATAs reported in the NCBI database (Fig. 2B). The results showed that BmGATAβ4, BmGATAβ5 and BmGATAβ6 were clustered with GATAc transcription factors from *Drosophila melanogaster*. BmGATA-A-X3 and BmGATA-A-X5 were clustered with GATA-A from *Nilaparvata lugens*. In contrast, BmGATA6 was closer to GATAc from *Culex quinquefasciatus*.

3.3. *BmGATAβ4 upregulated the activity of the BmVg promoter*

To analyze whether these GATA transcription factors were involved in the transcriptional regulation of *BmVg*, we constructed overexpression vectors of these GATA transcription factors. These GATA transcription factors were then cotransfected with pGL3-VgP1642 Luc into BmE-SWU1 cells, and the expression level of the GATA transcription factor was significantly higher in the cells transfected with the corresponding GATA transcription factor than in the control group respectively (Fig. 3A–F). Luciferase activity analysis showed that the activity of the *BmVg* promoter was significantly upregulated only when BmGATAβ4 was overexpressed at the cellular level which increased the promoter activity about 1.5 folds (Fig. 3G). This suggested that BmGATAβ4 had regulatory effects on the transcription of the *BmVg*.

To further confirm the regulation of BmGATAβ4, we first examined the subcellular localization of BmGATAβ4 using an anti-Flag tag antibody. The results showed that Flag-tagged BmGATAβ4 was localized in the nucleus (Fig. 4A), suggesting BmGATAβ4 could affect the transcriptional regulation of *BmVg*. Subsequently, we analyzed the expression of *BmGATAβ4* in the fat body during metamorphosis. The results showed that the expression of *BmGATAβ4* was consistent with that of *BmVg* (Fig. 4B). Accordingly, from BmGATAβ4 expression profile and subcellular localization, BmGATAβ4 may be involved in the transcriptional regulation of *BmVg*.

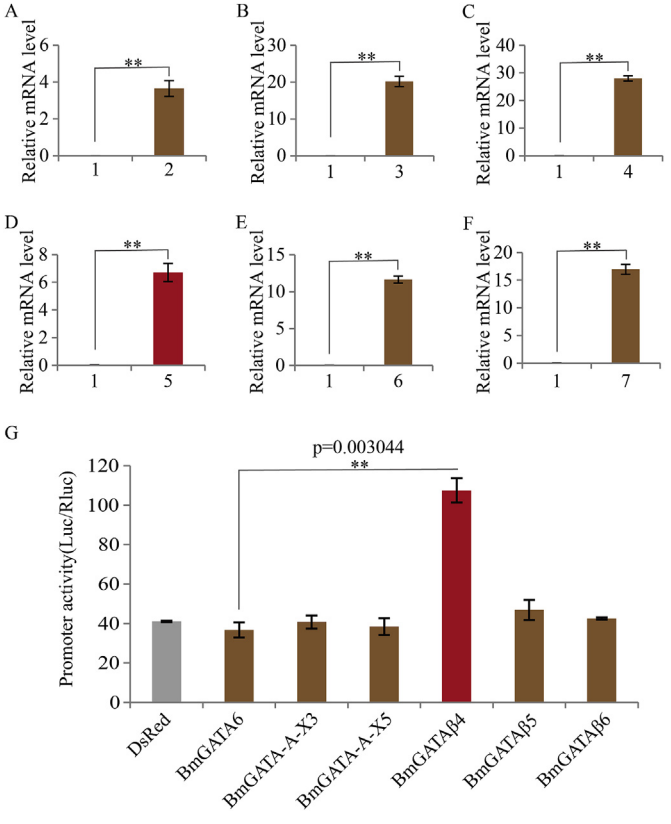


Fig. 3. Analysis of the cloned GATA transcription factors on the activity of the *BmVg* promoter. A-E. qRT-PCR analysis of different GATA transcription factors transcripts in transfected BmE-SWU1 cells. A. The mRNA level of BmGATA6; B. The mRNA level of BmGATA-A-X3; C. The mRNA level of BmGATA-A-X5; D. The mRNA level of BmGATAβ4; E. The mRNA level of BmGATAβ5; F. The mRNA level of BmGATAβ6. Different GATA transcription factors were co-transfected with *BmVg* promoter to BmE-SWU1 cells. 1, DsRed; 2, BmGATA6; 3, BmGATA-A-X3; 4, BmGATA-A-X5; 5, BmGATAβ4; 6, BmGATAβ5; 7, BmGATAβ6. G. Effects of different GATA transcription factors on the activity of the *BmVg* promoter.

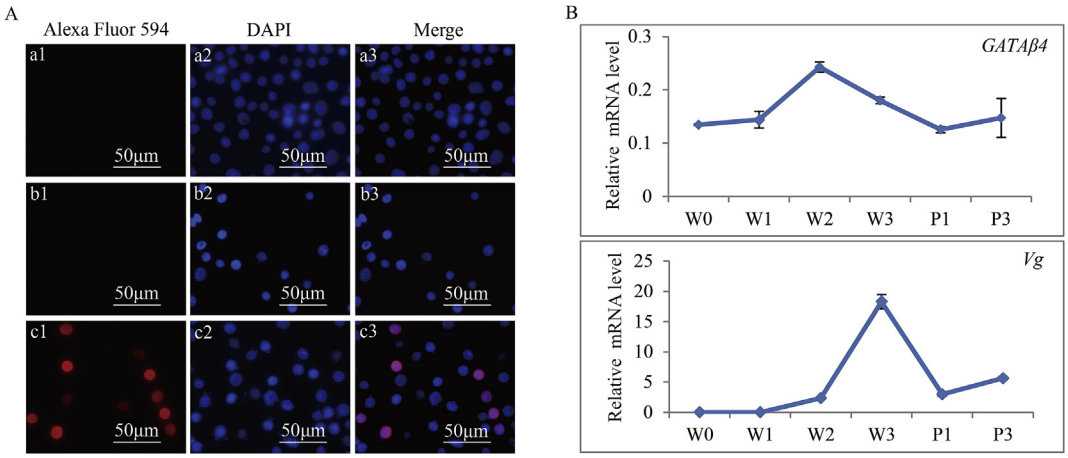


Fig. 4. Localization and expression pattern of BmGATAβ4. A. The subcellular localization of BmGATAβ4 in BmE-SWU1 cells. BmGATAβ4 fused with a Flag tag (BmGATAβ4-Flag) was transfected into BmE-SWU1 cells. The red fluorescence signal (Alexa Fluor 594) represents BmGATAβ4-Flag protein in the nucleus; DAPI was used to stain nuclei. Cells were transfected with BmGATAβ4-Flag in a and c, while cells were transfected with enhanced green fluorescent protein (EGFP) in b as a negative control; anti-IgG was used as a control in a, and anti-Flag was used in b and c. B. qRT-PCR analysis of *BmGATAβ4* and *BmVg* transcripts during metamorphosis in female silkworms. W0-W3, wandering stage (numbers are days); P1, P3, pupa stage (numbers are days). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. BmGATAβ4 enhanced the activity of the BmVg promoter through GATA-like CRE1 and CRE2

To analyze whether BmGATAβ4 increased the activity of the *BmVg* promoter via GATA-like CREs and to determine which GATA-like CRE groups were involved, we shortened the promoter to two lengths with or without the GATA-like CRE group to construct the cell transfection vector. The vectors were then cotransfected with the psl1180-Hr3-A4 BmGATAβ4 SV40 overexpression vector into BmE-SWU1 cells. After BmGATAβ4 was overexpressed, the activities of the promoter with GATA-like CRE1 and CRE2 were significantly higher than those without these elements (Fig. 5A). These demonstrated that BmGATAβ4 enhanced the activity of the *BmVg* promoter throngh GATA-like CRE1 and CRE2.

In order to better analyze the effects of GATA-like CRE1 and CRE2, excluding the influences of other CREs, we ligated GATA-like CRE1 and CRE2 into the basal promoter 78 ML to construct corresponding cell transfection vectors. These vectors were then cotransfected with the psl1180-Hr3-A4 BmGATAβ4 SV40 overexpression vector. Luciferase activity analysis revealed that BmGATAβ4 also could upregulate the activity of the promoter through CRE1 and CRE2 (Fig. 5B). At the same time, we mutated the GATA-like CRE1 and CRE2 sites on the promoter to analyze the effects of BmGATAβ4 on the *BmVg* promoter. Luciferase activity showed that BmGATAβ4 still could increase the activity of the promoter when GATA-like CRE1 and CRE2 were each individually mutated alone; however, this effect was significantly lower than that in the control group. When both GATA-like CRE1 and CRE2 were mutated, BmGATAβ4 did not increase the activity of the *BmVgP499* promoter (Fig. 5C). These findings indicated that BmGATAβ4 induced the activity of the *BmVg* promoter through GATA-like CRE1 and CRE2.

3.5. BmGATAβ4 upregulated BmVg by directly binding to GATA-like CRE1 and CRE2 in the BmVg promoter

The main vitellogenic stages for silkworm is from the day 2 of wandering stage to the adult stage. To investigate whether BmGATAβ4 induced the expression of *BmVg* by binding to GATA-like CRE1 and CRE2, we synthesized GATA-like CRE1 and CRE2 probes labeled with biotin and used these probes to perform EMSAs with nucleoproteins on day 1 after pupation when BmVg and BmGATAβ4 were all expressed. The results showed obvious lag bands for both the GATA-like CRE1 and CRE2 probe sets (Fig. 6A and B). When the nucleoproteins were upregulated, the lag bands were gradually enhanced; in contrast, with the addition of competitive probes, the signal was gradually weakened, indicating that some nucleoproteins were able specifically to bind to GATA-like CRE1 and CRE2.

To further analyze whether the bound nucleoprotein was BmGATAβ4, BmGATAβ4 protein fused with the recombinant sumo tag was expressed and purified, incubated with GATA-like CRE1 and CRE2 DNA probes in vitro, and evaluated by EMSA. The results showed that obvious lag bands were formed; these bands became stronger when more recombinant BmGATAβ4 was added and weaker when a greater amount of competitive probe was added (Fig. 6C and D). These results confirmed that BmGATAβ4 could bind to GATA-like CRE1 and CRE2 in vitro. Accordingly, we then examined whether BmGATAβ4 could regulate the activity of the *BmVg* promoter by binding to GATA-like CRE1 and CRE2 using ChIP with anti-Flag antibody in BmE-SWU1 cells in which Flag-tagged BmGATAβ4 protein was overexpressed. The results of ChIP assays and sequencing further confirmed that BmGATAβ4 bound to GATA-like CRE1 and CRE2 in the *BmVg* promoter (Fig. 6E and F) and suggested that BmGATAβ4 enhanced the transcription of the *BmVg* by binding directly to GATA-like CRE1 and CRE2.

3.6. Downregulation of BmGATAβ4 affected BmVg expression and egg formation

To analyze the role of BmGATAβ4 in the regulation of *BmVg*, dsRNAs targeting *BmGATAβ4* and the *EGFP* gene (as a control) were synthesized. We performed RNAi experiments in BmE-SWU1 cells to detect the effectiveness of the dsRNAi fragments. We added dsRNA fragments to cells transfected with pGL3-VgP78 ML + CRE1 Luc and psl1180-Hr3-A4 BmGATAβ4 SV40 overexpression vector. Cells were collected after 48 h to analyze gene expression and luciferase activity. The results showed that compared with the control, *BmGATAβ4* mRNA levels were significantly downregulated after the addition of dsGATAβ4 fragments (Fig. S1A). After downregulation of *BmGATAβ4*, the expression of endogenous *BmVg* was significantly decreased (Fig. S1B), and the transcription level of externally transferred *Luc* was also significantly decreased (Fig. S1C). Dual luciferase activity was significantly reduced after knockdown of *BmGATAβ4* (Fig. S1D). These results indicated that the selected interference fragment for *BmGATAβ4* was effective and that BmGATAβ4 affected the expression of *BmVg* at the cellular level.

Subsequently, dsRNAs targeting *BmGATAβ4* and *EGFP* were injected

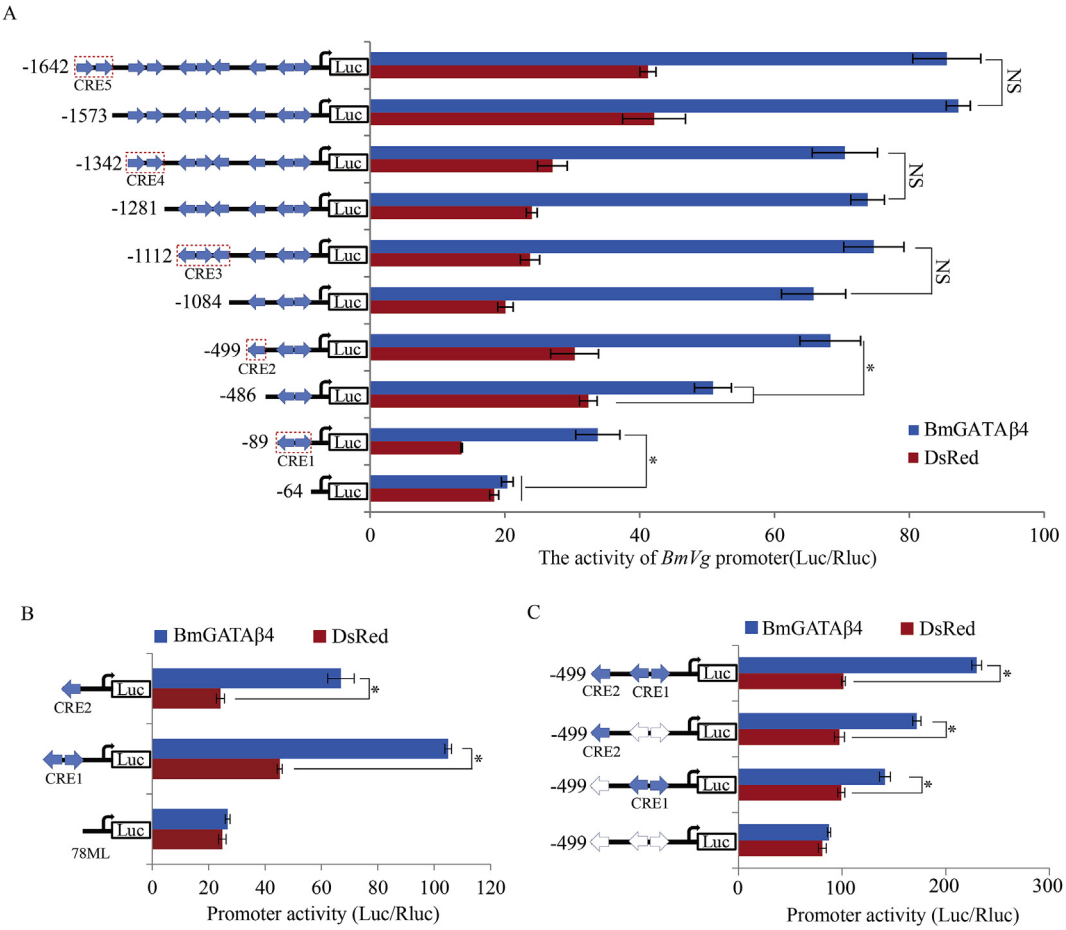


Fig. 5. BmGATAβ4 increased the activity of the *BmVg* promoter through GATA-like CRE1 and CRE2 in BmE-SWU1 cells. A. Effects of GATA-like CRE groups on the activity of the *BmVg* promoter. Different length *BmVg* promoter were cotransfected with the BmGATAβ4 expression plasmid psl1180-Hr3-A4 BmGATAβ4 SV40 into BmE-SWU1 cells. B. The effects of BmGATAβ4 on the activity of the 78 ML basal promoter linked with GATA-like CRE1 and CRE2. C. Effects of mutation of GATA-like CRE1 and CRE2 on the luciferase activity of the pGL3-VgP499 Luc reporter. The left panel shows a schematic representation of the various reporter constructs, and the right graph shows the means ± standard deviations (n = 3). The significance of the difference between data sets was calculated using two-tailed Student's *t*-tests; NS, no significant difference; *, $P < 0.05$.

twice into female silkworms on day 2 of the wandering stage and day 2 of the pupae, respectively. The differentiation of the ovary was incomplete, and the egg ducts were shorter compared with that in dsEGFP-treated pupae (Fig. 7A). Eggs in moths treated with dsBmGATAβ4 were smaller than those in moths treated with dsEGFP (Fig. 7A). Meanwhile, the eggs also weighed less in moths treated with dsBmGATAβ4 (Fig. 7B). Subsequently, silkworms were sacrificed at 48 h after the first injection, and qRT-PCR showed that the expression of *BmGATAβ4* in the fat body was downregulated by RNAi of *BmGATAβ4* (Fig. 7C). Additionally, we found that the transcript levels of *BmVg* were also significantly decreased in the fat body (Fig. 7D) and that the content of BmVg protein was also significantly reduced in the fat body (Fig. 7E). Western blotting showed that the content of BmVg in eggs was decreased by treating with dsRNAs of *BmGATAβ4* (Fig. 7F). These results indicated that BmGATAβ4 regulated *BmVg* transcription in the fat body of female silkworms and played an important role in egg formation and female reproduction.

4. Discussion

The transcriptional regulation of *BmVg* is a complex process that is mainly regulated by nutrients, hormones, and tissue-specific transcription factors. Our previous studies focused on the regulation of *BmVg* by ecdysone, which was found to regulate the expression of *BmVg* through its early response factor BRC-Z2 combined with the hox family transcription factor POUM2 (Lin et al., 2017; Yang et al., 2014a). However, we predicted a number of other transcription factor binding sites in the promoter of *BmVg*, including sites for GATA, DNA replication-related element factor, boundary element-associated factor, Fushi tarazu and so on. The potential effects of these transcription factors on the regulation of *BmVg* are still unclear. In order to further elucidate the transcriptional regulation of *BmVg*, we here focused on GATA transcription factors.

GATAs play important roles in the proliferation, differentiation, and development of eukaryotes. Multiple different GATAs are expressed in eukaryotes and have various functions; however, few studies have examined the functions of these proteins in silkworms. Ten GATA-like CREs were predicted on the promoter of *BmVg*; therefore, we speculated that GATA transcription factors may be involved in the regulation of *BmVg*. In order to test this hypothesis, six transcripts for three GATA genes were cloned in the fat body on days 1 and 3 during wandering. Only BmGATA6 had been reported in silkworms previously. Many forms of GATA transcription factors exist in the fat body during metamorphosis, thus further studies are needed to determine their functions and whether these proteins participate in metamorphosis. Only BmGATAβ4 could enhance the activity of the *BmVg* promoter in BmE-SWU1 cell line. Accordingly, in this study, we focused on the roles of GATAβ4 in the transcriptional regulation of the *BmVg*. However, whether other GATA transcription factors have effects on the transcriptional regulation of *BmVg* at the individual level requires further

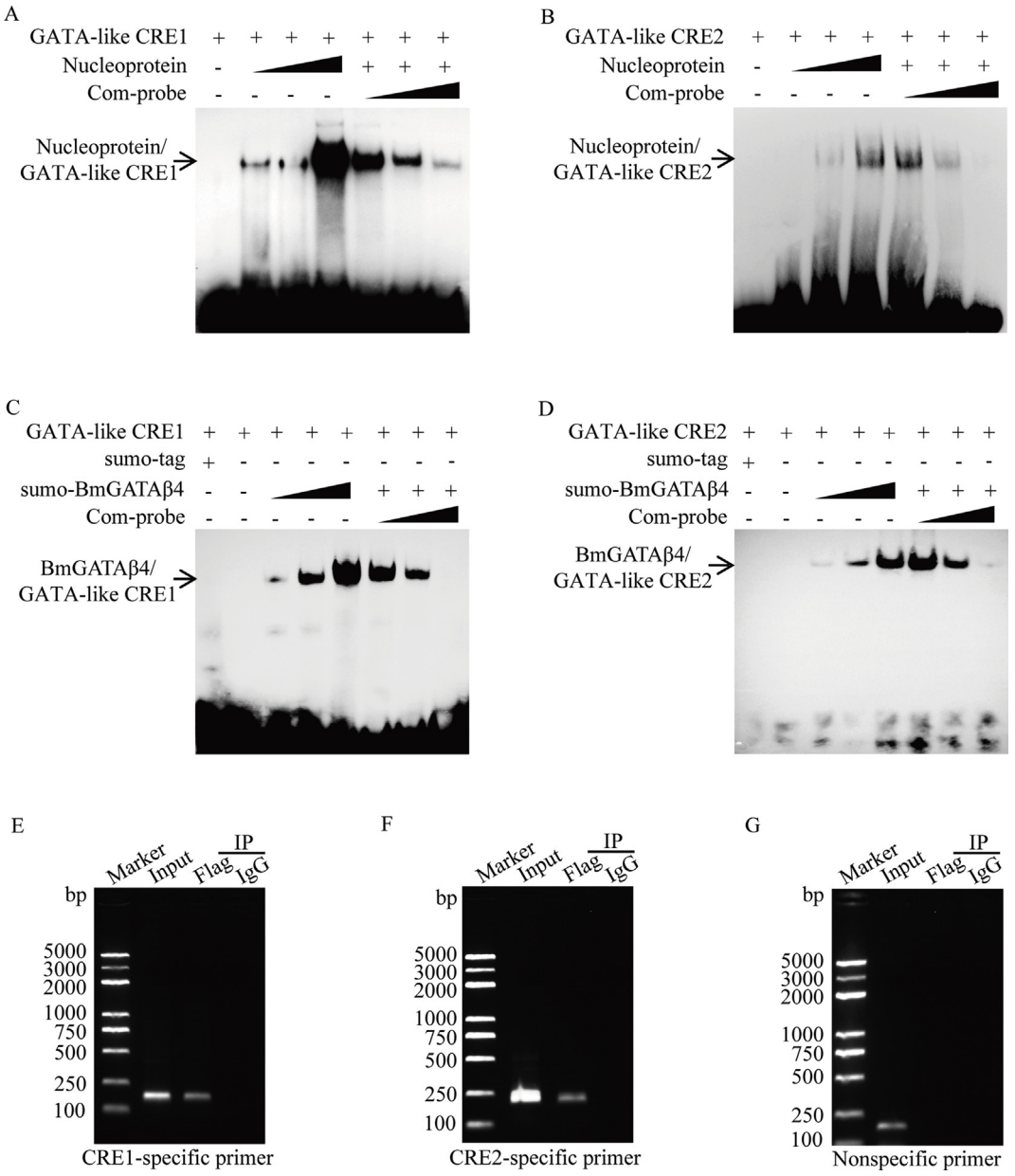


Fig. 6. BmGATAβ4 transcription factor bound to GATA-like CRE1 and CRE2 of the *BmVg* promoter. A, B. Nucleoproteins bound to GATA-like CRE1 (A) and CRE2 (B) in female silkworm fat body on day 1 after pupation. C, D. Electrophoretic mobility shift assays showed that GATA-like CRE1 (C) and CRE2 (D) probes bound to recombinant sumo-fused BmGATAβ4 proteins. GATA-like CRE1 and CRE2 probe concentrations were 100 nM; the sumo-BmGATAβ4 protein concentrations were 1, 10, and 100 nM; the concentrations of competing probes were 200, 1000, and 5000 nM. E-G. Chromatin immunoprecipitation of BmE-SWU1 cells lysates with BmGATAβ4-Flag overexpression. Precipitated DNA sequences were amplified by PCR using CRE1-specific (E), CRE2-specific (F), and nonspecific (G) primers. IP, immunoprecipitation; IgG, immunoglobulin G.

study. Due to the high similarity of the peptide sequence of various GATAβ subtypes (Fig. S2), we did not obtain a sufficiently specific antibody for BmGATAβ4, so we failed to detect a correlation between BmGATAβ4 and BmVg at the protein level. Thus, additional studies are needed to optimize the conditions to prepare specific antibodies targeting BmGATAβ4 in order to perform further functional studies of BmGATAβ4.

In anautogenous mosquitos, yolk protein precursor genes expression were regulated by four-way signaling pathways: juvenile hormone, ecdysone, nutrient, and insulin-like peptide (Hansen et al., 2014). After a blood meal, the nutrient signaling pathway is activated and induced *YPP* gene expression. The nutrient signal stimulates the release of ovarian ecdysiotropic hormone in the brain to promote the synthesis of ecdysone in the ovaries, which in turn acts on the fat body to upregulate the *Vg* (Attardo et al., 2005; Hansen et al., 2014). Alternatively, the amino acid signal can activate the transcription and translation of the downstream GATA transcription factor in the fat body and subsequently induces the expression of *Vg* (Attardo et al., 2005; Park et al., 2006). In *H. longicornis* ticks, the target of rapamycin (TOR) signaling pathway is activated after a blood meal, and the synthesis of GATA transcription factors is then increased, thereby inducing the expression of *Vg* (Boldbaatar et al., 2010). In the red flour beetle *Tribolium castaneum*, JH and nutrient signals can induce the production of insulin-like peptides, leading to phosphorylation of the downstream transcription factor Forkhead box O and ultimately inducing the expression of *Vg* (Parthasarathy and Palli, 2011; Parthasarathy et al., 2010). In summary, nutritional signals regulate the expression of *BmVg* by participating in the hormone signaling pathway or by downstream target

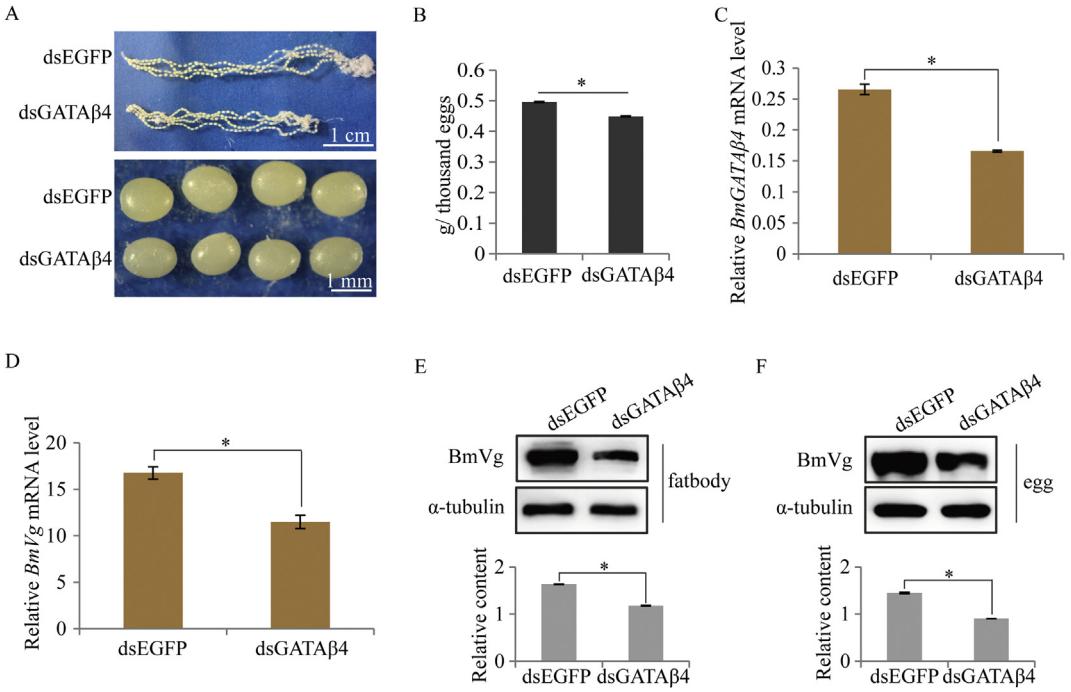


Fig. 7. Effects of double-stranded RNAi knockdown of *BmGATAβ4* in female silkworms. A. Phenotypes of the ovary differentiation and egg size after treatment with dsRNAi targeting BmGATAβ4 in female silkworms. dsRNAi targeting enhanced green fluorescent protein (dsEGFP) was used as a control. B. Weight of eggs after treatment with dsGATAβ4 in female silkworms. C. qRT-PCR analysis of *BmGATAβ4* transcription in dsRNA-treated female pupal fat body on day 2 after treatment with dsRNA. D. qRT-PCR analysis of *BmVg* transcription in dsRNA-treated female pupal fat body on day 2 after treatment with dsRNA. E-F. Analysis of BmVg expression in dsRNA-treated female moth fat body (E) and eggs (F). The upper was western blotting analysis of the BmVg expression, the below is the grayscale analysis of the western results. The data represent means ± standard deviations (n = 3). The significance of the differences between data sets was calculated using two-tailed Student's *t*-tests; *, $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

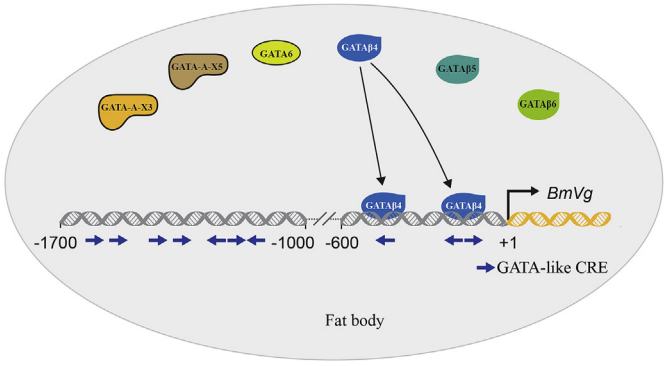


Fig. 8. Schematic diagram of a hypothetical mechanism by which BmGATAs regulate *BmVg* expression. Six inform GATA transcription factors were identified in the fat body of silkworms during vitellogenesis, and only BmGATAβ4 regulated the expression of *BmVg* by directly binding to GATA-like CRE1 and CRE2 on *BmVg* promoter.

transcription factors, including GATA. Indeed, nutrient signaling has not been shown to be involved in the transcriptional regulation of *BmVg* in silkworms. Notably, *BmVg* is expressed after wandering, when the silkworm is no longer fed. Accordingly, additional studies are needed to assess whether the nutrient signal participates in the regulation of *BmVg* through regulating BmGATAβ4 expression and whether this process is related to the spinning act of silkworms.

Multiple GATA-type transcription factors encoded by alternative splicing are involved in developmental regulation in silkworms, and all splicing products are expressed in the ovaries during the late stage of vitellogenesis (Drevet et al., 1995). These findings demonstrated that BmGATAβ may be involved in vitellogenesis and chorionic formation. BmGATAβ together with C/EBP can regulate the expression of chorion genes in a time-specific manner during choriogenesis (Lecanidou and Papantonis, 2010; Papantonis et al., 2008; Sourmeli et al., 2003; Tsatsarounos et al., 2015). We found, for the first time, that BmGATAβ4 participates in the expression of *BmVg* in silkworms. Thus, BmGATAβ is critical for vitellogenesis and choriogenesis in silkworms and plays a vital role in the reproduction of silkworms. Based on the expression model of *BmGATAβ4*, we found a certain level of *BmGATAβ4* expression before the expression of *BmVg*, implying that this protein had other functions in the fat body as well. At the same time, we found that when the fat body nucleoprotein was incubated with the GATA-like CRE1 element, an additional weak lag band above the lag band formed by BmGATAβ4/GATA-like CRE1 complex was shown (Fig. 6A). And the lag band was gradually weakened with the addition of competitive probes, indicating this protein was also specifically bind to the GATA-like CRE1 site. Whether this lag band is formed by other protein alone binding to the GATA-like CRE1 probe, or the superlag band is formed by other proteins/BmGATAβ4/GATA-like CRE1 complex, needing our further study. These findings also suggested that other transcription factors coordinated with BmGATAβ4 or antagonized BmGATAβ4 to regulate the transcription of BmVg in the fat body in silkworms.

In summary, six subtypes of GATA transcription factors were identified in the fat body of silkworms during metamorphosis, and only BmGATAβ4 regulated the expression of *BmVg* by binding directly to GATA-like CRE1 and CRE2 on *BmVg* promoter (Fig. 8). Additionally, we found that BmGATAβ4 participated in the formation of eggs. However, there are still many problems need to be solved in this study. In the future work, we will first prepare specific antibodies for these GATA transcription factors to explore the function of them during metamorphosis. We will also explore the activation mechanism of the nutrient signals in the fat body and the regulation mechanism of nutrient signaling on *BmVg* transcription in the silkworm. At the same time, we will identify other transcription factors that bind to GATA-like CRE1 and then analyze how the factor together with BmGATAβ4 regulate the of

transcription of *BmVg*. These will improve our understanding of the transcriptional regulation mechanism of *BmVg* and established a basis for the interpretation of the temporal-specific expression of *BmVg*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.01.004.

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