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Research

Effects of Certain *cis*-Regulatory Elements on Stage-Specific *vitellogenin* Expression in the *Bombyx mori* (Lepidoptera: Bombycidae)

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Abstract

Bombyx mori vitellogenin (*BmVg*) is highly upregulated during pupation, and the 20-hydroxyecdysone and amino acids may regulate stage-specific *BmVg* expression. However, previous studies showed that other factors may also affect stage-specific *BmVg* expression. Here, we characterized effective *BmVg* transcription factors by identifying the corresponding *cis*-regulatory elements (CREs). We prepared transgenic *B. mori*, in which *DsRed* was driven by various lengths of *BmVg* promoter. qRT-PCR analysis showed that *DsRed* expression driven by a 1.0-kb *BmVg* promoter (VgP1.0K) was consistent with endogenous *BmVg*. VgP1.0K specificity was closer to the endogenous *BmVg* promoter than that of VgP0.8K. These results suggest that CREs affecting stage-specific *BmVg* expression were localized to the 1.0-kb *BmVg* promoter. We investigated the effects of certain CREs that could influence the stage specificity of *BmVg* promoter on *BmVg* expression in transgenic *B. mori*. The relative *DsRed* expression was significantly reduced in transgenic female *B. mori* and the peak in *DsRed* expression was delayed after E-box CRE mutation. These results demonstrate that the E-box element enhanced *BmVg* expression and also affected stage-specific *BmVg* expression. Moreover, the relative *DsRed* expression was significantly increased in transgenic female of *B. mori* after 3×BD CRE mutation in *BmVg* promoter. However, the stage specificity of the mutated promoter was consistent with that of the endogenous *BmVg* promoter. The 3×BD element downregulated *BmVg* but had no effect on stage-specific *BmVg* expression. The present study promoted the process of elucidating the regulatory network for stage-specific *BmVg* expression and furnished a theoretical basis for the application of *BmVg* promoter.

Key words: vitellogenin, promoter, regulation, transgenic, *B. mori*

The Vg gene is unique in oviparous animals, which plays a significant role in yolk protein synthesis during the embryo formation (Valle 1993). Vg is essential for insect reproduction. When the Vg content is reduced, the embryo fails to develop normally (Lin et al. 2013, Yang et al. 2014a). Vg is synthesized mainly in the fat bodies of female insects at specific developmental stages (Valle 1993, Hirai et al. 1998, Raikhel et al. 2002). The mechanisms regulating Vg expression have been widely investigated. The stages at which Vg is expressed vary with insect species (Hirai et al. 1998, Tufail et al. 2010, Yang et al. 2014a). Stage-specific Vg expression is relatively complex and regulated mainly by juvenile hormones, ecdysone, amino acids, and insulin (Bownes 1982, Hansen et al. 2014, Gujar and Palli 2016, Lu et al. 2016, Roy et al. 2018, Asad et al. 2020).

Bombyx mori (*B. mori*) is an economically important lepidopteran insect that synthesizes and secretes abundant silk proteins (Luan et al. 2018). It is completely metamorphic. Previous studies showed that *B. mori Vg* (*BmVg*) expression has definite sex, tissue, and stage specificity (Yang et al. 2014a). *BmVg* expression starts in the fat body on day 2 after spinning in female *B. mori* (Yang et al. 2014a). Stage-specific *BmVg* expression is regulated mainly by nutritional and 20-hydroxyecdysone (20E) signals (Yang et al. 2014b; Lin et al. 2017; Shen et al. 2018; Liu et al. 2019a,b). After spinning is complete, the increase of amino acid in the hemolymph induces BmGATA β4 to upregulate *BmVg* (Liu et al. 2019a,b). 20E titer is also increased in the hemolymph at this time to induce the early gene *BmBrC-Z2* and the homodomain transcription factor *BmPOU-M2*, then BmBrC-Z2 and BmPOU-M2 to upregulate the expression of *BmVg* (Lin et al. 2017). During spinning, *BmGATAβ4* is expressed at a certain level and 20E has a small titer peak but *BmVg* is not expressed. Therefore, other unidentified transcription factors may regulate stage-specific *BmVg* expression.

Gene expression is regulated mainly by promoters including their *cis*-regulatory elements and their corresponding *trans*-acting factors. There are many predicted *cis*-regulatory elements in the *BmVg* promoter (Yang et al. 2014b). The regulatory regions −42 to −152 bp and −752 to −952 bp in the *BmVg* promoter significantly influence its activity. There is a predicted binding site for the *Drosophila* transcriptional repressor hairy in the −39 to −54 region of the *BmVg* promoter. Hairy belongs to the bHLH family, which could recognize E-box or N-box sites in target gene promoter (Sasai et al. 1992). Hairy could recruit a co-suppressor to inhibit target gene expression (Davis and Turner 2001, Jindra 2016, Saha et al. 2016). Thus, hairy may also affect *BmVg* expression. Moreover, three tandem repeat sequences in the −836 bp to −887 bp region of the *BmVg* promoter are the predicted binding sites for DREF (DNA replication-related element factor) or BEAF (boundary element-related factor). DREF elements promote gene expression (Hart et al. 1999, Fernández-Moreno et al. 2009). BEAF requires three tandem repeats to block the enhancer upstream of the gene promoter (Hart et al. 1999). The core motifs of DREF and BEAF are similar; therefore, DREF and BEAF may be antagonistic (Hart et al. 1999). However, there are no prior reports on DREF or BEAF in *B. mori*. It remains to be determined whether DREF or BEAF affects stage-specific *BmVg* expression.

Previous studies used ~0.8-kb upstream region of the *BmVg* promoter to drive green fluorescent protein expression and prepare transgenic *B. mori*. RT-PCR assays revealed that the promoter of 0.8 kb in length had the same sex, tissue, and stage specificity as the endogenous *BmVg* promoter (Xu et al. 2014). This discovery may imply that the *cis*-regulatory elements influencing stage-specific *BmVg* expression mainly reside within this 0.8-kb *BmVg* promoter.

For this reason, here we focused on the *cis*-regulatory elements in the *BmVg* promoter to identify those regulating stage-specific *BmVg* expression. We used the promoter 0.8 kb in length as a control and extended it. We first prepared transgenic *B. mori* with *BmVg* promoters of various lengths to drive *DsRed* expression and analyzed the expression characteristics of the different *BmVg* promoters. And we comprehensively analyzed the regulatory regions affecting stage-specific *BmVg* expression. Then we applied this transgenic method to explore the effects of certain *cis*-regulatory elements in the *BmVg* promoter on stage-specific *BmVg* expression. This study initiated the process of elucidating the regulatory network of stage-specific *BmVg* expression and provided a theoretical basis for the application of the *BmVg* promoter.

Materials and Methods

Construction of Vectors for Cell Line Transfection

Various lengths of *BmVg* promoter were amplified from the *B. mori* genome and ligated to the pGL3-basic vector (Promega, Madison, WI) using the restriction endonucleases XhoI and HindIII. The vector constructs were named pGL3-VgP0.8K Luc, pGL3-VgP1.0K Luc, and pGL3-VgP1.7K Luc. The *BmVg* promoter was truncated and used to construct the pGL3-VgP54 Luc vector with the E-box element, the pGL3-VgP39 Luc vector without the E-box element, the pGL3-VgP888 Luc vector with the 3×BD element, and the pGL3-VgP834 Luc vector without the 3×BD element. The E-box element was connected to the upstream regulatory sequence from −38 to +40 of *BmVg* (basic promoter VgP78ML), the VgP78ML was unresponsive to the induction of ecdysone, juvenile hormone, or their transcription factors (Liu et al. 2019b) stored in the laboratory and the product was named VgP78ML+E-box Luc. The amplification primers are listed in Table 1.

Cell Transfection

The *B. mori* embryonic cell line BmE-SWU1 stored in the laboratory was maintained at 27°C in Grace's insect medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS; HyClone, Thermo Fisher Scientific, Waltham, MA). X-tremeGENE HP DNA transfection reagent (Roche, Basel, Switzerland) was used for the cell transfection assays. The PRL-VgP78M vector was transferred as an internal control (Liu et al. 2018). After 36 h transfection, the cells were harvested and assayed for luciferase activity. The cell transfection experiments and luciferase activity measurements were conducted as previously described (Deng et al. 2012).

Construction of Vectors for Germline Transformation

BmVgP1.0K with a mutant E-box element was named as BmVgP1.0M. The BmVgP1.0M sequence was amplified with a unique primer containing the mutated E-box sequence. The core site of the E-box element, CGCGTG, was changed to TATACA. Three repeated DREF and BEAF elements in series on the *BmVg* promoter were named 3×BD. BmVgP1.7K with a deletion 3×BD element was named BmVgP1.7K-3×BD. The BmVgP1.7K-3×BD sequence was amplified with a unique primer deleting the 3×BD element.

The pBac [3xp3-EGFP-SV40] stored in the laboratory served as a framework vector expressing enhanced green fluorescent protein (EGFP) in the *B. mori* eyes (Chen et al. 2018). It is used to screen for positive transgenic *B. mori*. *DsRed* was the target gene driven by the *BmVg* promoter. The *BmVg* promoter was ligated with BamHI and EcoRI to the plasmid pSL1180-A4-DsRed-SV40 stored in the laboratory. The vector product was pSL1180-VgP-DsRed SV40. The plasmid containing VgP-DsRed-SV40 was digested with AscI and inserted into the vector pBac [3xp3-EGFP-SV40] to generate pBac [3xp3-EGFP-SV40; VgP-DsRed-SV40]. Transgenic vectors were constructed with promoters of various length and mutated CRE and named pBac [3xp3-EGFP-SV40; VgP0.8K-DsRed-SV40], pBac [3xp3-EGFP-SV40; VgP1.0K-DsRed-SV40], pBac [3xp3-EGFP-SV40; VgP1.7K-DsRed-SV40], pBac [3xp3-EGFP-SV40; VgP1.0M-DsRed-SV40], and pBac [3xp3-EGFP-SV40; VgP1.7K-3×BD -DsRed-SV40]. The primers used are listed in Table 1.

Production of Transgenic *B. mori*

The nondiapause *B. mori* dazao strains were used to prepare transgenic *B. mori* and the transgenic *B. mori* were generated as previously described (Chen et al. 2018). The injected G0 embryos were reared to the moth stage for G1 oviposition. The G1 embryos were screened under a fluorescence microscope (Olympus SZX12, Olympus, Tokyo, Japan) after growth for ~6–7 d. The G1 individuals on the basis of green fluorescence signal in compound eyes were selected and reared to adult stage. Individuals positive were interspecifically crossed over multiple generations to obtain pure stable transgenic *B. mori*.

Inverse PCR

DNA was extracted from transgenic adults and 15 μg was digested with HaeIII at 37°C for 4 h and purified. Then 1 μg purified product was circularized with T4 DNA ligase at 16°C overnight and used as template for PCR reaction with primers designed from the arm region of the piggyBac vector. The amplified PCR product was cloned into the pMD19-T vector for sequencing (TaKaRa Bio, Dalian,

Table 1. Primers used in the study

Purpose/primer name	Nucleotide sequence (5′–3′)
Cell transfection	
VgPcell-0.8K-F (XhoI)	CCGCTCGAGCCCGATCCATTAACAGTGCT
VgPcell-1.0K-F (XhoI)	CCGCTCGAGAGCACAGCTTACTAACAAGA
VgPcell-1.7K-F (XhoI)	CCGCTCGAGATCGAGAAGATAAAAAACTCGCAC
VgPcell888-F (XhoI)	CCGCTCGAGCATTCCCGATACAGCTCTAGGA
VgPcell834-F (XhoI)	CCGCTCGAGCATACCCGATCCCGTAGACCGA
VgP54-F (XhoI)	CCGCTCGAGTGGGACGCGTGTACCCTCCCTA
VgP39-F (XhoI)	CCGCTCGAGCTCCCTATATAAAGGGGGTGA
78ML+E-box-F (XhoI)	CCGCTCGAGTGGGACGCGTGTACCCGGTCTCGATCAGCGGGT
E-box-M1-F (XhoI)	CCGCTCGAGTGGGATATACATACCCTCCCTA
VgPcell-R (HindIII)	CCCAAGCTTTGTACTAGCTCCGCTGTC
Transgenic vectors	
VgP0.8K-F (EcoRI)	CCGGAATTCCCCGATCCATTAACAGTGCT
VgP1.0K-F (EcoRI)	CCGGAATTCAGCACAGCTTACTAACAAGA
VgP-R (BamHI)	CGCGGATCCTGTACTAGCTCCGCTGTC
E-box-M1-F	AGTTCTGGGATATACATACCCTCCCTA
E-box-M1-R	TAGGGAGGGTATGTATATCCCAGAACT
3×BD-M1-F	ACCGTATTGGCTTACGACACATACCCGATCCCGT
3×BD-M1-R	ACGGGATCGGGTATGTGTCGTAAGCCAATACGGT
RT-PCR	
DsRed-F	AACGGCCACGAGTTCGA
DsRed-R	GGTGTAGCCTCGTTGTGGG
BmVg-F	CCACCCTCAATAACTTCTAC
BmVg-R	AGGTATGTATCCTTGTGCC
BmActin3-F	AACACCCCGTCCTGCTCACTG
BmActin3-R	GGGCGAGACGTGTGATTTCCT
qRT-PCR	
Bmsw22934-F	TTCGTACTGGCTCTTCTCGT
Bmsw22934-R	CAAAGTTGATAGCAATTCCCT
DsRed-F	TTCGCCTGGGACATCCTG
DsRed-R	TGGTCTTCTTCTGCATTACGG
BmVg-F	AGTCACGACGAATACCAAGAAGAT
BmVg-R	TACGATAGTCCTGTGTGAAAACG
Inverse PCR	
pBacL-F	ATCAGTGACACTTACCGCATTGACA
pBacL-R	TGACGAGCTTGTTGGTGAGGATTCT
pBacR-F	TACGCATGATTATCTTTAACGTA
pBacR-R	GTACTGTCATCTGATGTACCAGG

China). The sequencing results were blasted in the *B. mori* genome database SilkDB (http://www.silkdb.org/silkdb/) to identify the insertions in the transgenic *B. mori*. The primers are listed in Table 1.

Collection of Fat Body Samples and RNA Isolation

Numerous factors affect exogenous *DsRed* expression in transgenic *B. mori* such as the *DsRed* copy number and the homozygous and hybrid transgenic lines. To compare *BmVg* promoter activity accurately and eliminate the influences of the aforementioned factors, the transgenic lines with single insertion site were selected. The transgenic *B. mori* lines were also backcrossed with the wild type to generate more hybrid transgenic *B. mori* for subsequent experiments.

The last-instar *B. mori* larvae began spinning silk on days 7–8. The period from spinning to pupation is the wandering stage and it lasts for 2~3 d. The fat bodies of 5–10 *B. mori* were collected for each group between day 1 of wandering to day 3 of pupation. TRIzol reagent (Invitrogen) was used to extract total RNA according to the manufacturer's protocol.

RT-PCR and qRT-PCR

The corresponding cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (Promega). For the RT-PCR analysis, the *BmVg* and *DsRed* fragments were amplified from the cDNA samples using gene-specific primers. *BmActin3* was the housekeeping gene. The PCR program was as follows: 95°C, 4 min; 95°C, 30 s; 55°C, 30 s; 72°C, 30 s, *n** cycles; and 72°C, 10 min. The amplified samples were maintained at 4°C.

*Here, n = 20 for *BmVg* in females, n = 25 for *DsRed* in females, *BmVg* in males, and *BmActin3*, and n = 30 for *DsRed* in males. The PCR products were separated by agarose gel electrophoresis.

For qPCR, SYBR Green kits (TaKaRa Biotech, Osaka, Japan) were used according to the manufacturer's instructions. The relative mRNA levels of the target gene were normalized to the housekeeping gene translation initiation factor 4A. The expression level was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Three independent replicates were performed for each experiment. The RT-PCR and qPCR primers are listed in Table 1.

Statistical Analysis

The data are presented as means ± standard error. The relative *DsRed* expression values were calculated as follows: *DsRed* mRNA level/*BmVg* mRNA level. Independent-sample *t*-tests were applied to evaluate the promoter activity and gene expression data. Differences

between group means were considered significant at $P < 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$.

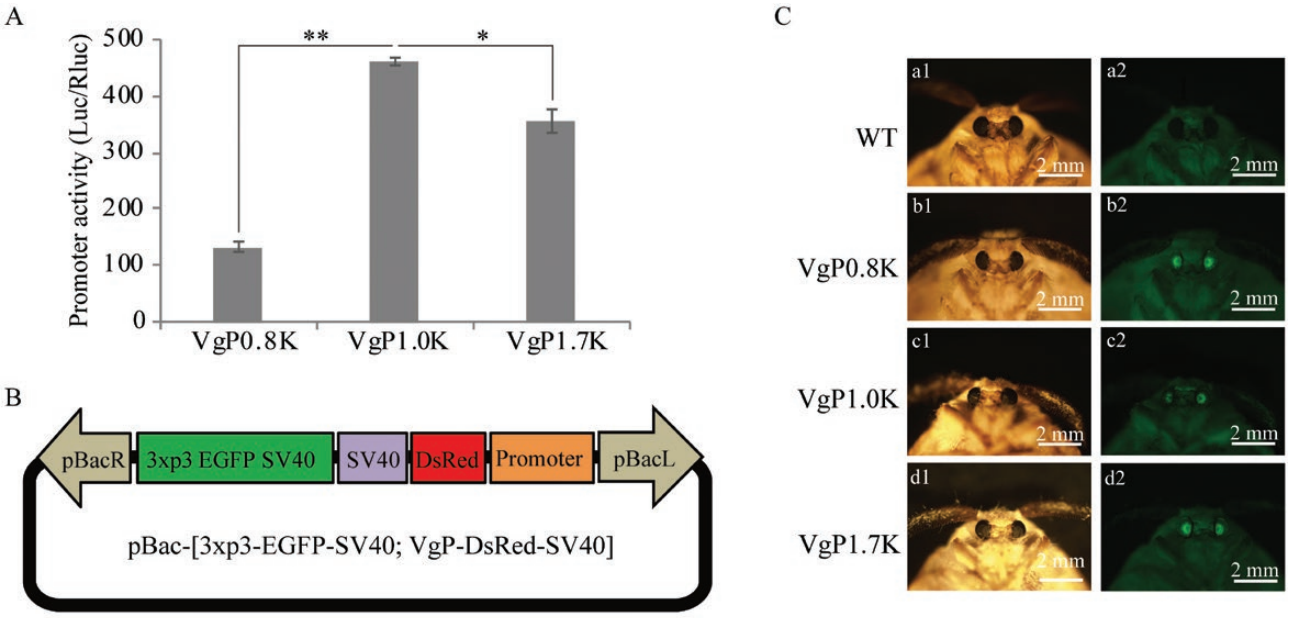
Results and Discussion

BmVgP1.0K Stage Specificity Was Closer to the Endogenous *BmVg* Promoter

Previous studies showed that BmVgP0.8K expression is tissue-, stage-, and sex-specific (Xu et al. 2014). To verify whether all elements determining stage-specific *BmVg* expression are located in the 0.8-kb region of the *BmVg* promoter, we used BmVgP0.8K as a control to analyze promoters of different lengths. The luciferase activity assay in cells revealed that BmVgP1.0K activity was significantly higher than those of BmVgP0.8K and BmVgP1.7K (Fig. 1A). Thus, there are some elements upregulate promoter activity from –0.8 to –1.0 kb.

We then constructed the transgenic vector for *BmVg* promoters of different lengths (Fig. 1B). These vectors were injected into *B. mori* embryos to gain transgenic *B. mori*. The compound eyes of the positive moths presented with specific EGFP emission (Fig. 1C) and the transgenic insects with only one insertion site (Fig. 1D) were backcrossed of wild-type *B. mori* to collect fat bodies for detection. RT-PCR showed that exogenous *DsRed* was not expressed in female or male wild-type *B. mori* (Fig. 2A). The expression patterns of *DsRed* were consistent with those of *BmVg* in BmVgP0.8K (Fig. 2B), BmVgP1.0K transgenic *B. mori* (Fig. 2C), and BmVgP1.7K transgenic *B. mori* (Fig. 2D). *DsRed* was highly expressed only in the fat bodies of transgenic *B. mori* (Fig. 2E). This discovery was consistent with previous reports demonstrating that BmVgP0.8K had *BmVg* promoter specificity (Xu et al. 2014). Our results showed that BmVgP1.0K and BmVgP1.7K also had *BmVg* promoter specificity. These results indicated that the elements that determine the stage-specific expression are mainly in the 0.8-kb *BmVg* promoter. In this region, the DSX, BrC-Z2, POUM2, ERR, and GATA elements could regulate *BmVg* expression (Suzuki et al. 2003, Yang et al. 2014b, Lin et al. 2017, Shen et al. 2018, Liu et al. 2019b). Moreover, ABDB, FTZ, and hairy elements have been predicted for this region (Yang et al. 2014b). However, we found that the onecut, ABDB, and FTZ elements had no apparent impact on *BmVg* promoter activity (data not shown). Therefore, we selected a *Drosophila* hairy binding site E-box element that could inhibit the *BmVg* promoter in theory (Davis and Turner 2001) for analyzing the effects on *BmVg* stage-specific expression in this study.

Then we used qPCR to further compare promoter activity among the three types of transgenic *B. mori*. In BmVgP0.8K transgenic *B. mori*, the *DsRed* expression peak was delayed (Fig. 3A).



D

Transgenic lines	Chromosome	Genomic DNA sequences surrounding *piggyBac* insertions
VgP0.8K	9	AGACATACAA**TTAA**--*piggyBac*--**TTAA**TATATGTTGT
VgP1.0K	15	CTTAAGTTCT**TTAA**--*piggyBac*--**TTAA**TAGAAATTCG
VgP1.7K	23	ATATATAGAT**TTAA**--*piggyBac*--**TTAA**AATAACTAAA

Fig. 1. Generation of transgenic *B. mori* with *DsRed* driven by the *BmVg* promoter. (A) Activity of *BmVg* promoters with different lengths. (B) Map of transgenic vector; pBacR and pBacL are the right and left arms of the transposon, respectively; 3xp3 EGFP SV40 is the transgene marker; and Promoter is the *BmVg* promoter. (C) Screening of positive transgenic *B. mori* under fluorescence microscope; (a1–d1) white light image of wild-type *B. mori* (a1), VgP0.8K transgenic *B. mori* (b1), VgP1.0K transgenic *B. mori* (c1), and VgP1.7K transgenic *B. mori* (d1); (a2–d2) fluorescence image of wild-type *B. mori* (a2), VgP0.8K transgenic *B. mori* (b2), VgP1.0K transgenic *B. mori* (c2), and VgP1.7K transgenic *B. mori* (d2). (D) Analysis of insertion sites for transgenic *B. mori*. Significant differences between data sets were calculated by two-tailed Student's *t*-tests; $^{*}P < 0.05$; $^{**}P < 0.01$.

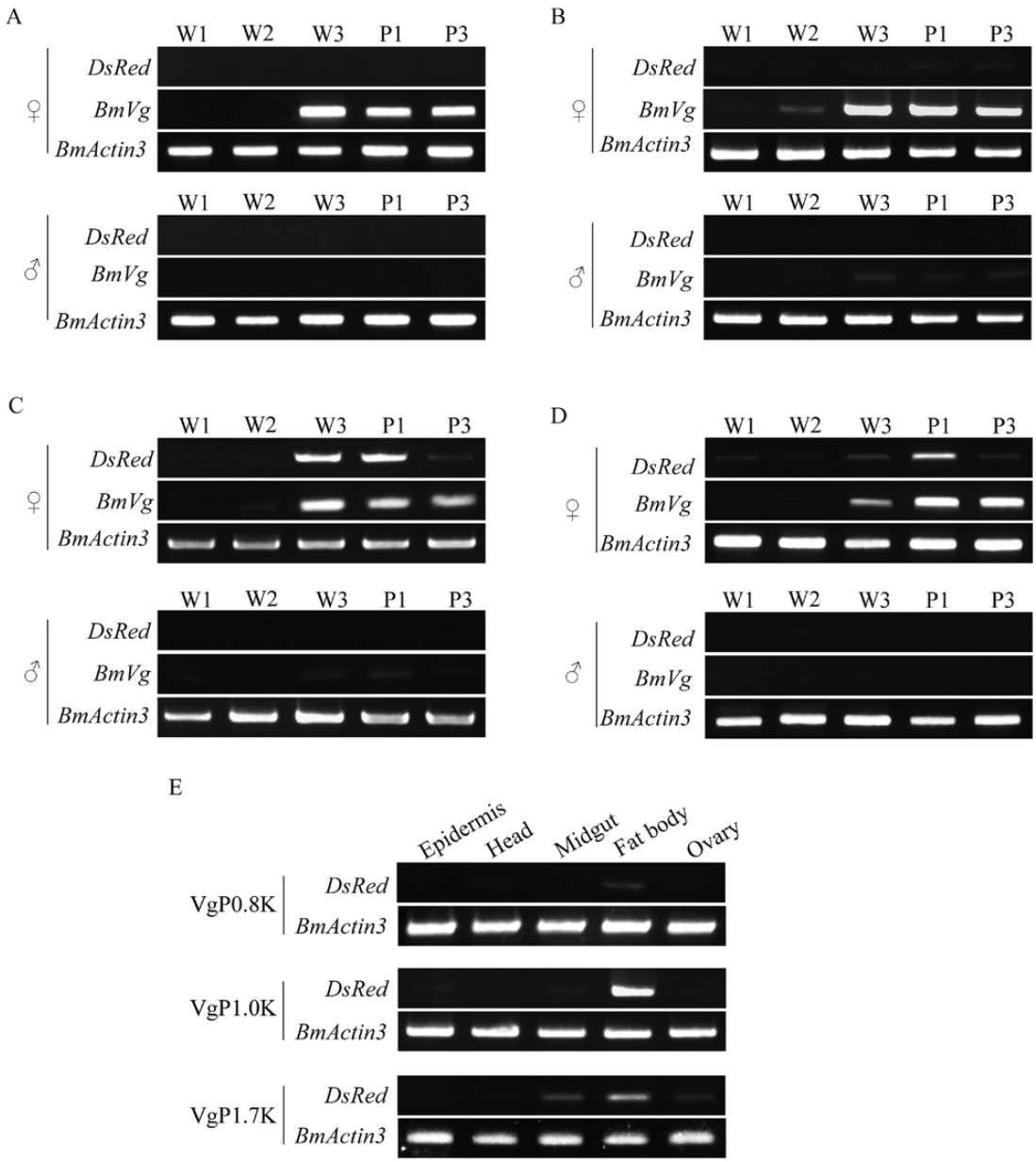


Fig. 2. Detection of exogenous *DsRed* expression in various *B. mori* by RT-PCR. (A–D) *DsRed* expression in fat bodies at different *B. mori* stages. (A) Wild-type *B. mori*. (B) BmVgP0.8K transgenic *B. mori*. (C) BmVgP1.0K transgenic *B. mori*. (D) BmVgP1.7K transgenic *B. mori*. (E) *DsRed* expression in various *B. mori* tissues. *BmVg* was a positive control and *BmActin3* was a housekeeping gene. (W1–W3) Wandering stage (d); (P1, P3) pupal stage (d).

However, *DsRed* expression was consistent with endogenous *BmVg* expression in BmVgP1.0K (Fig. 3B) and BmVgP1.7K (Fig. 3C) transgenic *B. mori*. These results suggest that the elements determining stage-specific *BmVg* expression resided mainly within 0.8 kb. However, there were some elements between −0.8 and −1.0 kb of the *BmVg* promoter that affected stage-specific *BmVg* expression. The typical elements in the range from −0.8 to −1.0 kb are three tandem repeats of the BEAF or DREF element (Yang et al. 2014b), so we then detected the effect of 3×BD CREs on the stage-specific expression of *BmVg* promoter. And we calculated relative exogenous *DsRed* expression value and found that it was significantly higher in BmVgP1.0K transgenic *B. mori* than it was in BmVgP0.8K and BmVgP1.7K transgenic *B. mori* on day 3 after wandering (Fig. 3D), suggesting the BmVgP1.0K activity was higher than that of BmVgP0.8K and BmVgP1.7K. Therefore, some *cis*-regulatory elements may enhance *BmVg* promoter activity in the region from −0.8 to −1.0 kb and inhibit *BmVg* promoter activity in the region from −1.0 to −1.7 kb.

The E-Box Element Affects the Stage-Specific Expression of *BmVg* Promoter

The E-box element was between −39 and −54 bp in the *BmVg* promoter (Fig. 4A). We transfected the vectors for *BmVg* promoter with and without E-box into cells and found that the E-box element could significantly enhance *BmVg* promoter activity (Fig. 4B). We then connected the E-box element to the basic promoter VgP78ML and also confirmed that the E-box element enhanced promoter activity (Fig. 4C). While the promoter activity was significantly downregulated after mutating the E-box element (Fig. 4D).

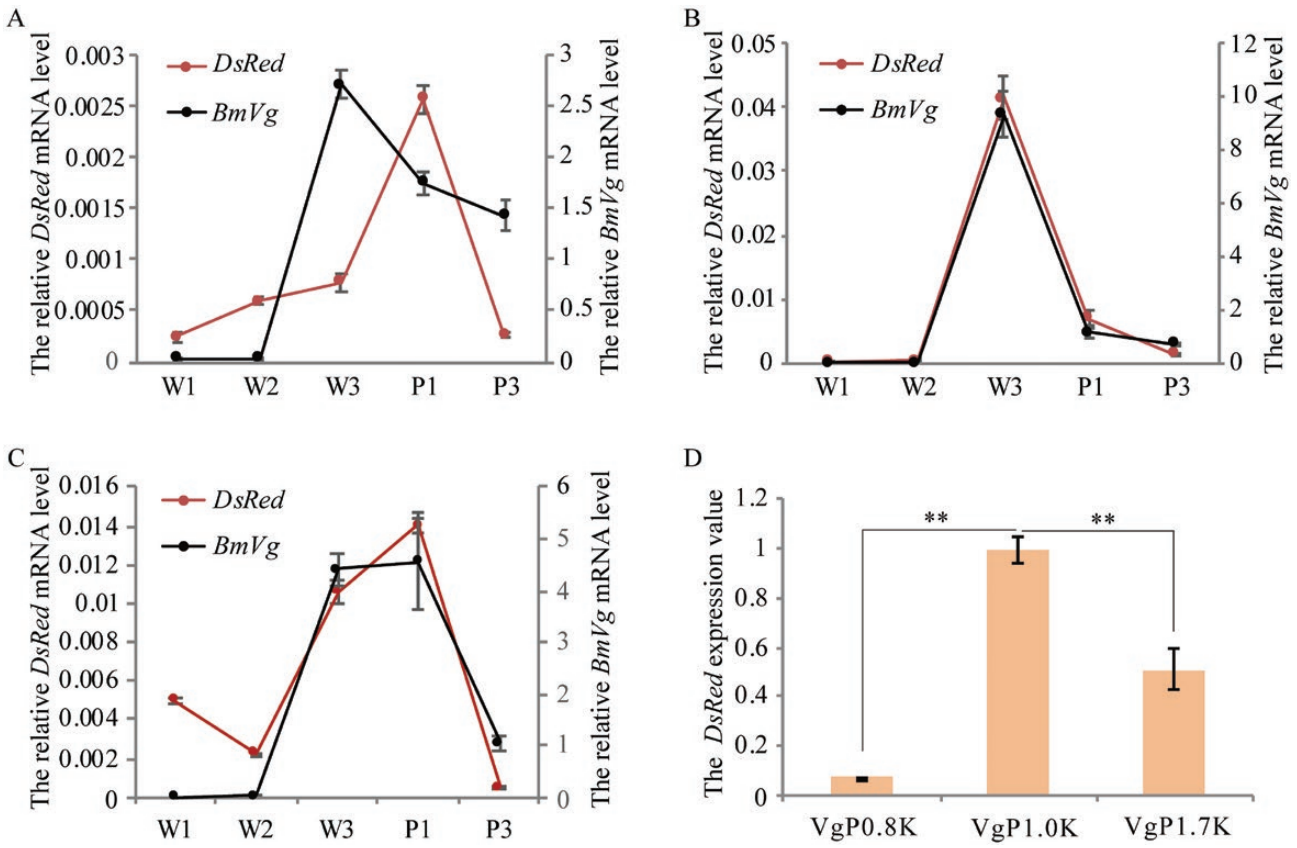


Fig. 3. Detection of exogenous *DsRed* expression in various transgenic *B. mori*. (A) *DsRed* expression trend in BmVgP0.8K transgenic *B. mori*. (B) *DsRed* expression trend in BmVgP1.0K transgenic *B. mori*. (C) *DsRed* expression trend in BmVgP1.7K transgenic *B. mori*. (D) Relative expression of *DsRed* in various transgenic *B. mori*. Significant differences between data sets calculated by two-tailed Student's *t*-tests; $^{**}P < 0.01$.

We then constructed a transgenic vector by VgP1.0K with the E-box mutation (Supp Fig. S1A [online only]) and screened the stable heritable transgenic individuals (Supp Fig. S1B [online only]). We selected single-insertion moths in subsequent analyses (Supp Fig. S1C [online only]). RT-PCR revealed that the expression profile of exogenous *DsRed* driven by VgP1.0M was consistent with that of endogenous *BmVg* (Supp Fig. S1D [online only]). *DsRed* was also highly expressed in the fat bodies (Supp Fig. S1E [online only]). Therefore, VgP1.0M had essentially the same expression characteristics as the *BmVg* promoter.

Similarly, we also analyzed *DsRed* expression by qPCR. The *DsRed* expression peak was delayed and occurred on day 1 after pupation in VgP1.0M transgenic *B. mori* (Fig. 4E), indicating the E-box element affected the stage specificity of *BmVg* expression. Meanwhile, we found that the relative *DsRed* expression value was significantly lower after mutating the E-box element (Fig. 4F). This finding was consistent with the observed results in the transfected cells. Nevertheless, this E-box element was predicted to be the binding site for the hairy which inhibits promoter activity (Saha et al. 2016). Therefore, we speculated that it was not the hairy that bound the E-box element in *BmVg* promoter. At the same time, we overexpressed the hairy in cells and found that the E-box element does not respond to the hairy (data not shown). However, the E-box element nonetheless stimulates promoter activity and must, therefore, bind a transcription factor upregulating *BmVg* expression. Our findings indicated that the mutated E-box element impeded *BmVg* upregulation on day 3 after wandering and could alter *BmVg* promoter stage specificity. But the mechanism of the effect of the E-box element on *BmVg* expression remains unknown. To this end, we must determine which transcription factor(s) binds this site and have regulatory functions there in the further.

3×BD Elements Have No Effects on the Stage-Specific Expression of *BmVg* Promoter

Previous studies showed certain elements might affect the stage specificity of the promoter in the upstream 0.8- to 1.0-kb region of the *BmVg* promoter. There are three tandem repeat elements in this sequence (Fig. 5A). They may be the binding elements of BEAF and DREF (Fig. 5B). DREF could significantly upregulate the promoter activity and BEAF could suppress upstream enhancer in theory (Hart et al. 1999). To confirm whether 3×BD affects *BmVg* promoter stage-specific expression, we firstly analyzed its effects on promoter activity in cells and found the 3×BD element could inhibit promoter activity (Fig. 5C). So, the 3×BD element may be a boundary blocking the enhancer upstream of this element. Thus, we choose the 1.7-kb *BmVg* promoter to examine the effects of 3×BD CRE in transgenic individuals. The transgenic vector VgP1.7K-3×BD (Supp Fig. S2A [online only]) was injected into *B. mori* embryos and obtained stable heritable transgenic individuals (Supp Fig. S2B [online only]). Similarly, the single-insertion transgenic individuals were selected for the subsequent analyses (Supp Fig. S2C [online only]). RT-PCR revealed that the stage and tissue expression profiles of *DsRed* driven by VgP1.7K-3×BD were consistent with endogenous *BmVg* (Supp Fig. S2D and E [online only]). Thus, it retained the *BmVg* promoter expression characteristics even after the 3×BD element was deleted.

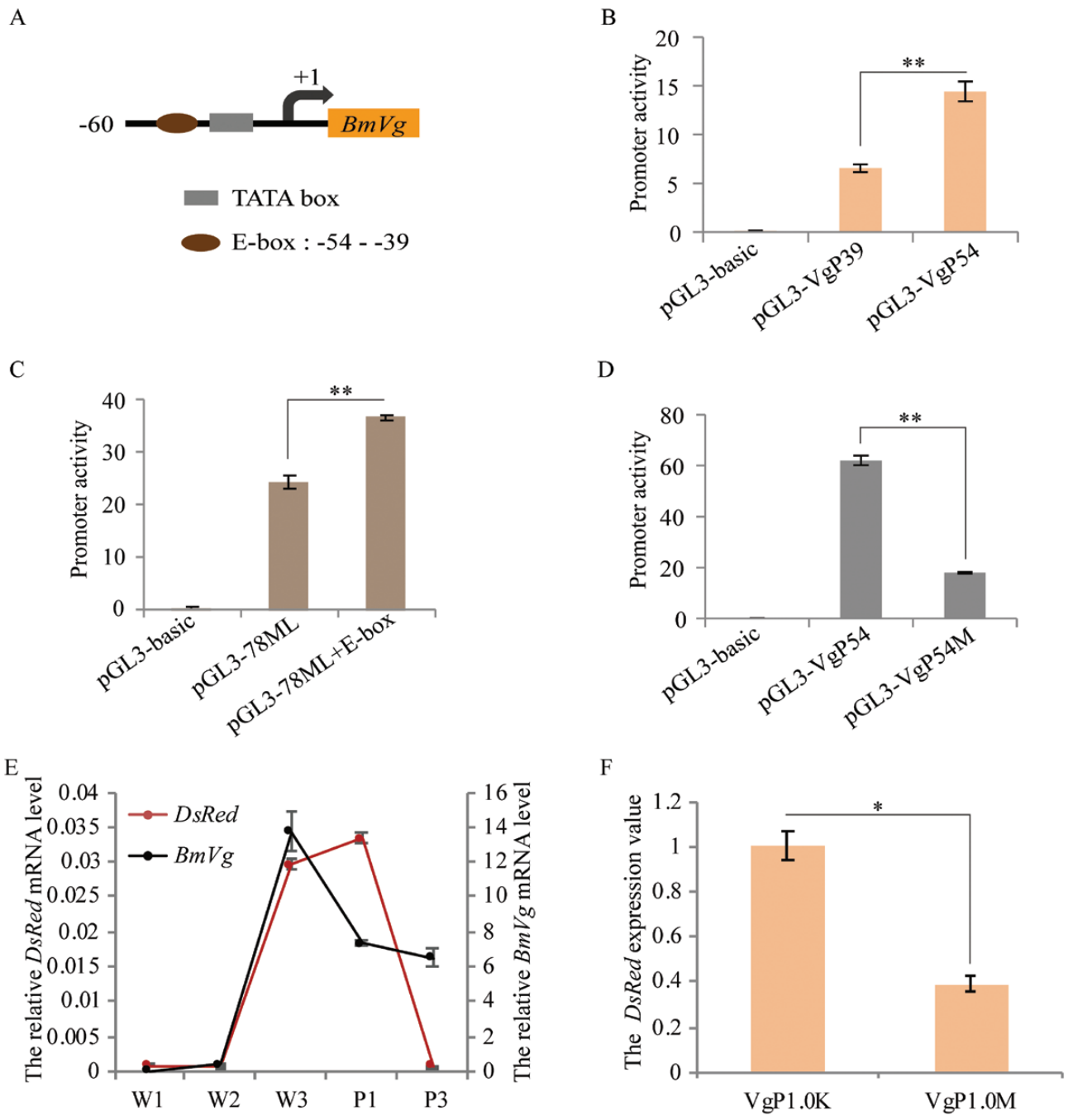


Fig. 4. Effect of E-box on *BmVg* promoter activity. (A) Position of E-box element on *BmVg* promoter. (B) Effect of E-box element on *BmVg* promoter activity in BmE-SWU1 cell. (C) Activity of 78ML basal promoter linked with E-box CRE. (D) Effects of E-box CRE on *BmVg* promoter activity after mutation. (E) qPCR of *DsRed* expression trend in BmVgP1.0M transgenic *B. mori*. (F) Comparison of *DsRed* expression on day 3 after wandering in female of transgenic *B. mori*. For more visual representation relative fold change in promoter activity after mutation, the *DsRed* expression driven by BmVgP1.0K promoter without mutation was normalized as 1. Significant differences between data sets calculated by two-tailed Student's *t*-tests; $*P < 0.05$; $**P < 0.01$.

We ran qPCR to analyze the VgP1.7K-3×BD expression characteristics and found that the expression of *DsRed* driven by it was consistent with that of endogenous *BmVg* (Fig. 5D). Therefore, the 3×BD element did not affect *BmVg* promoter stage specificity. Consistent with cell results, *DsRed* expression was significantly increased after 3×BD CRE deletion in transgenic *B. mori* (Fig. 5E). Thus, the transcription factor binding 3×BD CRE was not related to DREF. 3×BD CRE does not affect *BmVg* promoter stage specificity, but only inhibited *BmVg* expression. The stage specificity and the activity of the 1.0-kb *BmVg* promoter were greater than those of the 0.8-kb *BmVg* promoter. These results suggest that in this sequence interval, there were other elements influencing *BmVg* promoter activity and stage specificity. Nevertheless, these elements remain to be identified. However, physiological significance of the element inhibiting *BmVg* expression during vitellogenesis is obscure.

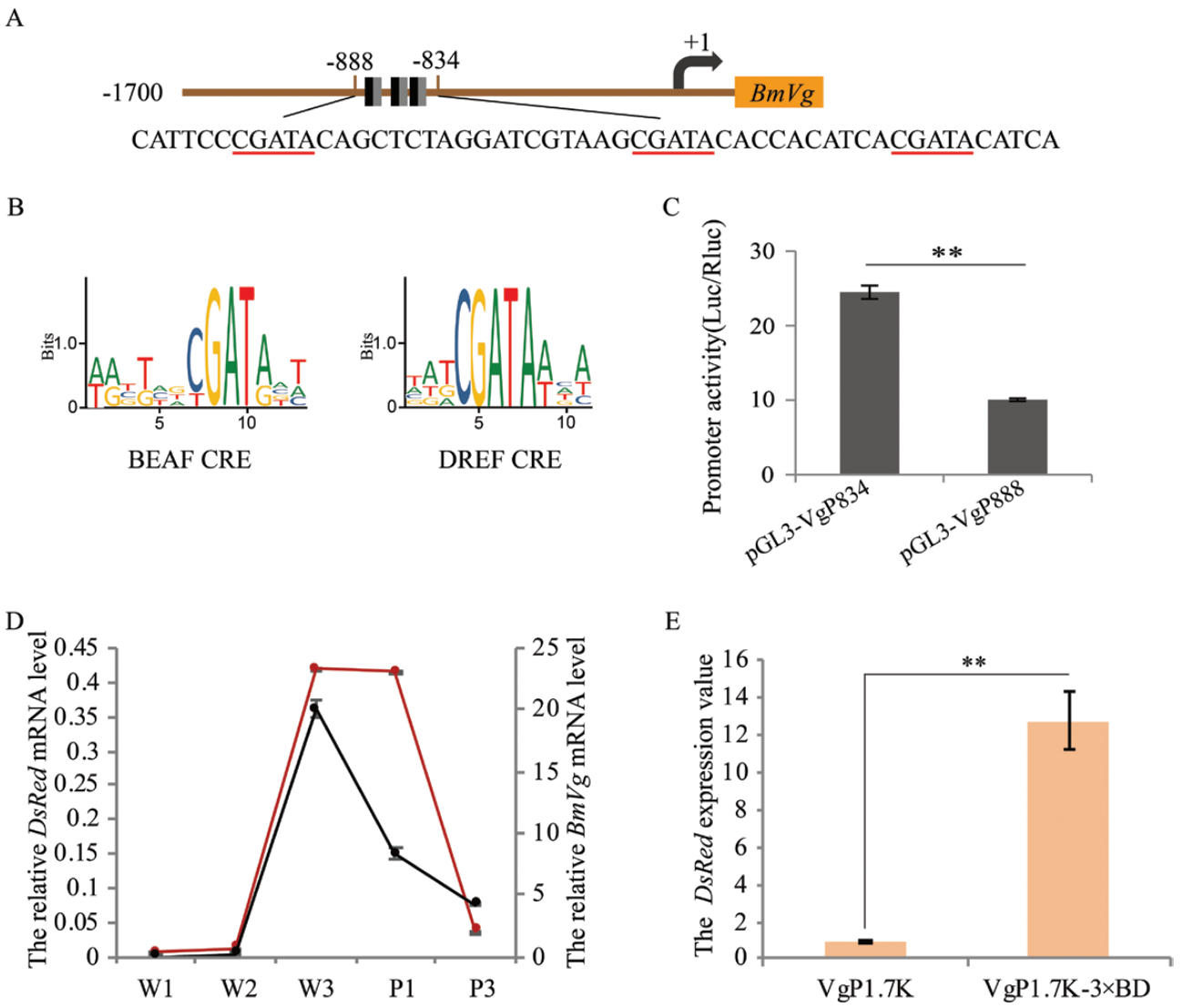


Fig. 5. Effects of 3×BD CRE on *BmVg* promoter activity. (A) Position of 3×BD CRE in *BmVg* promoter. (B) Conservative core motif of DREF and BEAF CRE. (C) Effect of 3×BD CRE on *BmVg* promoter activity in BmE-SWU1 cell. (D) qPCR of *DsRed* expression trend in BmVgP1.7K-3×BD transgenic *B. mori*. (E) Comparison of *DsRed* expression on day 3 after wandering in female transgenic *B. mori*. *DsRed* expression in W3 of BmVgP1.7K female transgenic *B. mori* was normalized as 1. Significant difference between data sets calculated by two-tailed Student's *t*-tests; $^{**}P < 0.01$.

Conclusion

The present study compared the activity of *BmVg* promoters of different length and examined the effects of certain elements on *BmVg* promoter stage specificity and activity in transgenic *B. mori*. This study indicated that the elements that affect the stage-specific *BmVg* expression were basically localized to the 1.0-kb *BmVg* promoter and the E-box element could affect the stage-specific *BmVg* expression, while the 3×BD element could not. This information forms the theoretical basis for the transformation and application of the *BmVg* promoter.

Supplementary Data

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

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