Bombyx mori transcription factors FoxA and SAGE divergently regulate the expression of wing cuticle protein gene 4 during metamorphosis

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Stage-specific gene expression governs metamorphosis of the silkworm, Bombyx mori. B. mori wing cuticle protein gene 4 (BmWCP4) is an essential gene for wing disc development expressed specifically during pupation. BmWCP4 transcription is suppressed at the larval stage by unknown mechanisms, which we sought to elucidate here. Bioinformatics analysis predicted that BmFoxA suppressed activity at the retic mobility shift (EMSA) and DNA pull-down assays revealed discs and up-regulated that of in the seven potential Forkhead box (Fox) transcription factors FoxA and SAGE in the BmWCP4 promoter region, and we found that Fox CRE6 contributes to suppression of BmWCP4 expression. Electrophoretic mobility shift (EMSA) and DNA pull-down assays revealed that BmFoxA suppressed activity at the BmWCP4 promoter by specifically binding to the Fox CRE6. The expression level of BmFoxA in the wing discs was higher during the larval stage than at the pupal stage. In contrast, expression of another transcription factor, BmSAGE, increased over the course of development. Of note, the hormone 20-hydroxyecdysone (20E), which governs molting in insects, suppressed BmFoxA expression in the wing discs and up-regulated that of BmSage. EMSA and cell co-transfection assays indicated that BmSAGE interacted with BmFoxA and suppressed its binding to the Fox CRE6, thereby releasing BmFoxA-mediated suppression of BmWCP4. In summary, higher BmFoxA expression during the larval stage suppresses BmWCP4 expression by binding to the Fox CRE6 on the BmWCP4 promoter. During metamorphosis, BmSAGE forms a complex with BmFoxA to relieve this repression, initiating BmWCP4 expression. Taken together, this study reveals a switchlike role for BmFoxA in regulating BmWCP4 expression and provides new insights into the regulatory regulation of wing disc development in insects.

Molting is an important developmental process regulated by 20-hydroxyecdysone (20E), whereas the outcome of molting (stationary and metamorphic) is determined by juvenile hormone (JH) titer in insects. 20E binds to its receptor complex of ecdysone receptor (EcR) and ultraspirecule (USP) in the target cells and initiates the process of molting or metamorphosis that is involved in a cascade action of transcription factors (1). Met and Kr-h1 have been shown to participate in the JH signal pathway in regulation of molting and metamorphosis, but the details are still largely unknown (2, 3).

Bombyx mori wing cuticle protein gene BmWCP4 is a pupal stage–specific gene essential for wing disc development. It is expressed during the prepupal and midpupal period and up-regulated by 20E but inhibited by JH III (4). During the prepupal stage, transcription factor BmPOUM2, which is induced by another transcription factor BmBR-C-Z4, interacts with transcription factor BmA-bd-A to up-regulate the expression of BmWCP4 in response to 20E signal (5, 6). However, the mechanism for BmWCP4 suppression in the larval stage is not clear. Our result of bioinformatics analysis showed that there are seven potential Forkhead box (Fox) cis-regulatory elements on the BmWCP4 promoter, which implies that the expression of BmWCP4 may be mediated by Fox proteins.

The Fox protein family, containing a highly conserved DNA binding domain that consists of three α helices, three β folds, and two ring connections, plays key roles in cell cycle, apoptosis, proliferation, and other cellular processes (7, 8). The FoxA subfamily plays a key role in the metabolism and the early organ development (9, 10). Moreover, FoxA proteins might act as the pioneer factors to open the chromatin or recruit RNA polymerase II to activate the transcription (11, 12). In insects, Fkh is the first FoxA protein identified in Drosophila, participating in the differentiation of embryo cells, autophagy, phospholipid metabolism, and hormone signal pathway (13, 14). It is also required for the salivary gland development in Drosophila melanogaster and inhibits apoptosis in salivary glands or activates the expression of some important genes, such as Senseless (Sens) (15–17). Har-FoxA regulates diapause in Helicoverpa armigera by up-regulating the expression of DH-PBRAN (18). In Spodoptera litura, SIFoxA regulates the expression of ABCC2 enhanced GFP, aa amino acids; qRT-PCR, quantitative real-time PCR; ANOVA, analysis of variance; EMSA, electrophoretic mobility shift assay; NP-40, Nonidet P-40.
and ABCC3, receptors of Cryl Ac-toxin, to enhance the susceptibility of SI-HP cells to Bt toxin (19). In Nilaparvata lugens, NlFoxA is a functional factor that participates in ovary development and affects the number of offspring through regulating the expression of Vg protein (20). Silk gland factor-1 (SGF-1) of B. mori is a homologue of D. melanogaster Fkh and plays a key role in the silk gland development by regulating the expression of ser-I, P25, fib-h, and other genes by binding to the Fox cis-regulatory element (CRE) (21–24). SGF-1 is also important for the neurodevelopment (25). The specific binding between SGF-1 and the CRE in its target genes is influenced by the neighboring bases, and the efficient Fox elements may affect the promoter activity of the genes that express in the labial gland (26).

The FoxA subfamily has been shown to cooperate with another transcription factor, SAGE, in many cellular activities. The SAGE protein contains a basic helix-loop-helix domain (bHLH) and regulates the expression of the target genes via binding to their E-box element (27). In D. melanogaster, SAGE cooperates with Fkh to regulate the expression of PH4αSG1 and PH4αSG2, maintaining a uniform salivary gland lumen (28). In addition, SAGE achieved the tissue specificity for Fkh function, and this SAGE-Fkh collaboration was important for the gland development (28). Besides interacting with Fkh, DmSAGE interacts with CrebA to regulate and maintain the expression of fkh (29). Meanwhile, DmSAGE and Daughterless (Da) form a complex to maintain the expression of senseless, initiating apoptosis in salivary glands (17, 30). In B. mori, BmSAGE plays a crucial role in silk gland development, and it forms the heterologous complex with SGF-1 to activate the expression of the fib-H gene by binding to element A and B of the fib-H gene promoter (23, 31). BmSAGE also interacts with BmDmm to up-regulate the expression of fib-H by binding to the E-box of the fib-H gene promoter (32).

In this study, we found that BmFoxA suppressed the promoter activity and the expression of BmWCP4 by binding to the Fox CRE6 of the BmWCP4 promoter. BmSAGE interacted with BmFoxA and inhibited the binding of BmFoxA to the Fox CRE6, thus relieving the suppression of BmWCP4 expression by BmFoxA. Our results revealed the molecular regulation mechanism of the pupal-specific expression of BmWCP4 by BmFoxA and BmSAGE during metamorphosis.

Results

Identification of potential Fox CREs in the BmWCP4 promoter

To analyze the regulation mechanism of BmWCP4, the potential cis-elements in the BmWCP4 promoter were analyzed, and seven potential Fox CRE elements (CRE1, nt –1857 to –1874; CRE2, nt –1612 to –1628; CRE3, –535 to –551; CRE4, nt –445 to –473; CRE5, nt –370 to –387; Fox CRE6, nt –277 to 294; CRE7, nt –18 to –35 nt) were found in the upstream promoter and the Fox CRE7, which contained a TATA box, located closest to the transcription start site (Fig. 1A). The sequences of these Fox CREs were aligned, and the conserved A(G)T(A/C)(G)A(T)A-(C)(T)AAT(C)A(G) sequence was identified (Fig. 1B).

To determine which Fox CRE participates in the regulation of BmWCP4, the vectors containing the Fox CREn and the core promoter of BmWCP4 were constructed and transfected into the Spli221 cells to measure the CRE activity in regulating the luciferase expression. The results showed that the Fox CRE7 could increase the activity of BmWCP4 promoter by 3.0-fold over the core promoter that did not contain any Fox CRE. On the contrary, the Fox CRE1, Fox CRE4, Fox CRE5, and Fox CRE6 decreased the activity significantly, whereas the Fox CRE2 and Fox CRE3 had no effect on the activity of BmWCP4 (Fig. 1C). These results suggested that the Fox CRE1, Fox CRE4, Fox CRE5, Fox CRE6, and Fox CRE7 might be involved in the regulation of the BmWCP4 expression.

Then Fox CRE6 and Fox CRE7, which had the optimal effect on the activity of BmWCP4 promoter (Fig. 1C), were mutated to further determine their effects on the BmWCP4 promoter (–1455/+30 bp). The core sequences of Fox CRE6 (AATA-ATAGGG) and Fox CRE7 (TATAAAA) were mutated to GCGGGCGGGG and CGCGGGCGG, respectively. The mutated BmWCP4 promoter Fox CRE6M-pGL3-Luc or BmWCP4 promoter Fox CRE7M-pGL3-Luc plasmid, as well as the WT BmWCP4 promoter-pGL3-Luc plasmid, was transfected into Spli221 cells, and the luciferase activity was detected 48 h after transfection. The luciferase activity of BmWCP4 promoter Fox CRE7M-pGL3-Luc decreased by ~9.0-fold, compared with the WT construct. However, the luciferase activity increased significantly when the Fox CRE6 was mutated (Fig. 1D). The results indicated that Fox CRE6 suppressed the activity of BmWCP4 promoter, whereas Fox CRE7 positively regulated the BmWCP4 promoter activity.

BmFoxA represses the BmWCP4 promoter activity

There are at least 18 potential BmFox proteins in the silkworm (33), and 14 of them were cloned. To find out which BmFox protein regulates the expression of BmWCP4, the overexpression vectors IE1-BmFox-EGFP that express different BmFox proteins were constructed and co-transfected with the BmWCP4 promoter-pGL3 vector into the Spli221 cell, respectively, and the luciferase expression was measured. The results showed that only BmFoxL2-1 increased the activity of BmWCP4 promoter, compared with the EGFP control. On the contrary, BmFoxC-2, BmFoxA, BmFox-1A, and BmFoxO-2 significantly reduced the activity of the BmWCP4 promoter (Fig. 2A), indicating that these five BmFox proteins might be involved in repression of the BmWCP4 expression. Among them, BmFoxA had the optimal suppression role. To further determine whether BmFoxA suppresses the BmWCP4 promoter activity, BmFoxA RNAi was performed in the spli-221 cells. When the overexpression of BmFoxA was suppressed by dsBmFoxA (Fig. 2B), the BmWCP4 promoter activity was significantly recovered (Fig. 2C), as compared with the dsEGFP treatment (Fig. 2C). These results suggested that the nuclear protein BmFoxA down-regulated the BmWCP4 promoter activity.

Expression of BmFoxA in the wing discs and its relationship with insect hormone

To further understand the relationship between BmFoxA and BmWCP4, the expression of BmFoxA in the wing disc was examined. The result of Western blotting showed that BmFoxA had a high expression level during the fifth instar larval stage, but its expression became lower at the prepupal period. How-
ever, BmFoxA expression increased again from the pupal stage (Fig. 3A), which was opposite to the expression of BmWCP4 (34). The response of BmFoxA to JH III and 20E treatments in the wing disc and BmN cells was detected. The results of quantitative real-time PCR (qRT-PCR) showed that the expression of BmFoxA decreased by 3.0–4.0-fold 4 h after the treatment with 20E both in the wing discs and BmN cells, whereas no significant change was detected after treatment with JH III (Fig. 3B). These results suggested that the expression pattern of BmFoxA in the wing discs and BmN cells and its response to 20E were negatively related to BmWCP4 (4, 34), implying that BmFoxA might repress the expression of BmWCP4 during the larval stage.

**BmFoxA binds to the Fox CRE6 of BmWCP4**

To clarify how BmFoxA repressed the expression of BmWCP4, the BmFoxA-EGFP was overexpressed in the Spli22I cells, and the nuclear protein was extracted to incubate with the biotin end-labeled double-stranded oligonucleotide containing the putative Fox CRE1 and CRE6, respectively, which repressed the promoter activity of BmWCP4 most obviously. The electrophoretic mobility shift assay (EMSA) results showed that the nuclear proteins in BmFoxA-EGFP–overexpressing cells bound to the Fox CRE6 (Fig. 4A, lane 3), but not the Fox CRE1 (Fig. 4A, lane 8). When the 200°C cold probe of Fox CRE6 was added, the binding signal disappeared (Fig. 4A, lane 4), indicating that cold probe of Fox CRE6 could competitively suppress this binding. When the WT core sequence (AATAATAGGT) of the Fox CRE6 was mutated to GGCGGCCGGG, no binding signal was observed (Fig. 4A, lane 5). The above results suggested that the binding pattern of BmFoxA in the wing discs and BmN cells and its response to 20E were negatively related to BmWCP4 (4, 34), implying that BmFoxA might repress the expression of BmWCP4 during the larval stage.

**Figure 1. Identification and characterization of seven potential Fox CREs in the promoter of BmWCP4 in B. mori.** A, location of the seven potential Fox CREs in the upstream promoter of BmWCP4. B, sequence alignment of the seven potential Fox CREs. The conserved nucleotides are indicated with a black background. C, luciferase assay analysis of the effect of the seven potential Fox CREs on the promoter activity of BmWCP4. D, luciferase activity assay for the effect of Fox CRE7M and Fox CRE6M on the BmWCP4 promoter activity. Fox CRE6 and Fox CRE7 in the BmWCP4 promoter were mutated by overlap extension PCR, respectively. The core sequences of Fox CRE6 (AATAATAGGT) and Fox CRE7 (TATAAAAA) were mutated to GGGCGGCCGGG and CGCGCGCGC, respectively. 1 µg of the Fox CREn-BmWCP4 core promoter-pGL3 constructs and 0.1 µg of pRL-SV40 plasmid DNA were used to transfect the Spli221 cell line. The pRL-SV40 was used as an internal plasmid. The cells were harvested at 48 h post-transfection for the luciferase assay. Each data point is the mean ± S.E. (error bars) of three independent assays. Different letters above the columns indicate significance in the difference of luminescence at P < 0.05 by ANOVA.
to the Fox CRE6. This was further confirmed by using purified recombinant His-BmFoxA protein, which also specifically bound to the Fox CRE6 (Fig. 4C). To further confirm the specificity of BmFoxA protein binding in vivo to the Fox CRE6, a DNA-pulldown assay was performed. After incubating the BmFoxA-EGFP–overexpressing nuclear proteins with Fox CRE6 or the Fox CRE6 mutant probe, respectively, the elutions were analyzed by Western blotting using the anti-BmFoxA polyclonal antibody. The result showed that the anti-BmFoxA polyclonal antibody recognized the BmFoxA-EGFP protein in
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Figure 3. Expression pattern and 20E– or JH III–induced expression of BmFoxA in the wing discs. A, Western blot analysis of expression of BmFoxA protein in the wing discs. A total of 30 μg of protein of the wing disc from day 5 of the fifth instar larval stage to adult was used for Western blotting, and β-tubulin was used as a marker for equal protein loading. B, qRT-PCR analysis of induced expression of BmFoxA in the wing discs in the presence of 20E or JH III. 2 μl of a solution of 20E (2 μg/μl) and JHIII (2 μg/μl) were injected into 5-day-old fifth instar larvae at the thoracic region. An equal volume of DMSO at a concentration of 0.1% (v/v) was used as control. After 4 h, the wing discs were dissected in PBS buffer. C, qRT-PCR analysis of induced expression of BmFoxA in BmN cells in the presence of 20E or JH III. BmN cells were treated with JH III or 20E at a final concentration of 1 μM for 4 h, and then the cells were harvested. BmRp49 was used as an internal marker. Each data point is the mean ± S.E. (error bars) of three independent assays. Significance of the differences between the treatment and control was statistically analyzed at p < 0.05 using a t test. nD, day of development; nL, stage of larval instar; W, wandering stage; P, pupal stage.

the elution that was incubated with the Fox CRE6, but not the mutant one (Fig. 4D). Thus, either in vivo or in vitro, BmFoxA could bind to the Fox CRE6 of the BmWCP4 promoter.

The alignment of the sequence of Fox CRE6 with the DNA sequences that FoxA proteins bound to in other insects (18, 21–23, 35–37) showed that the whole sequence of the Fox CRE was relatively conserved (Fig. 4E), implying that the Fox CRE6 in the promoter of BmWCP4 is the target binding site of FoxA.

All of the above results suggested that as a direct regulator of BmWCP4, BmFoxA protein specifically bound to the conserved Fox CRE6.

Interaction of BmFoxA with BmSAGE and the expression pattern of BmWCP4 in the wing discs

It was reported that FoxA interacts with SAGE and regulates the expression of the downstream genes (23, 28, 31). To understand whether or not BmSAGE was involved in the regulation of BmWCP4 by interacting with BmFoxA, the interaction between BmFoxA and BmSAGE was examined using far-Western blotting. The results showed that anti-BmFoxA polyclonal antibody recognized His-BmSAGE protein after incubating with purified His-BmFoxA protein, but not with the negative protein SIGST (Fig. 5, A and B), which implied that BmFoxA protein interacted with BmSAGE to form a complex.

To further investigate the function of the BmSAGE and BmFoxA complex, the expression pattern of BmSage in the wing disc and the response to the insect hormone were analyzed. The mRNA expression level of BmSage decreased from day 2 of fifth instar larvae to day 1 of the wandering stage, followed by an increase at day 2 of the wandering stage and maintenance at the pupal period (Fig. 5C). The results of Western blotting showed a similar result. The BmSAGE expression was higher at the wandering and pupal periods than at the larval stage (Fig. 5D). Furthermore, the expression of BmSage was up-regulated by ~2.5-fold at 4 h after 20E treatment at day 5 of fifth instar larva (Fig. 5E). The expression pattern and the 20E-induced expression of BmSage were opposite to the expression of BmFoxA (Fig. 3A) but similar to that of BmWCP4 (4). This indicates that there is a negative relationship in expression pattern between BmSage, as well as BmWCP4, and BmFoxA, implying that BmSAGE interacts with BmFoxA to regulate the repression of BmWCP4.

BmFoxA suppression of BmWCP4 was inhibited by BmSAGE

To explore how BmSAGE is involved in the regulation of BmWCP4 by interacting with BmFoxA, BmFoxA-EGFP and BmSAGE-EGFP were co-transfected with BmWCP4-pGL3 into Spli221 cells, and the luciferase activity was measured. The results showed that the luciferase activity of BmWCP4 promoter decreased significantly after overexpression of BmFoxA-EGFP, whereas the luciferase activity increased after the over-expression of BmSAGE-EGFP, compared with the control EGFP (Fig. 6A and Fig. S1). The luciferase activity could be recovered after co-transfection with BmFoxA-EGFP and BmSAGE-EGFP (Fig. 6A), implying that BmSAGE released the BmFoxA suppression of BmWCP4 by interacting with BmFoxA. In addition, BmSAGE-EGFP protein alone also increased the luciferase activity of the BmWCP4 promoter (Fig. 6A). It has been reported that BmSAGE binds to the E-box to regulate the target gene expression (27), and we speculated that BmSAGE might also bind to the E-box (nt −1101 to −1089) element of the BmWCP4 promoter to promote the expression of BmWCP4.

To further demonstrate whether BmFoxA or BmSAGE can bind the Fox CRE6 in the BmWCP4 promoter, a ChIP assay was performed. An EGFP tag was fused to BmFoxA or BmSAGE, and anti-EGFP tag antibody was used in the ChIP assays (38). The cells were transfected with the recombinant plasmid
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BmFoxA, BmSAGE, or EGFP (as a control) and BmWCP4 promoter-pGL3-Luc, respectively. Then the cells were collected for the ChIP assay 48 h post-transfection. The results showed that the anti-EGFP antibodies, but not IgG (as a negative control), precipitated the Fox CRE6 in the cells transfected with the BmFoxA-EGFP–expressing plasmid, but not in the cells transfected with EGFP or BmSAGE-EGFP alone (Fig. 6B). The enriched Fox CRE6 sequence was amplified by PCR and confirmed by sequencing (Fig. 6, C and D). This experiment demonstrated that BmFoxA did bind to the Fox CRE6 in the nt –294 to –277 region of the BmWCP4 promoter ex vivo, whereas BmSAGE did not bind to the Fox CRE6.

Then purified His-BmSAGE protein, nuclear proteins isolated from the BmFoxA-overexpressing cells, and Fox CRE6 probe were used for EMSA to clarify the relationship of BmSAGE and BmFoxA in binding with Fox CRE6. The results showed that the binding signal between BmFoxA-EGFP and Fox CRE6 gradually was weakened with the increase in concentration of His-BmSAGE, but not with control protein SIGST (Fig. 6D), whereas BmSAGE alone could not bind to the Fox CRE6 (Fig. 6D, lane 4). This implied that His-BmSAGE antagonized the binding ability of BmFoxA-EGFP with Fox CRE6 in a dose-dependent manner. These results suggested that BmSAGE interacted with BmFoxA and then suppressed the binding of BmFoxA with the Fox CRE6 and eliminated its repression action on the BmWCP4 promoter.

Discussion

In our previous studies, we have found that 20E increased the expression of BmWCP4 during pupation through a cascade of transcription factors, such as BmBR-CZ4 and BmPOUM2, and that JH III inhibited the 20E induction action (4–6). In this study, we further elucidated the regulatory mechanism for the expression of BmWCP4, in particular during the larval stage.

BmFoxA suppressed the expression of BmWCP4 during the larval stage

EMSA, DNA pulldown, ChIP assay, and luciferase assay showed that BmFoxA specifically bound to the Fox CRE6 and then suppressed the promoter activity of BmWCP4 (Figs. 2, 4, and 6 and Fig. S1A). In addition, BmFoxA had high expression during the larval stage (Fig. 3). BmFoxA also had the higher...
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Figure 5. Interaction analysis of BmFoxA and BmSAGE and expression pattern of BmSage in the wing discs at different stages of B. mori. Shown is far-Western blot analysis of the interaction between BmFoxA and BmSAGE. The purified recombinant BmSAGE and SIGST proteins (2 μg) were separated using 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were used either for direct immunoblotting using anti-BmFoxA antibody (A) or for far-Western blot analysis (B), where the recombinant BmFoxA was used to incubate with BmSAGE on the membrane before immune detection by anti-BmFoxA antibody. Positive bands were observed when BmFoxA–BmSAGE complex was present. SIGST protein was used as a negative control. C, qRT-PCR analysis of expression pattern of BmSage in the wing discs from day 1 of the fifth instar larval stage to the pupal stage. D, Western blot analysis of the expression of BmSAGE in the wing discs. A total of 30 μg of protein isolated from the wing disc from day 1 of the fifth instar larval stage to day 5 of the pupal stage was used for Western blotting, and β-tubulin was used as a marker for equal protein loading. E, qRT-PCR analysis of 20E-induced expression of BmSage in the wing discs. 2 μl of a solution of 20E (2 μg/μl) were injected into 5-day-old fifth instar larvae at the thoracic region. An equal volume of DMSO at a concentration of 0.1% (v/v) was used as a control. After 4 h, the wing discs were dissected in PBS buffer. BmRp49 was used as an internal marker. Each data point is the mean ± S.E. (error bars) of three independent assays. Different letters above the columns indicate significance in the difference of luminescence between the treatments and control at p < 0.05 (*) by ANOVA. Significance of the differences between the treatment and control was statistically analyzed at p < 0.05 using a t test. nD, day of development; nL, stage of instar larvae; W, wandering stage; P, pupal stage.

expression during the fifth instar larval stage than in metamorphosis to activate fibroin gene expression (24). The JH maintained the larval characteristics, and 20E promoted the molting and metamorphosis (39, 40). The expression of BmWCP4 was induced by 20E pulse, and the action of 20E was inhibited by JH III (4). However, both of the treatments with 20E or JH III had no significant effect on it. The expression of sf7/BmFoxA was not induced by 10 μM JHA (32). Thus, the high expression of BmFoxA suppresses the expression of BmWCP4 during the larval stage, and the high titer of 20E prior to pupation inhibited the expression of BmFoxA to relieve the suppression of BmWCP4 during the larval–pupal metamorphosis. Determination of whether the suppression of BmWCP4 by BmFoxA during the larval stage is related to the JH signal pathway requires further investigation.

EMSA showed that in addition to BmFoxA, there may be other proteins binding (Fig. 4 (A and B), P2 and P3) to the Fox CRE6. The luciferase assay also showed that BmFoxC-2, BmFoxJ-1A, and BmFoxO-2 inhibited the promoter activity of BmWCP4 (Fig. 2A). Thus, the binding bands P2 and P3 might represent other BmFox proteins that bind to the Fox CRE6. The mRNA expression of BmFoxC-2 and BmFoxO-2 was higher during the larval stage than during the pupal stage (Fig. S2), implying that they might also be involved in the suppression of the expression of BmWCP4. In Caenorhabditis elegans, DAF-16/FOXO and PHA-4/FOXA co-regulated a set of downstream transcription factor genes (41). In HepG2 cells, the interdependent binding of FoxO1 and FoxA1/A2 influenced the IGFBP1 promoter activity to regulate the glucose metabolism of hepatic patients (42). Both FoxC and FoxJ were involved in the regulation of individual development (43, 44). However, whether BmFoxC-2, BmFoxJ-1A, and BmFoxO-2 were involved in the regulation of BmWCP4 by cooperating with BmFoxA remains to be analyzed.

Interaction of BmSAGE and BmFoxA relieved the BmFoxA suppression of BmWCP4

BmSAGE, a member of the bHLH transcription factor family, interacted with BmFoxA, as detected by far-Western blotting (Fig. 5, A and B). In D. melanogaster, SAGE cooperated with Fkh, playing an important role in regulating the expression of PH4aSG1 and PH4aSG2 to maintain the normal development of the salivary gland (28) and the expression of transmem-
brane proteins (45). In B. mori, BmSAGE interacted with SGF-1 to activate the expression of the fib-H gene by binding to elements A and B of the fib-H promoter (23).

Forkhead domain was the key binding domain with DNA and proteins. FoxA2 interacted with HNF-6 through the Forkhead domain to stimulate the FoxA2 transcription and to inhibit HNF-6 DNA binding (46). In the FoxP subfamily, FoxP2 bound via the Forkhead domain with FoxP3 to form a homodimer, which bound to the target DNA sequence (47, 48). The results of SMART and PredictProtein program analysis showed that BmSAGE contains a bHLH domain (aa 157–210) and a low-complexity region (aa 20–35). However, most of the predicted protein-binding sites are located in the C terminus, but not in these two regions, whereas the predicted DNA binding sites are located in the bHLH domain (Fig. S3). The results of EMSA in this study showed that BmSAGE suppressed the binding of BmFoxA with the Fox CRE6, and the luciferase assay of the promoter activity showed that overexpression of BmSAGE relieved the BmFoxA suppression of BmWCP4 promoter activity (Fig. 6). Thus, it is speculated that the predicted protein-binding sites in the C terminus of BmSAGE interacted with the Forkhead domain of BmFoxA to form a complex, which blocked the binding of BmFoxA to the Fox CRE6, resulting in the expression of BmWCP4, which otherwise is suppressed by BmFoxA during the larval stage.

Luciferase assay showed that BmSAGE increased the promoter activity of BmWCP4 (Fig. 6A). The members of the bHLH superfamily bound to the E-box element (CANNTG) to activate the expression of their target genes in Drosophila (27). Bioinformatics analysis showed that a predicted E-box element locates between nt 11002 to 11019 in the BmWCP4 promoter. Thus, it was suspected that BmSAGE at a high expression level might bind to the E-box element, enhancing the promoter activity of BmWCP4.

qRT-PCR and Western blotting showed that BmSAGE had the higher expression during the wandering and midpupal stage than the fifth instar feeding larval stage, and its expression was induced by 20E in the wing discs (Fig. 5, C–E), which is opposite to the BmFoxA expression and similar to the BmWCP4 expression. It seems that, on one hand, 20E represses the expression of BmFoxA. On the other hand, 20E induced the expression of BmSAGE, which interacts with BmFoxA to inhibit the binding between BmFoxA and the Fox CRE6 during the pupal metamorphosis.

Figure 6. BmSAGE relieved the BmFoxA suppression of BmWCP4 expression. A, effect of overexpression of BmFoxA and BmSAGE on the BmWCP4 promoter activity. The vectors of BmFoxA-EGFP, BmSAGE-EGFP, and pGL3 luciferase under the control of the BmWCP4 promoter were used to co-transfect Spl221 cells, and the activity of reporter gene luciferase was measured. Different letters above the columns indicate significance of the differences of luminescence at p < 0.05 by ANOVA. B, ChIP assay of the binding between Fox CRE6 and BmFoxA or BmSAGE. Spl221 cells were transfected with EGFP vector as a control or with BmFoxA-EGFP and BmSAGE, respectively. ChIP target sequences were detected by qRT-PCR. The enrichment of the promoter sequences in immunoprecipitated DNA samples was normalized with DNA present in the 10% input material. Data are means ± S.E. (n = 3). Different letters above the columns indicate significance of the difference of luminescence at p < 0.05 by ANOVA. C, reverse strand sequence of the nt -308 to -267 region of the BmWCP4 promoter. The reverse sequence of Fox CRE6 is boxed in red. D, sequencing atlas of the enriched RT-PCR product of Fox CRE6 in the ChIP assay.
In summary, the evidence of this study suggests that BmFoxA is involved in the regulation of BmWCP4 as a switch. During the larval stage, BmFoxA binds to the Fox CRE6 of the BmWCP4 promoter to suppress its expression. During the pupal metamorphosis, 20E suppresses the expression of BmFoxA and induces the expression of BmSAGE. BmSAGE and BmFoxA interact to form a complex, inhibiting the binding of BmFoxA to the Fox CRE6, relieving its suppression of BmWCP4 (Fig. 7).

**Materials and methods**

**Insects, cells, and treatments**

Silkworm strain Dazao was reared on fresh mulberry leaves in the laboratory at 25 °C under a 12-h light/12-h dark cycle. Under this condition, larval wandering occurred on day 6 of the fifth larval instar, and pupation took place 3 days after. The beginning (0 h) of the wandering stage was defined as the time at which the larvae stopped feeding. 0 h of the pupal stage was defined as the time at which the larvae just shed the remnants of the larval integument, marking the end of the prepupal stage. Silkworm BmN cells were maintained at 27 °C in TC-100 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (Gibco).

For hormone treatments, 20E and JH III (Sigma) were first dissolved in DMSO and then diluted with distilled water to the tested concentrations with a final concentration of DMSO at 0.1% (v/v) in water. 2 μl of a solution of 20E (4 μg) and JH III (4 μg) were injected into larvae at the thoracic region on the fifth day of the fifth instar stage, respectively. An equal volume of DMSO at a final concentration of 0.1% (v/v) was used as control. For Western blotting, proteins (30 μg) from wing discs at different stages were mixed with 5× loading buffer (250 mM Tris-HCl, pH 6.8, 10% SDS, 0.5% bromphenol blue, 5% glycerol, 5% β-mercaptoethanol), separated on 12% SDS-PAGE,
and then transferred to nitrocellulose membranes. The membranes were washed in 1× TBS-Tween 20 (TBST), pH 8.0, for 1 h with three buffer changes, 20 min each to remove SDS, and blocked with 3% (w/v) BSA in TBST at 37 °C for 2 h. The membranes were washed in TBST three times with each wash lasting 10 min and then incubated with anti-BmFoxA antibody under a gentle rocking at 30 °C for 1 h. After washing with TBST, the membranes were incubated with the alkaline phosphatase–conjugated goat anti-rabbit IgG secondary antibody under a gentle rocking at 30 °C for 1 h. The anti-BmFoxA antibodies were diluted to 1:2000 with TBST (150 mM NaCl, 0.05% Tween 20, 20 mM Tris–HCl, pH 8.0) containing 1% (w/v) BSA, and the secondary antibodies were diluted to 1:5000 (Boster Biological Technology Co. Ltd.) in the same buffer.

**Construction of expression vectors and transfection**

The ORFs of BmFox and BmSage were cloned into IE1-pEGFP-N1 plasmid vector (Clontech), and the BmWCP4 promoter-pGL3-Luc was constructed (4). The mutants of Fox CRE6 (mutated sites from nt –294 to –277) and Fox CRE7 (mutated sites from nt –35 to –18) in the BmWCP4 promoter (bp –1455 to +30 bp) were made with the method of overlap extension PCR using the BmWCP4 promoter (bp –1455 to +30)-pGL3-Luc plasmid as template. These mutants were confirmed by DNA sequencing. For the Fox CRE6 mutant, the nucleotide sequence TTTAAATAATAGGTAT was mutated to GTTACGCGCGCGGACAG. The mutated fragments were then inserted into the luciferase reporter plasmid, pGL3-basic vector (Promega, Madison, WI) between KpnI and HindIII restriction sites, to control the reporter expression. The recombinant vectors, named IE1-BmFoxn-EGFP, IE1-BmSage-EGFP, Bm WCP4 promoter Fox CRE6M-pGL3-Luc, Bm WCP4 promoter Fox CRE7M-pGL3-Luc, and Bm WCP4 promoter-pGL3-Luc, respectively, were used for co-transfection.

**Cell transfection and co-transfection were conducted using Lipofectamine™ 2000 (Invitrogen) as described previously (4).** All reporter constructs were prepared using the HiPeruge plasmid/BAC EF microkits (Magen, Shanghai, China). To normalise the firefly luciferase activity, the Renilla luciferase vector, pRL-SV40, was co-transfected with each of the pGL3-derived reporter constructs. Plasmid DNA (1 μg of IE1-BmFoxn-EGFP, 0.2 μg of Bm WCP4 promoter-pGL3-Luc, and 0.2 μg of pRL-SV40) was mixed with 4 μl of Lipofectamine™ 2000 in the Opti-MEM reduced serum medium. Fresh SF-900 medium with 10% fetal bovine serum was added at 6 h post-transfection. The cells were cultured for 48 h at 27 °C before the promoter activity assay.

Co-transfection was repeated three times (n = 3), and the average expression levels of the target genes were represented as mean ± S.E. The measurement of luciferase activity was carried out according to the manufacturer’s protocol (Promega, Madison, WI). Statistical significance of the differences between the treatments and control was analyzed by t test (for comparison of two means) and one-way analysis of variance (ANOVA; Systat, Inc., Evanston, IL) followed by a least significant difference test for multiple comparisons. The data were analyzed using GraphPad Prism version 5.0 and presented as mean ± S.E.

**Nuclear extract and in vitro expression and purification of recombinant BmFoxA and BmSAGE protein**

Nuclear proteins of the BmFoxA-overexpressing BmN cells were extracted according to the instructions for the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Waltham, MA).

The BmFoxA ORF DNA fragment was subcloned into the pET-28a vector in fusion with a His6 tag between EcoRI and Sall restriction enzyme sites, generating BmFoxA-pET-28a recombinant expression vectors. BmSage ORF DNA fragment was subcloned into the pPROEXHTa expression vector (Life Technologies, Burlington, Canada) in fusion with a His6 tag at the N-terminal end between the BamHI and HindIII restriction enzyme sites, generating BmSage-pPROEXHTa recombinant expression vectors. Recombinant BmFoxA and BmSAGE proteins were expressed in Escherichia coli cells and purified with nickel-chelating affinity chromatography using the His-Bind® kit according to the manufacturer’s protocol (Novagen).

**EMSA**

To verify the binding of BmFoxA to the putative Fox CRE6 response element in the promoter of Bm WCP4, EMSA was conducted using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific). The oligonucleotides were labeled with biotin at the 5′ end and incubated at 95 °C for 10 min in 50 mM Tris acetate buffer, pH 4.1, and then annealed to generate the probe by cooling at room temperature. WT and mutant probes were used as competitors to each other. The oligonucleotide probes were synthesized by Invitrogen (Shanghai, China), and the sequences are as follows: WT probe, 5′- AATGCTTTAAATAGGTATCAGA-3′; mutant probe (the mutant sites are underlined), 5′- AATGCTTTAAATAGGTATCAGA-3′.

Binding reactions were performed in a 20-μl mixture containing a 1× binding buffer (50 ng of poly(dI-dC), 2.5% glycerol, 0.05% NP-40, 50 mM KCl, 5 mM MgCl2, 4 mM EDTA, 10 μg of nuclear extracts/2 μg recombinant protein, and 20 fmol of a biotinylated end-labeled double-stranded probe) for 30 min at 28 °C. For the competition assay, 200-fold cold probes were added to the binding mix. For the supershift assay, 2 μg of Bm FoxA antiserum or 2 μg of rabbit IgG was added and incubated for another 30 min at 28 °C prior to the binding reaction. For the protein competition assay, 10, 20, 40, and 80-fold BmSAGE protein was added into the DNA- and BmFoxA-binding reaction. The gels were run at 100 V for 2 h using a 4% polyacrylamide gel on ice. After electrophoresis, the gel was blotted onto a positively charged nylon membrane (Hybond-N; Amersham Biosciences). Membranes were developed using the LightShift chemiluminescent EMSA kit (Thermo Scientific) according to the manufacturer’s protocol.

**DNA–protein pull-down**

For the DNA–protein pulldown assay, 20 μg of the biotinylated Fox CRE6 or mutated Fox CRE6M probes were incubated with streptavidin-coated Dynabeads (Invitrogen) for about 20 min in the binding buffer containing 5 mM Tris-base, 0.5 mM...
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EDTA, 1 m NaCl, and 0.003% NP-40. Then the oligonucleotide-conjugated beads were incubated for 30 min with the blocking buffer, which contained 2.5 mg/ml BSA, 10 mM HEPES, 10 mM glutamate potassium, 2.5 mM DTT, 10 mM magnesium acetate, 5 mM EGTA, 3.5% glycerine, 0.003% NP-40, and 5 mg/ml pvp-k30. The oligonucleotide-bead complex was then incubated with 100 μg of BmFoxA-EGFP overexpression nuclear protein at 4°C for 4 h in protein-binding buffer containing 10 mM HEPES, 100 mM glutamate potassium, 2.5 mM DTT, 10 mM magnesium acetate, 5 mM EGTA, 3.5% glycerine, 0.001% NP-40, 80 mM KCl, and 1 μg of nonspecific DNA. Then the DNA–protein complex was washed three times with washing buffer containing 100 mM glutamate potassium, 10 mM HEPES, 2.5 mM DTT, 10 mM magnesium acetate, 5 mM glycerin, 5 mM DTT, 0.05% NP-40, and 0.5 mg/ml BSA. SDS-PAGE sample buffer, which contains 50 mM Tris-base, 10 mM HEPES, 100 mM glutamate potassium, 2.5 mM DTT, 0.5% SDS, was incubated with the complex to elute the bound protein. The eluted target protein was detected by Western blotting using BmFoxA antibody.

**ChIP assay**

The ChIP experiment was performed to detect the effects of BmFoxA and BmSAGE on the promoter activity of BmWCP4 in Spli221 cells. Cells were cross-linked with 1% formaldehyde for 10 min at room temperature prior to being co-transfected with 0.5 μg of BmFoxA-EGFP or 0.5 μg of BmSAGE-EGFP with BmWCP4 promoter plasmid for 48 h. Glycine was added to terminate the fixation, and cells were washed and collected with ice-cold PBS. Cells were collected, and the cell pellet was broken up with membrane extraction buffer containing protease-/phosphatase inhibitors. Nuclei were collected and digested with MNase. Digested chromatin was collected by centrifuge. An immunoprecipitation experiment was conducted by following the manufacturer’s instructions for the Pierce™ Magnetic ChIP kit (Thermo Scientific). 5 μg of either rabbit anti-GFP antibody or normal rabbit IgG were used in immunoprecipitation reactions for 2 h at 4°C with mixing. Antibody–protein–DNA complex was enriched by incubation with ChIP Grade Protein A/G magnetic beads for 1.5 h at 4°C with mixing. The bound DNA was purified following the kit’s instructions. Immunoprecipitated genomic DNA fragments were amplified and quantitated by qRT-PCR.

**RNAi**

The RNAi assay for BmFoxA was performed in Spli221 cells. A 355-bp fragment in the ORF region of BmFoxA was chosen as a template for the synthesis of gene-specific dsRNA as described by Liu et al. (25). Double-stranded RNA was synthesized from linearized templates by using the T7 RiboMAX™T Express RNAi system (Promega, Madison, WI). A total of 4 μg of dsRNA were co-transfected with BmWCP4 promoter or BmFoxA-EGFP into Spli221 cells with 4 μl of Fugene HD transfection reagent (Promega) in the Opti-MEM reduced serum medium (Invitrogen). The cells were collected 48 h post-transfection. Total RNA was extracted according to the protocol of the Total RNA Kit II (Omega, Norwalk, CT). Reverse transcription was done using the PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China) following the manufacturer’s instructions. qRT-PCR was performed using specific primers of BmFoxA and cDNA obtained above as template.

**Author contributions**—Q. H. formal analysis; Q. H. visualization; Q. H., Z. Z., and D. Z. methodology; B. Z. software; S. Z. and Q. F. resources; Q. S. and Q. F. writing-review and editing; H. D. and Q. F. supervision; H. D. writing-original draft.

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