Basic Helix-Loop-Helix Transcription Factor Bmsage Is Involved in Regulation of fibroin H-chain Gene via Interaction with SGF1 in Bombyx mori

Xiao-Ming Zhao1,2*, Chun Liu1,2, Qiong-Yan Li1,2, Wen-Bo Hu1,2, Meng-Ting Zhou1,2, Hong-Yi Nie1,2, Yin-Xia Zhang1,2, Zhang-Chuan Peng1,2, Ping Zhao1,2, Qing-You Xia1,2*

1 State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing, China, 2 Key Sericultural Laboratory of the Ministry of Agriculture, College of Bio-Technology, Southwest University, Chongqing, China

Abstract
Silk glands are specialized in the synthesis of several secretory proteins. Expression of genes encoding the silk proteins in Bombyx mori silk glands with strict territorial and developmental specificities is regulated by many transcription factors. In this study, we have characterized B. mori sage, which is closely related to sage in the fruitfly Drosophila melanogaster. It is termed Bmsage; it encodes transcription factor Bmsage, which belongs to the Mesp subfamily, containing a basic helix-loop-helix motif. Bmsage transcripts were detected specifically in the silk glands of B. mori larvae through RT-PCR analysis. Immunoblotting analysis confirmed the Bmsage protein existed exclusively in B. mori middle and posterior silk gland cells. Bmsage has a low level of expression in the 4th instar molting stages, which increases gradually in the 5th instar feeding stages and then declines from the wandering to the pupation stages. Quantitative PCR analysis suggested the expression level of Bmsage in a high silk strain was higher compared to a lower silk strain on day 3 of the larval 5th instar. Furthermore, far western blotting and co-immunoprecipitation assays showed the Bmsage protein interacted with the fork head transcription factor silk gland factor 1 (SGF1). An electrophoretic mobility shift assay showed the complex of Bmsage and SGF1 proteins bound to the A and B elements in the promoter of fibroin H-chain gene (fib-H), respectively. Luciferase reporter gene assays confirmed the complex of Bmsage and SGF1 proteins increased the expression of fib-H. Together, these results suggest Bmsage is involved in the regulation of the expression of fib-H by being together with SGF1 in B. mori PSG cells.

Introduction
The silkworm, Bombyx mori, which is a key economic insect often used as a model for lepidopteran insects, has huge economic value for its potential applications in industry and medical technology [1–3], as well as for studying the fundamental mechanisms underlying gene regulation and organ differentiation [4]. The B. mori silk gland is the focus of this study because silk production is the major, if not the only, purpose for domestication of this insect. The B. mori silk gland is a specifically differentiated silk-producing organ composed of the anterior silk gland (ASG), middle silk gland (MSG) and posterior silk gland (PSG). The ASG is responsible for silk spinning, the MSG secretes sercin and the PSG produces fibroin. The sercin protein is a complex containing at least five sercin proteins encoded mainly by ser1, ser2 and ser3 through alternative splicing of mRNA. Each is expressed in certain parts of the MSG during embryonic and larval development [5]. Fibroin is composed of three main protein components, fibroin heavy chain (fib-H), light chain (fib-L) and P25, encoded by the corresponding genes, which forms a hexameric structure with a fib-H/fib-L/P25 ratio of 6:6:1 [6]. Among them, the fib-H gene is highly expressed in PSG cells but is repressed in MSG cells. The 5′ flanking sequence of B. mori fib-H, which is required for maximal transcription in vitro, contains five regions that bind protein factors from the PSG extract [7]. However, details of the mechanism underlying the ability to be synthesized effectively in silk glands and the precise regulation of how fib-H is highly expressed in PSG cells but is repressed in MSG cells is not fully understood.

Several transcription factors involved in transcriptional regulation of the fib-H have been identified, including Bombyx Fkh/SGF-1 [8], which is a homologue of the protein encoded by the Drosophila region-specific homeotic gene fork head [9], SGF-2 [10,11], POU-M1/SGF-3 [12] which is a homologue of Drosophila Ci-f, and FMBP-1 [13,14]. The gene expression profiles of those factors in the silk glands have been characterized individually [15] or by genome-wide analysis [16], expression of the fib-H gene
seems to be controlled directly by the co-ordination of these factors, which are expressed differentially during silk gland development. However, whether other factors or partners are involved in the regulation of silk genes needs further study.

A superfamily of transcription factors containing a basic helix-loop-helix domain (bHLH) has important roles in the control of cell proliferation, determination and tissue differentiation during the development of animals and plants [17,18]. The bHLH domain, which is approximately 60 amino acids in length, comprises a DNA-binding basic region of 15 amino acids residues followed by two α helices separated by a variable loop region [19]. Besides binding to DNA, the bHLH domain also promotes dimerization, allowing the formation of homodimer or heterodimer complexes [20,21]. Previous studies showed that the cycle homolog (Bmal1) belongs to the bHLH-PAS subfamily and is expressed in all silkworm tissues tested [22]. The 52 bHLH genes identified in B. mori belong to 59 bHLH families in groups A, B, respectively [23]. In D. melanogaster, sage is a salivary gland-specific bHLH protein that works with Fkh protein to regulate expression of SG2 directly as well as to express sage itself, and Fkh and sage regulate SG1 indirectly [24]. The salivary glands of Drosophila and the silk glands of B. mori are likely to be homologous organs [25,26], and specialized for the massive production of several tissue-specific secretory proteins. Presumably, they might have a similar function in B. mori silk glands. In this study, we identified a Mesp subfamily bHLH transcription factor termed Bmsage and demonstrated it is expressed specifically in the silk glands of B. mori.

In addition, we confirmed that the Bmsage protein interacted with a fork head protein (SGF1) and formed a complex that bound to the A and B elements in the promoter of fib-H to increase expression of the fib-H gene.

Materials and Methods

Insects

The wild type strain Dazao (low silk strain which produces little silk proteins) and strain 872 (high silk strain which produces much more silk proteins) were obtained from the Gene Resource Library of Domesticated Silkworm, Southwest University, China. The cocoon shell weight and the cocoon shell rate of strain 872 is higher than strain Dazao. The silkworms were reared on fresh mulberry leaves or on artificial diets at 25°C under a photoperiod of 12 h light/12 h dark with 75% relative humidity.

Bioinformatics analysis of candidate genes

Scanning and identification of candidate genes were done from the B. mori microarray database (http://silkworm.swu.edu.cn/silkdb/) and verified by domain prediction using SMART (http://smart.embl-heidelberg.de/). The prediction of an open reading frame and translation of amino acid sequences were preformed in ExPaSy (http://www.expasy.org/tools/). Phylogenetic analysis was conducted with the candidate gene and that of invertebrate and vertebrate species belonging to the bHLH superfamily, including D. melanogaster, Glossina morsitans, Aedes aegypti, Mus musculus, Danio rerio and Brachioptera floridana. Amino acid sequences were aligned using Clustal X using default parameters [27] and the phylogenetic analysis was carried out using a neighbor-joining (NJ) method with programs in MEGA version 5.0 [28].

RT-PCR and qPCR

Total RNA was prepared using TRIzol reagent according to the manufacturer’s protocol. Reverse transcription was carried out with 1 μg of total RNA in 10 μL reactions with a PrimeScript RT reagent Kit (Takara) according to the manufacturer’s recommendations. Primer sets for RT-PCR of Bmsage is given in Table S2. The semi-quantitative RT-PCR conditions were; 94°C for 30 s followed by 20 cycles at 94°C for 10 s, 60°C for 15 s then at 72°C for 90 s. Finally, an elongation step at 72°C for 7 min. The semi-quantitative RT-PCR reaction under the following conditions: denaturation at 95°C for 10 min followed by 40 cycles of treatment at 95°C for 10 s, 60°C for 30 s then at 72°C for 35 s. The gene encoding ribosomal protein L3 (BmRpl3) was used as a control gene. The primers for qPCR reaction are given in Table S2. The threshold cycle of each target product was determined and set in relation to the amplification plot of BmRpl3. The detection threshold was set to the log linear range of the amplification curve and kept constant (0.05) for all data analysis. Difference in Ct values (ΔCt) of two genes was used to calculate the relative expression [29]. The transcript abundance value of each individual was calculated as the mean of three replicates.

Recombinant expression, purification and western blot

The coding region of Bmsage was PCR-amplified using a sense primer (5'-CCCGGATCCGATGATCTGAATCTAACCATAC-3') with BamHI site upstream of the first amino acid residue and an antisense primer (5'-CGCAACCTTATGTTACTCGTTGACG-3') with a HindIII site downstream of the amino acid residue. The purified PCR product was digested with BamHI/HindIII and ligated into pET28a vector (Novagen), resulting in a recombinant expression vector pET-28a/Bmsage. The resultant plasmid was sequenced and transformed into Escherichia coli strain BL21 (DE3) competent cells (TransGen) and grown at 37°C in Luria-Bertani medium containing kanamycin (final concentration, 20 μg/mL), then induced with 0.2 mM isopropyl β-D-1-Thiogalactopyranoside (IPTG). Purification of the recombinant Bmsage protein was achieved using Ni-NTA affinity column (GE Healthcare) according to the manufacturer’s instructions. Phenylmethlysulfonyl fluoride (PMSF, 100 mM) was used as a proteinase inhibitor (Roche) during the purification. The purified protein was then subjected to the G-25 column (GE Healthcare) for desalting and exchanging the buffer with 20 mM Tris-HCl, 500 mM NaCl, pH 8.0. The separated lysate was detected using SDS-PAGE, (15% (w/v) polyacrylamide gel) and the concentrations of proteins were measured using a BCA kit (Beyotime, China). The purified proteins were injected into New Zealand White rabbits for preparation of antibodies.

The Malpighian tubule, fat body, head, midgut, and posterior midgut were collected from 1 day-of age larva and homogenized in 10 mM sodium phosphate-buffered saline (PBS, pH 7.4) containing a mixture of protease inhibitors (Roche). The supernatant of homogenates was collected by centrifugation (12 000 g, 4°C, and 10 min) and the protein concentrations were measured using a Bradford Assay Kit (Tiangen, China) with bovine serum albumin (BSA) used as the standard. The proteins were separated by SDS-PAGE, (15% (w/v) polyacrylamide gel) and electrophoresed onto polyvinylidene difluoride (PVDF) membrane (Roche). The membranes were blocked in 5% (v/v) skim milk overnight at 4°C and incubated with a primary antibody for 1 h at 37°C. The membranes were washed, incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma) as a secondary antibody for another
DNA constructs and Cell culture

The promoter of fib-1 (f-1bp) [forward primer: 5'-GGGGTTACAGCTTTTGAATTCGCTCAAACTG-3'; reverse primer: 5'-CTAGCTAGCGCTGATTTGAAAAAGTTGAA-3', set as described [30]) was cloned into the luciferase reporter plasmid pGL3-fibH-Luc (pGL3 basic vector from Promega) at the N-terminal end of the pBamHI and pNdI restriction sites. Three different 5′-truncated fragments were created by PCR amplification from the pGL3-fibH-Luc reporter plasmid and inserted into pGL3-Basic as described above. For expressing vectors, the primer sequences of Bmsage and SGF1 were set as its open reading frame and contained a BamHI and a Not restriction site, respectively. The target fragments were obtained by electrophoresis in a 1% (w/v) agarose gel and then cloned into a BamHI and Not-digested 1180 [Hrs1000-BmAclt4-LUC-Ser1PA] expression vector (maintained in our lab). Highly purified plasmid DNA was prepared using the QIAGEN Plasmid Midi KIt (Qiagen, Germany). B. mori cell line BmE (developed originally from an embryonic cell) [31], which express endogenous Bmsage and SGF1 genes (data not shown), was maintained at 27°C in Grace medium supplemented with 10% fetal bovine serum (HyClone). The transfection vectors were transfected into BmE cells using X-tremeGENE HP DNA Transfection Reagent (Roche) as described by the manufacturer’s instructions using the enhanced green fluorescent protein (EGFP) transfection vector as the control. Cotransfection was repeated three times, and the average expression levels of a target gene is represented as mean ± SE.

Far-western blot, ELISA

To obtain soluble proteins for protein-protein interaction studies, the full-length Bmsage-glutathione S-transferase (GST) fusion protein expression plasmid was constructed by subcloning the full-length Bmsage cDNA into the GST gene fusion vector pGEX-4T-1 (Amersham Biosciences) in the correct reading frame of the full-length Bmsage cDNA into the GST gene fusion vector pGEX-4T-1 (Amersham Biosciences) in the correct reading frame of the full-length Bmsage cDNA into the GST gene fusion vector pGEX-4T-1 (Amersham Biosciences). The purified GST proteins were used as negative controls. 

Electrophoretic Mobility Shift Assay (EMSA)

To test the binding of proteins to the regulatory sequences, electrophoretic mobility shift assay (EMSA) was performed according to the method of Kethidi et al. [35]. The labeled probe used in EMSA were the A element at nucleotide positions −64 to −39 (5′-CGAAAGTAAAATACGTGAAAATCCTG-3′) and the B element at positions −87 to −63 (5′-AATGTGTA-GATGTTTTATCTTCG-3′) of the fib-1 gene promoter [30]. The oligonucleotides were labeled using Cy3 from the 5′-end and then annealed to produce a double-stranded probe. DNA-binding reactions were carried out in a volume of 10 µL containing 10 µg of nuclear protein extracts or 1 µg of purified recombinant proteins and 2 µL of 5× binding buffer (Beyotime, China). Labeled probe (5 µM) was added after incubation for 20 min at 25°C and the incubation was continued for a further 25 min. For the competition experiments, unlabeled double-stranded probe was added to the reaction mixture at the same time as the labeled probe and mixtures were then loaded onto 4% polyacrylamide gel and electrophoresed in 1×TBE buffer (45 mM Tris/borate, 1 mM EDTA, pH 8.3). After electrophoresis, the gels were scanned and photographed with a TYPHON scanner (Amersham).
Figure 1. Bioinformatics analysis of Bmsage. A. The location of Bombyx sage in the silkworm genome (left), the structure (sketch map), and its nucleotide and deduced amino acid sequences. B. The bHLH motif of Bmsage is compared with those of other bHLH proteins, including Mesp subfamily of vertebrates, and Dmsage, Gmsage, Aasage of invertebrates, respectively. Dots indicate identical amino acids. Dashes represent gaps introduced to maximize the alignment.

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Statistical analysis

All data were statistically analyzed by independent sample t-test. Asterisks indicate significant differences (*, p<0.05; **, p<0.01).

Results

Bioinformatics analysis of the Bmsage gene

According to the tissue chip data of the whole silkworm genome [36], we found a gene (ID: BGIBMGA005127) with a higher level of expression in the silk gland compared to other tissues (Fig. S1). It was located on chromosome 25 in silkworm precise mapping [37], which includes a single copy of a completed 705 bp coding frame, and consists of four exons spanning a 2.9 kb fragment in nscf2823 (Fig. 1A). The predicted Bmsage protein consists of a single 234 amino acid with a bHLH domain (AA 157-210) (Fig. 1A). A blast search with the deduced amino acid sequence revealed several closely related Mesp subfamily of bHLH family members including vertebrate and invertebrate (Fig. 1B). The Bmsage bHLH domain showed 74%, 74%, 50%, 44.8%, 40.3% and 40.3% identity to those of Gmsage, Dmsage, Asagse, DrMesp, BmMesp and MmMesp, respectively (Fig. S2, Table S1). Based on this similarity and the phylogenetic tree, we concluded that Bmsage belongs to the Mesp-related subfamily of bHLH transcription factors.

Bmsage expressed specifically in silk glands and closely relative to fib-H gene of silkworm

To verify Bmsage is expressed specifically in the silk glands, its level of expression was examined in different tissues of B. mori larvae on day 3 of the 5th instar larval by semiquantitative RT-PCR (qPCR). BmRpl3 expression is shown as a control. Different tissues are shown as: Hd, Head; ASG, Anterior silk gland; MSG, Middle silk gland; PSG, Posterior silk gland; Mg, Midgut; Fb, Fatbody; Mp, Malpighian; Gd, Gonad; Ep, Epidermis.

Bmsage interacts with SGF1

As mentioned above, SGF1/fork head is the Bombyx homologue of the Drosophila fork head protein (Fkh), which co-regulates directly the expression of SG-specific genes with sage in the salivary gland. In B. mori, the SGF1 protein existed in MSG and PSG cells of day 3 of 5th instar larvae, as found for the Bmsage protein (Fig. S4). To determine whether Bmsage and SGF1 can interact directly in vitro, a far-western blot assay was used with purified full-length His-SGF1 and Bmsage-GST fusion proteins. The results showed that Bmsage could bind to SGF1 on the PVDF membrane (~41 kDa) (Fig. 4A-a2, lane 2), but the control could not (Fig. 4A-a1, lane 2). An ELISA binding assay showed that absorbance at 450 nm was significantly different between Bmsage/SGF1 and Bmsage/BSA (Fig. 4B), suggesting Bmsage is able to bind to SGF1 in vitro. Based on the data presented above, we asked whether SGF1 and Bmsage can interact in vivo. To address the issue, we expressed HA-tagged SGF1 and Flag-tagged Bmsage in BmE cells using a BmA4 promoter-driven construct. The results of immunohistochemical analysis showed that SGF1 and Bmsage were localized in the nucleus (Fig. S5) and colocalized within the same cells (Fig. 4A, A-D). Nuclear extracts from BmE cells over-expressing Flag-sage and HA-SGF1 were immunoprecipitated with anti-Bmsage antibody or anti-SGF1 antibody followed by western blot analysis using HA antibody or Bmsage antibody. As shown in Fig. 5, the SGF1 protein was present in an anti-Bmsage immunoprecipitate (Fig. 5C, left). Vice versa, Bmsage protein was present in an anti-SGF1 immunoprecipitate (Fig. 5C, right). These results indicated the Bmsage protein was able to interact with the SGF1 protein to form a complex in the cell nucleus.

Complex of Bmsage and SGF1 binds to the A and B elements in the fib-H gene promoter and up-regulates expression of the fib-H gene

Earlier studies showed there are two regions (the A element between positions –64 and –39 and the B element between –87 and –63) in the fib-H promoter and SGF1 can bind to the A and B elements in vitro [4]. However, evidence of this interaction in vivo is not available. In order to study the functional role of SGF1 in transcription, the SGF1 protein and different 5’ truncated fragments of the fib-H regulatory sequence were prepared. Unsurprisingly, recombinant SGF1 protein alone could bind directly to the A and B elements of the fib-H promoter and the binding was inhibited competitively when the amount of cold probe was increased gradually (Fig. 6A, B). In addition, the
luciferase expression activity of these constructs was assessed by transfecting the BmE cell line. Expression activity was increased dramatically compared to the control by over-expressing SGF1. However, when the region between positions $-2400$ and $-237$ containing A and B elements was deleted, the expression activity was not significantly different from the control (Fig. 6C). These results indicated that the A and B elements were involved in the regulation of fib-H expression in the BmE cell line. Whether Bmsage works with SGF1 involved in the regulation of fib-H by the elements, however, remains poorly understood. Thus, to determine whether a complex of Bmsage and SGF1 proteins binds to the A and B elements of the fib-H promoter directly, purified Bmsage and SGF1 proteins were incubated with the A or B element DNA probe in vitro and then analyzed by EMSA. The recombinant Bmsage protein alone was not able to bind to the A and B elements probe (Fig. 6D, lane 2), but the complex of Bmsage and SGF1 protein bound to the A and B regions of the fib-H promoter and retarded the mobility of this complex compared to recombinant SGF1 protein (Fig. 6D, lane 3–4), and the binding was partially inhibited by a 50-fold excess of the cold probe (Fig. 6D, lane 5). Therefore, we hypothesized Bmsage could be involved in the regulation of fib-H by making contact with SGF1. To test this, we used the luciferase reporter assay to examine whether Bmsage and SGF1 regulate the fib-H promoter activity. As shown in Fig. 6, the level of expression of the luciferase gene under the control of the fib-H promoter increased about 3.0-fold compared to the control in cells by over-expressing Bmsage and SGF1 (Fig. 6E). Together, these results suggest that the Bmsage protein is able to interact with the SGF1 protein to form a complex and regulate expression of the fib-H gene.

**Discussion**

In the present study, by scanning the microarray database of the silkworm B. mori we identified an Mesp subfamily bHLH transcription factor that shares 74% amino acid sequence identity...
with sage from *D. melanogaster* [38]. This transcription factor has been found in vertebrates and invertebrates and has important roles in cell type-specific gene expression and cell fate determination; e.g. *D. rerio* and *D. melanogaster* [24,39]. Here, Bmsage was shown to be expressed only in the silk glands of *B. mori* and has a low level of expression in the 4th instar molting stages, increasing gradually to a high level in feeding stages of the 5th instar (Fig. 2 and Fig. 3). The expression patterns of Bmsage and silk protein-encoding genes are similar. Previous studies showed several transcription factors were involved in transcriptional regulation of the silk genes in the silk glands of *B. mori*. Besides binding to the proximal upstream elements of both the *fibroin* and *sericin-1* genes, the SGF1/fork head acts as a crucial transcription activator of the *P25* gene in PSG cells by binding to the promoter element [40]. SGF-2, which is a 1.1 MDa heteromeric complex containing Awh, Ldb, Lcaf and fibrohexamerin proteins [10], bound three distal regions of the upstream modulator of the *fibroin* gene, which is composed of AT-rich repeats [7]. SGF-3, which was identified as POU-M1 [12], regulates the *sericin-1* gene negatively [41]. The Bmsage gene, which has a distinct spatial specificity in *B. mori*, also plays an important role in the silk glands.

The members of the bHLH superfamily have been classified into various families: the bHLH-Per-Arnt-Sim domain, the Hairy-Enhancer of split (HES), the Myc/Upstream Transcription Factor (USF), Atonal, Mesp, Hand, p48, NeuroD/Neurogenin family, Shout and Achaete-scute (AS-C) [42]. The major finding of this work was the discovery that Bmsage, which belongs to the Mesp subfamily (Fig. 1C), existing specifically in the silk glands of *B. mori*. The members of the bHLH superfamily contain bHLH mainly activates expression of the target via bind to E-box (CANNTG) sequence [43]. The level of expression of the luciferase gene under the control of the *fib-H* promoter (−865 - +1bp) increased by

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**Figure 4. Bmsage protein interacts with SGF1 by far-western blot and ELISA analysis.**

A. Interaction analysis of Bmsage with SGF1. (a1) Purified Bmsage-GST as prey protein was incubated with PBS (as a control) and detected by anti-GST antibody. (a2) purified SGF1 as prey protein was incubated with purified Bmsage-GST (bait protein) and detected by anti-GST antibody. The purified GST protein was used as a positive control. BSA protein was used as a negative control. B. The binding of Bmsage and SGF1 was determined by ELISA. The BSA protein was used as a negative control. The results are expressed as the means ± SD of three independent experiments. Asterisk indicate that the value is significantly different from control (**p<0.01). doi:10.1371/journal.pone.0094091.g004
1.5-fold over the control in cells through over-expressing Bmsage (Fig. 6E). However, we have not found a specific bind site (E-box sequence) in the proximal region of the 5′ flanking sequence of the fibroin gene. It is possible that Bmsage interacts with endogenous SGF1 protein and is involved in the regulation of the fib-H gene.

The silk glands of B. mori produce vast amounts of several silk proteins secreted mainly in the MSG and PSG cells [5]. The genes encoding these silk proteins are expressed actively in the feeding stages but are repressed during the molting stages [44]. Among them, the fibroin genes are highly expressed in the PSG cells but are repressed in MSG cells. The Bmsage protein was detected only in the MSG and PSG cells, not in ASG cells (Fig. 2B), indicating Bmsage might be correlated with synthesis of silk proteins in B. mori MSG and PSG cells. In D. melanogaster, the salivary glands are specialized also for the massive production of several tissue-specific secretory proteins; sage is a salivary gland-specific bHLH protein that works with Fkh to regulate expression of SG2 directly [24]. In B. mori, SGF1 is a fork head factor, which is a homologue of the Drosophila fork head protein, and is present in the silk gland nuclei during the whole course of larval life [45]. Furthermore, the B. mori silk glands are regarded as organs homologous with the D. melanogaster salivary glands [25]. Previous studies showed that SGF1 was able to bind to the A and B elements of the fibroin gene promoter in vitro [4], but we, for the first time, provided direct evidence for this interaction (Fig. 5).

Figure 5. Immunohistochemical co-localization of Bmsage and SGF1 proteins and co-IP assay. A. Immunohistochemical co-localization of Bmsage and SGF1 in BmE cells. HA-SFG1 and Flag-Bmsage were co-transfected into BmE cells. Primary antibody (anti-HA monoclonal antibody mouse (Sigma) and anti-Bmsage antibody rabbit) was for 1 h, followed by incubation with the secondary antibody (anti- rabbit IgG FITC and anti-mouse Alexa 555) for 30 min, both at room temperature. The samples were mounted using a mounting medium containing DAPI and photographed using confocal microscopy (Japan). The scale bar shows 5 μm. B. Nuclear extracts from BmE cells over-expressing Flag-Bmsage and HA-SGF1 were analyzed by Western blot (WB) using HA antibody (left) or Bmsage antibody (right). C. Nuclear extracts were immunoprecipitated (IP) with anti-Bmsage antibody or anti-SGF1 antibody followed by Western blot (WB) analysis using HA antibody (left) or Bmsage antibody (right). doi:10.1371/journal.pone.0094091.g005
evidence that SGF1 interacted with Bmsage and regulated expression of the fibroin gene by binding to the 2400 and 237 region containing A and B elements (Fig. 6). However, we found that the expression activity was reduced when the region between 2865 and 2400 of the fib-H gene regulatory sequence was deleted. This result indicated the region between 2865 and 2400 contained cis-regulatory elements (CREs) possibly involved in the regulation of fib-H expression. In addition, the 59 flanking sequence of the B. mori fibroin gene, which is known to be required for a maximal level of transcription in vitro, contains five regions (A to E) that bind protein factors from the PSG extract [4]. Besides the proximal A and B regions, the promoter of fib-H gene contains three distal regions (C, D and E) which bind one posterior silk gland factor (SGF-2) and two ubiquitous factors (SGF-3 and -4). These factors have an important role in the expression of silk protein genes, and the clustering of the C, D and E regions in the fibroin gene promoter might be necessary to create a high-affinity site for these silk gland proteins [7,10,19].

On the basis of data in the literature and our own results, we propose a new model for the regulation of the fib-H gene (Fig. 6). The nuclear bHLH transcription factor Bmsage interacts with fork head transcription factor SGF1, and this complex binds to the A and B elements in the promoter of fib-H involved in the expression of fibroin H-chain gene in B. mori PSG cells. This is the first report that the transcription factor containing a bHLH domain is expressed specifically in silk glands and is involved in the regulation of the expression of fib-H gene in B. mori. Thus, our findings provide a new insight into the regulation of other genes encoding B. mori silk proteins.

Supporting Information

Figure S1 Expression of Bmsage in multiple silkworm tissues on day 3 of the fifth instars based on microarray database. (TIF)

Figure S2 A phylogenetic tree of Bmsage. A phylogenetic tree of bHLH transcription factors is constructed using the MEGA5 program with the neighbor-joining algorithm. GenBank accession numbers were shown in Table S2. (TIF)

Figure S3 Cocoon shell rate of Dazao and 872. A. Expression of fib-H in different stages of B. mori. BmRpl83 expression is shown as a control. Developmental stages are shown as: IV-molting: 4th instar molting, V: 5th instar feeding stages (1 day, 3 day, 5 day, and 7 day), and prepupa. B. The cocoon picture (left) and the cocoon shell rate (right) of 872 and Dazao. The scale bar
shows 1 cm. The experiment was set as three groups independently and each group has five individuals. The result was showed as mean±SE. C. Expression of flb-H in MSG and PSG by qPCR analysis. BmRPl5 expression is shown as a control. Dz: Dazao, low silk strain; 672: high silk strain. The results are expressed as the means ± 2 SD of three independent experiments. Asterisk indicate that the value is significantly different from control (** p<0.01).

**Figure S5** Immunohistochemical localization of Bmsage and SGF1 proteins in BmE cells. HA-SGF1 and Flag-Bmsage were transfected into BmE cells. Primary antibody [anti-HA monoclonal antibody mouse (Sigma)] or anti-Flag monoclonal antibody M2 mouse (Sigma)] was for 1 h, followed by incubation with the secondary antibody (anti-mouse Alexa 555) for 30 min, both at room temperature. The samples were mounted using a mounting medium containing DAPI and photographed using confocal microscopy (Japan). The scale bar shows 5 μm.

**Table S1** Name of gene and accession number for phylogenetic analysis from NCBI.

| Gene Name | Accession Number |
|-----------|------------------|
| MyoD | NM_001005674.1 |
| Myogenin | NM_001006272.1 |
| Dp | NM_001005675.1 |
| MRF4 | NM_001005893.1 |
| MRF4 | NM_001005894.1 |
| MRF4 | NM_001005895.1 |

**Table S2** Primer sequences used in this study.

| Primer Name | Primer Sequence |
|-------------|-----------------|
| Forward | ACTCCGTGATCACTGCGGAA |
| Reverse | TGGTTCATGATCATGCGGAG |

**Author Contributions**

Conceived and designed the experiments: XMZ, CL, QYX. Performed the experiments: XMZ, QYL, CL. Analyzed the data: XMZ, CL, QYX.

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