RESEARCH

BmAly Is an Important Factor in Meiotic Progression and Spermatid Differentiation in *Bombyx mori* (Lepidoptera: Bombycidae)

Pengjie Zhang,[1] Jinfeng Zhong,[1] Guangli Cao,[1,2] Renyu Xue,[1,2] and Chengliang Gong[1,2,3]

[1]School of Biology and Basic Medical Science, Soochow University, Suzhou 215123, China
[2]National Engineering Laboratory for Modern Silk, Soochow University, Suzhou, People's Republic of China
[3]Corresponding author, e-mail: gongcl@suda.edu.cn

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ABSTRACT. The *Drosophila melanogaster* "*always early*" gene (*Dmaly*), which is required for G2/M cell-cycle control and spermatid differentiation, is one of the meiotic arrest genes. To study the *Bombyx mori aly* gene (*Bmaly*), the cDNA of *Bmaly* was cloned and sequenced, and the results showed that the open reading frame of *Bmaly* is 1,713 bp in length, encoding 570 amino acid residues, in which a domain in an Rb-related pathway was found. Phylogenetic analysis based on the amino acid sequence of conserved regions showed that Aly from different insects gathered together, except for DmAly and *Culex quinquefasciatus* Aly, which were not clustered to a subgroup according to insect order. The *Bmaly* gene was inserted into expression vector pGS-21a(+) and then the recombinant protein was expressed in *Escherichia coli* and used to immunize mice to prepare the antibody against BmAly. Immunofluorescence examination showed that BmAly was distributed in both the cytoplasm and nucleus of BmN cell. The *Bmaly* gene expression could not be detected in the silk gland, malpighian tubule, fat body, or midgut of the silkworm. Expression levels of the *Bmaly* gene were detected in the gonadal tissues, where the levels in the testes were 10 times higher than that in the ovaries. Moreover, *Bmaly* expression was detected by quantitative polymerase chain reaction at different stages of *B. mori* testis development, at which fifth instar was relatively grossly expressed. The result suggested *Bmaly* was abundantly expressed in primary spermatocytes and prespermatids. To further explore the function of *Bmaly, Bmaly* siRNA was injected into third and fourth instar silkworm larvae, which markedly inhibited the development of sperm cells. These results together suggest that *Bmaly* is a meiotic arrest gene that plays an important role in spermatogenesis.

Key Words: *always early* gene, *Bombyx mori*, meiosis, spermatogenesis

Spermatogenesis is one of the most interesting events in the animal development. Until recently, little has been known about the exact mechanisms of spermatogenesis, though some researchers have studied it in *Drosophila* (Olivieri and Olivieri 1965; White-Cooper et al. 1998, 2000; Jiang et al. 2003). There are likely to be many genes involved in spermatogenesis, from mitosis (*string*) (Edgar and O'Farrell 1990, 1994) to meiosis (*aly*, *can, mia,* and *sa*) (Alphey et al. 1992; Courtot et al. 1992; Lin et al. 1996; White-Cooper et al. 1998, 2000) and spermatid differentiation (*Mst87F*). In *Drosophila* spermatogenesis, the spermatogonium generates 16 primary spermatocytes through four mitotic divisions. All transcripts needed for spermatid differentiation are made at this stage, and most transcripts needed for spermatid elongation are shut off premeiotically (Gould-Somero and Holland 1974, Fuller 1993, Lin et al. 1996, White-Cooper et al. 2000). Transcription is not fully detected until spermatid differentiation has been switched on. Spermatid differentiation is controlled by meiotic arrest genes, mutation of which would cause spermatogenesis to be arrested before the G2/M transition, and ceased at the primary spermatocyte stage (Gould-Somero et al. 1974; Fuller 1993; Schäfer et al. 1995; White-Cooper et al. 1998, 2000).

At present, several meiotic arrest genes have been found, including always early (*aly*), cannonball (*can*), meiosis I arrest (*mia*), achintya/vismay (*achi/vis*) (Ayyar et al. 2003, Hyman et al. 2003), cookie monster (*comr*) (Jiang et al. 2003), spermatocyte (*sa*), matotopetli (*topi*) (Perezgazga et al. 2004), and tombola (*tom*) (Jiang et al. 2007). If any of the aforementioned genes mutated, sterile offspring would be produced, with testes containing spermatogenic cells morphologically normal up to the mature primary spermatocyte stage. Meiotic arrest genes can be split into two classes, the *aly*-class and the *can*-class, based on the mechanism by which they control the transcription activation of several cell cycle genes (*twine*, *cyclinB*, *boule*) (White-Cooper et al. 2000). The *aly*-class regulates transcription of the cell cycle genes required for progression toward meiotic divisions. In contrast, *can*-class genes only regulate translation of the cell cycle and encode testis-specific homologs of TATA-binding proteins and associated factors (Aoyagi et al. 2001, Hiller et al. 2001).

The *Dmaly* gene encodes a homolog of the *Caenorhabditis elegans* gene *lin-9*, a negative regulator of vulval development that acts in the same SynMuvB genetic pathway as the LIN-35 Rb-like protein. This gene coordinates meiotic cell-cycle progression and terminal differentiation during male gametogenesis by regulating chromatin conformation in primary spermatocytes. DmAly is required for cell-cycle progression through the G2/M transition of meiosis I in males for onset of spermatid differentiation and for maintenance of normal chromatin structure in primary spermatocytes (White-Cooper et al. 1998, 2000). DmAly might contain components of a complex paralogous to the *Drosophila* dREAM/Myb-MuvB and *C. elegans* DRM transcriptional regulatory complexes (White-Cooper et al. 2000).

In *Drosophila*, the function of several genes involved in spermatogenesis have been well characterized; however, little is known about the genes implicated in this process in other insects. The silkworm *Bombyx mori* is a model species in the order Lepidoptera. The sequencing of the whole genome of the silkworm has been completed (Xia et al. 2004, Xiang et al. 2008). Numerous genes relating to growth, development, metamorphosis, immunologic response, and synthesis of fibroin have been well studied, but little has been reported on the genes involved in spermatogenesis. To understand spermatogenesis in

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silkworms, the *Bmaly* gene, a homolog of *C. elegans lin-9* and *Drosophila Dmaly*, was cloned and identified.

Materials and Methods

RNA Isolation and cDNA Synthesis. Total RNA was isolated from the testes of fifth instar silkworm larvae (strain Dazao) using a total RNA Isolation Kit (TaKaRa, DaLian, China) and was treated by DNaseI to remove possible contamination of genomic DNA. cDNA was synthesized by reverse transcription, using a SuperScript III kit (Invitrogen, Carlsbad, California).

Cloning and Sequencing of the Bmaly Gene. The hypothetical coding sequence of *Bmaly* gene was obtained by comparing DmAly protein sequence (GenBank accession no. NP_524857) with both Bombyx genome sequence and Bombyx ESTs using the tBlastN program. *Bmaly* gene-specific primers *Bmaly*-1 (5′-TAGGATCCATGGCGG ATAAATCAG-3′) and *Bmaly*-2 (5′-TGCTCGAGCTATGTGCCTG CAGAATATTC-3′) contain *Bam*HI and *Xho*I restriction sites, respectively. The primers were designed according to the hypothetical *Bmaly* gene cDNA sequence, which was obtained through Silico cloning based on DmAly protein sequences (GenBank accession no. NP_524857), using the Blast program (http://www.ncbi.nlm.nih.gov/blast). Polymerase chain reaction (PCR) was carried out with synthesized cDNA as a template and the paired primers *Bmaly*-1/*Bmaly*-2. The PCR product was subjected to agarose gel electrophoresis, and the recombinant plasmids were sequenced after the recovered PCR product was cloned into vector pMD19-T (TaKaRa, Dalian, China).

Sequence Analysis. A homology search and multiple alignments were carried out with BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and the ClustalW software program (Thompson et al. 1994), respectively. A phylogenetic tree was constructed with MEGA4.1 using the maximum-likelihood method based on the Aly protein sequences, which were retrieved from GenBank. The validity of the various branches of the trees was tested by bootstrapping using 500 replicates. Conserved motif and protein function analyses were performed using the Conserved Domain Search Service program (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

BmAly Expression in *Escherichia coli* and BmAly Antibody Preparation. The *Bmaly* PCR product was ligated into vector pMD19-T, and the fragment of *Bmaly* was excised out with *Bam*HI/*Xho*I and ligated with the expression vector pGS-21a(+) (GenScript, Nanjing, China) to generate the recombinant plasmid pGS-21a(+)-*Bmaly*. Fusion proteins were expressed in *E. coli* strain BL21 and Rosetta, respectively. The recombinant protein purified using Ni-NTA agarose (Qiagene, Shanghai, China) was used to immunize Kunming mice (Soochow University, Suzhou, China) by subcutaneous injection. The prepared antibody was identified by western blotting.

Sodium dodecyl sulfate polyacrylamide gelelectrophoresis (SDS-PAGE) and Western Blotting. The bacterium transformed with pGS-21a(+)-*Bmaly* was mixed with 2× SDS loading buffer (0.1 mol/liter TrisCl, 0.2 mol/liter dithiothreitol, 4% SDS,20% glycerol, 0.2% bromophenol blue, 4% b-mercaptoethanol) and boiled in 100°C water for 5 min. After centrifugation at 12,000 *g* for 3 min, the supernatant was electrophoresed on acrylamide gels—the stacking gel and the separating gel were at 5% (v/v) and 12% (v/v), respectively. The gel for protein staining was treated with Coomassie Brilliant Blue R250. The gel for Western blotting was transferred to a polyvinylidene fluoride (PVDF) membrane using an electrophoretic transfer cell. Western blotting was then performed using a prepared mouse anti-BmAly and HRP-conjugated goat antimouse IgG (Biosynthesis Biotechnology, Beijing, China).

Subcellular Localization. The cultured BmN cells derived from the ovaries of silkworms were collected and fixed with 4% paraformaldehyde for 15 min, then rinsed with 0.01 M PBST (0.05% of Tween-20 in Phosphate Buffered Saline (PBS)) and incubated with a mouse anti-BmAly at 4°C overnight. After rinsing with 0.01 M PBST three times, the cells were then incubated with FITC-conjugated goat antimouse IgG (Tiangen, Beijing, China) at 37°C for 1 h. The cells were then observed by fluorescence microscopy after removing the noncombined fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG and staining with 4′,6-diamidino-2-phenylindole (DAPI). In negative controls, the BmN cells reacted with preimmune serum as the primary antibody.

Quantitative PCR. Quantitative PCR (qPCR) was used to determine the expression of *Bmaly* in the testis, ovary, silk gland, malpighian tubule, fat body, and midgut from Day 3 of fifth- instar larvae. In addition, qPCR was also used to determine the expression level of *Bmaly* in the testes at different stages. Before reverse transcription, the mRNA samples were fully digested with Rnase-free DnaseI to avoid genomic DNA contamination. Primers Realy-1 (GGATGCCTGAAGACTTT GAACG) and Realy-2 (GCCCTGGTGCAATTTGTTGA) were designed based on the cDNA sequences. Actin3-1 (CGGCTACTCGTT CACTACC) and Actin3-2 (CCGTCGGGAAGTTCGTAAG) were used as primers for amplifying the housekeeping gene *B. mori actin A3*, used as an internal control. A 20-µl volume containing 0.2 µg of cDNA, 5 pmol of each primer, and 10 µl of SYBR green real-time PCR master mix (Applied Biosystems, Foster City, CA) was used for each PCR. The PCR was then processed on a real-time RT-PCR System (ABI 7300) under the following program: one cycle at 50°C for 2 min, one cycle at 95°C for 15 min; then 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. This procedure was repeated three times.

RNAi. The *Bmaly*-specific siRNA-657 (sense strand 5′-GCCUCG ACGAUGUUCUCAAtt-3′, antisense strand 5′-ttCCGAGCUGCUAC AAGAGUU-3′), *Bmaly*-specific siRNA-96 (sense strand 5′-GGAAGA AGAAGAAGAAGAAtt-3′, antisense strand 5′-ttCCUUCUUCUUC UUCUUCUU-3′) were synthesized by the Gima Corporation (Shanghai, China), and was diluted to 1 µg/µl. On the first day of third and fourth instar, the larva was injected with 1 µg of *Bmaly* siRNA. The injected larvae were then reared on fresh mulberry leaves at 25°C. At the same time, negative control larvae were injected with random siRNA-NC (sense strand: 5′-UUCUCCGAACGUGUCACGUtt-3′; antisense strand: 5′-ttAAGAGGCUUGCACAGUGCA-3′). In addition, Diethy pyrocarbonate (DEPC) H_2O controls that were injected with DEPC H_2O were raised.

Histochemical Observations. The testes dissected from the injected larvae at the beginning of fifth instar were fixed in 4% paraformaldehyde and embedded in paraffin, and the section was stained with hematoxylin–eosin solution.

Results

Isolation and Identification of *Bmaly* Gene. Using the total RNA of the testes of fifth instar larvae as the template, real-time PCR was carried out with specific primers Realy-1 and Realy-2 designed from the hypothetical cDNA sequence of the *Bmaly* gene, which was obtained by Silico cloning based on the amino acid sequences of DmAly (GenBank accession no.: NP_524857.3). The sequencing result showed that the open reading frame of the *Bmaly* gene was 1,713 bp in size, encoding a putative protein of 570 amino acid residues. This sequence has been deposited in the GenBank database under the accession no. GQ999610.

The sequence of *Bmaly* cDNA was compared with the genomic sequence of *B. mori* using the BlastN program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The genomic sequence of *Bmaly* was completely located in a genomic DNA fragment (AADK01022835). The *Bmaly* gene had two exons, which were located at position 1,033→1,051 and 1,375→3,068, respectively. The border sequence of the first intron followed the GT–AG ruler; however, the second intron border sequence was GC–AG. A domain in Retinoblastoma (Rb) tumor suppressor gene-related pathway (DIRP) conserved in lin-9 of *C. elegans* and DmAly was found at position 174–278 in a deduced amino acid sequence of BmAly.

Sequence Comparison and Phylogenetic Tree analysis. The deduced amino acid sequence of BmAly was aligned with those of other species, the results revealed that Aly of *B. mori* shared 57, 57, 56, 52, 44, 43, 42, 41, 40, 27, and 21% identity with Aly (or lin-9) of *Apis mellifera* (XM_394339), *Nasonia vitripennis* (XM_001605133),

Tribolium castaneum (XM_970110), *Pediculus humanus corporis* (XM_002433084), *Danio rerio* (XM_002664628), *Cu. quinquefasciatus* (XM_001862992), *Mus musculus* (NM_001103182), *Homo sapiens* (BC045625), *Xenopus* (*Silurana*) *tropicalis* (XM_002941131), *C. elegans* (NM_001027844), and *Drosophila melanogaster* (AJ277307), respectively. Although the identity of the Aly protein from different species was not high, two conserved regions could be found (Fig. 1), one of which had a DIRP domain.

To investigate the evolutionary relationship between Aly of different species, a phylogenetic tree was constructed using the maximum-likelihood method based on the amino acid sequence of two conserved regions (Fig. 2). The result showed that the *aly* gene from different insects gathered together, except *Dmaly* and *Cu. quinquefasciatus aly*, which did not cluster in a subgroup according to insect order. *D. melanogaster* belongs to Insecta, but DmAly was markedly distant from Aly in other insects, suggesting that DmAly might have evolved in a unique way.

Prokaryotic Expression and BmAly Antibody Preparation. *E. coli* transformed with pGS-21 a(+)-*Bmaly* was induced with IPTG for 4 h at 37°C. To detect BmAly expression, harvested bacteria were subjected to SDS-PAGE, a specific band of about 97 kDa representing 6× His-GST-BmAly fusion protein could be detected (Fig. 3A) indicating that BmAly was correctly expressed in *E. coli*. The recombinant protein was purified by using Ni-NTA agarose (Qiagen, Shanghai, China) (Fig. 3A) and was used to immunize mice to prepare antibodies.

To assess the efficacy of prepared mouse anti-BmAly antibody, western blotting was carried out. A specific band representing 6× His-GST-BmAly fusion protein was observed (Fig. 3B), indicating that the prepared antibody can be used to detect BmAly.

Subcellular Localization of BmAly in BmN Cells and Testis. The subcellular localization of BmAly in BmN cells was performed with immunofluorescence, which indicated that BmAly was distributed uniformly both in the cytoplasm and nucleus. Otherwise, there was no green fluorescence was detected when using the preimmune serum (Fig. 4).

Expression Pattern of the Bmaly Gene. To determine the transcriptional levels of *Bmaly* in different tissues, qPCR was performed. The *Bmaly* gene expression could not be detected in the silk gland, malpighian tubule, fat body, or midgut of the silkworm. Expression levels of the *Bmaly* gene were detected in the gonadal tissues, where the levels in the testes were 10 times higher than that in the ovaries (Fig. 5A). The result were similar to the data analyzed by microarray and released in SilkDB (http://www.silkdb.org/microarray/), where *Bmaly* is mainly present in the gonads of *B. mori*, with little detected in other tissues (data not shown). Expression pattern of *Bmaly* in testis at different development stages was determined by Q-PCR, the result showed that *Bmaly* was relatively higher expressed at fifth instar larvae (Fig. 5B).

Effect of Injecting Bmaly siRNA into Silkworm Larvae on the Development of Sperm Cells. In the Lepidoptera, RNAi was successful in *B. mori* and other insects. For instance: Quan-injected dsRNA to knockdown a pigment gene in embryos (Quan et al. 2002); others targeted a putative *Bacillus thuringiensis* toxin receptor in early fifth instar Spodoptera litura larvae, and the RNAi effect could be inherited (Rajagopal et al. 2002).

To understand the functions of the *Bmaly* gene, *Bmaly* siRNA-657, *Bmaly* siRNA-96, and DEPC H2O were injected into silkworm third and fourth instar larvae to detect expression of the *Bmaly* gene. The level of *Bmaly* mRNA in testes of the third day of fifth instar larvae was estimated using qPCR. The level of *Bmaly* in the larvae injected with *Bmaly* siRNA-657 decreased by 86% compared to 26% in DEPC H_2O control group, respectively. In addition, *Bmaly* siRNA-96 decreased by 12%. Moreover, the effect of injecting *Bmaly* siRNA-657 into silkworm larvae on the development of sperm cells was investigated by histochemical observation (Fig. 6A and B). At the most apical tip of the testes, cellula apicalis (ca) are surrounded by germline stem cells. Further from the tip is a zone of larger primary spermatocytes organized into 64-cell cysts (ps). These cells remain in meiotic prophase, a specialized stage of the meiotic cell cycle that precedes meiotic division. However, sperm cells mostly develop into the mid-anaphase of the first

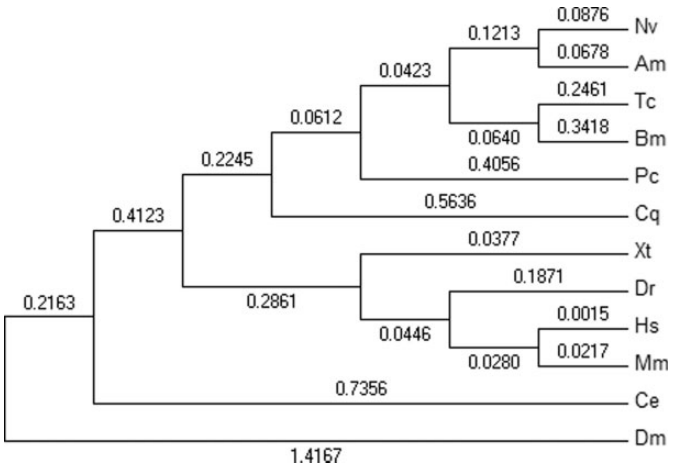

Fig. 1. Multialignment between the Protein BmAly and its analogs. Homologs amino acids are indicated with a dark grey shadow frame and similar amino acids with light gray shadow. Dark box indicates conserved region 1, dark box with dots indicates conserved region 2, and gray box indicates DIRP domain. Bm, *B. mori* Aly (GQ999610); Dm, D. *melanogaster* (AJ277307); Hs, *H. sapiens* (BC045625); Mm, *M. musculus* (NM_001103182); Ce, *C. elegans* (NM_001027844); Nv, *N. vitripennis* (XM_001605133); Cq, *Cu. quinquefasciatus* (XM_001862992); Pc, *P. humanus corporis* (XM_002433084); Dr, *D. rerio* (XM_002664628); Xt, *X.* (*Silurana*) *tropicalis* (XM_002941131); Am, *A. mellifera* (XM_394339); Tc, *T. castaneum* (XM_970110).

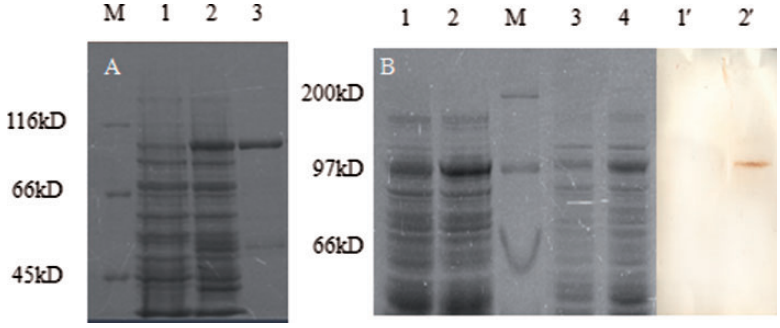

Fig. 2. The phylogenetic analysis of Aly protein. The conserved amino acid sequence of Aly was used for the construction of the tree by a maximum-likelihood program with bootstrapping using 500 replicates. The genetic distances are shown on each internal branches. Abbreviations are the same as those mentioned in Fig. 1.

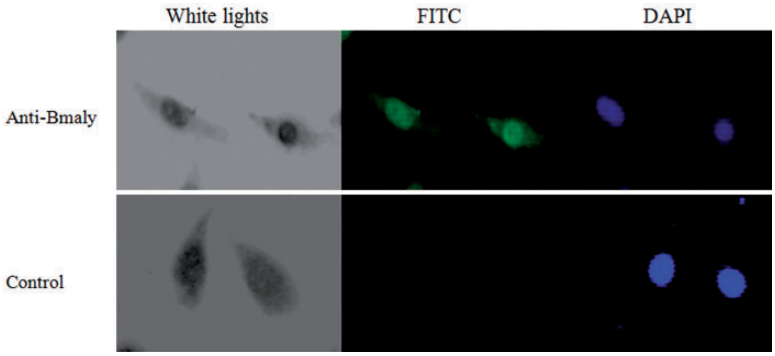

Fig. 3. Expression in *E. coli* and BmAly antibody preparation. (A) SDS-PAGE of BmAly expressed in *E. coli*. M, protein marker; lane 1, *E. coli* strain Rosseta transformed with pGS21a(+); lane 2, *E. coli* strain Rosseta transformed with pGS21a(+)-Bmaly; lane 3, purified recombinant protein. (B) Western blotting for specificity analysis of the BmAly antibody. Left: SDS-PAGE Right: Western blotting. M, protein marker; lanes 1 and 2, *E. coli* strain BL21 and Rosseta transformed with pGS21a(+)-Bmaly, respectively; lanes 3 and 4, *E. coli* strain BL21 and Rosseta transformed with pGS21a(+), respectively; Lanes 1′ and 2′, Western blots corresponding to lanes 1 and 2, respectively. The primary antibody was mouse anti-BmAly and the secondary antibody was HRP-conjugated goat antimouse IgG.

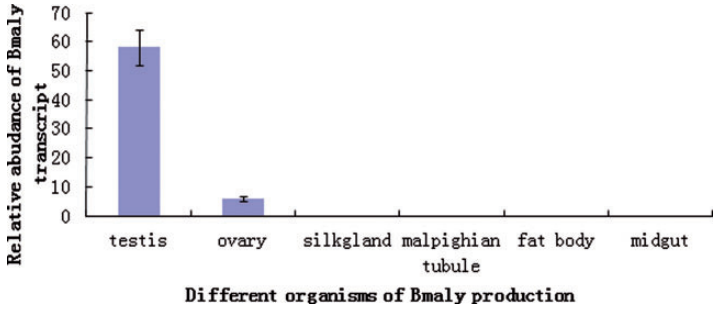

Fig. 4. Subcellular localization of BmAly. BmN cells were treated with anti-BmAly antibody, followed by treatment with FITC-conjugated goat antimouse IgG, and the nucleus was treated with DAPI (blue), which were examined under Inverted fluorescence microscope. From left to right, green fluorescence for FITC-treated BmAly, DAPI-treated nucleus. For the control, preimmune serum was used as the primary antibody. Original magnification for BmN cells =200×.

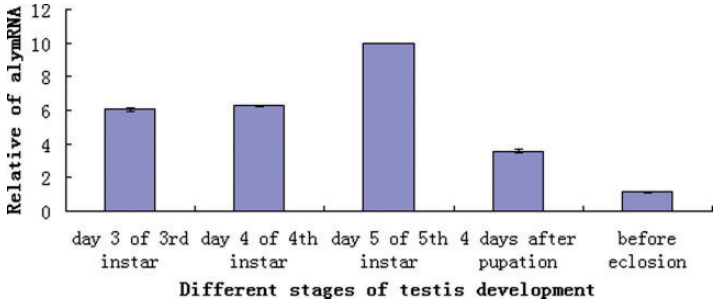

Fig. 5. Bmaly expression. Q-PCR analysis of *Bmaly* mRNA at different organisms, different stages of testis development. Testes, ovaries, silk gland, malpighian tubule, fat body, and midgut were dissected from silkworms at the times indicated, and cDNA was synthesized as described above.

meiosis, and secondary spermatocytes (ss) were observed far from the apical tip of the testes in fifth instar larvae, and prespermatids were also observed (pr) at the bottom of the chamber of testes. Instead, the region of cellula apicalis in the testes of the fifth instar larvae injected with *Bmaly* siRNA-657 was larger than that of larvae in DEPC H_2O control, and the primary spermatocytes and secondary spermatocytes were also larger than that in DEPC H_2O control. The schematic showing the progression of *B. mori* testis development was apparent in the article (Fig. 6C) (Zhang et al. 2012), at the apical tip of the testis, teloblast is surrounded by spermatogonial, then go to larger zone of primary spermatocytes, followed by bigger secondary spermatocytes and finally get sperm bundle which contain mature sperm. These results suggested that the mitotic divisions proceed normally in the testes of larvae injected with *Bmaly* siRNA, but meiotic division is arrested.

Discussion

Aly is a homolog of lin-9, which acts with Rb tumor suppressor protein LIN-35 to antagonize RTK-Ras-MAPK signaling in *C. elegans* vulval development (Kornfeld 1997, Beitel et al. 2000). In *Drosophila* spermatogenesis, *aly* is one of the most important meiotic arrest genes required in the progression of G2/M and spermatozoa formation (White-Cooper et al. 1998, 2000; Jiang et al. 2003). Mutations of *aly*

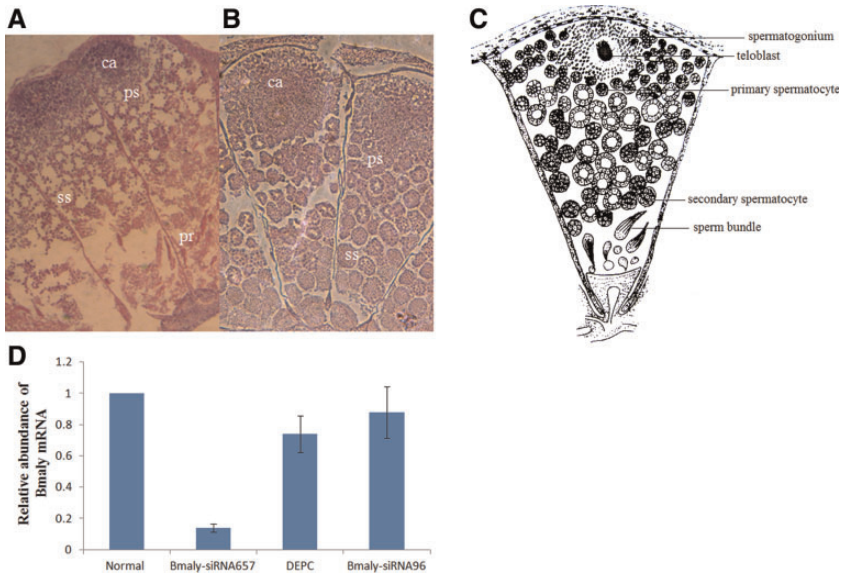

Fig. 6. Development of sperm cells. (A) Testis of the first day of fifth instar larvae. (B) Testis of the first day of fifth instar larvae, which was injected with *Bmaly* siRNA-657 at third and fourth instars. ca, cellula apicalis; ps, primary spermatocytes; ss, secondary spermatocytes; pr, prespermatid. (C) A schematic showing the progression of spermatogenesis in *B. mori.* Spermatogonial cell born at the apical tip surround the teloblast, they undergo six rounds of synchronous mitotic divisions to produce cysts of 64 primary spermatocysts, then they go to larger secondary spermatocytes, followed by sperm bundle which containing mature sperm. (D) The level of Bmaly siRNA-657 mRNA-injected silkworms compared with that in normal (no injection), DEPC H_2O, and Bmaly siRNA-96, respectively.

cause no meiotic or postmeiotic cysts except the primary spermatocytes in *Drosophila* testis development. This result has been ascribed to the numerous genes required for meiotic and spermatid differentiation that are not fully transcribed in the absence of the Aly protein (White-Cooper et al. 1998, 2000; Jiang et al. 2003).

The functions of several genes involved in spermatogenesis have been well characterized in *Drosophila*, but little is known about other types of insect. In this article, we characterized the structure and function of the *Bmaly* gene in relation to spermatogenesis for the first time in the silkworm. The *aly* gene family is conserved from plants to humans, suggesting that *aly* may play an important role in biological development. The open reading frame of the *Bmaly* gene was 1,713 bp in size, encoding a putative protein of 570 amino acid residues. Although the identity of Aly protein between silkworms and other species was not high, a DIRP domain, which is usually considered to have relationships with Rb in chromatin structure discovered in *C. elegans* and *Drosophila* (Hsieh et al. 1999, Lu and Horvitz 1998), was found in BmAly. Therefore, the functions of BmAly may be similar to those of Aly in other species. Phylogenetic analysis showed that Aly from different insects did not gather together according to insect order, suggesting that Aly may have evolved differently in different species.

DmAly regulates meiotic cell-cycle progression and terminal differentiation during male gametogenesis by regulating chromatin conformation in primary spermatocytes. The Aly protein is both cytoplasmic and nuclear in early primary spermatocytes, after which it resolves to a chromatin-associated pattern. It remains cytoplasmic in a loss-of-function missense allele, suggesting that nuclear localization is critical for Aly function (White-Cooper et al. 1998, 2000). Transcription of meiotic cell cycle and terminal differentiation genes depend on a conserved chromatin-associated protein, whose nuclear localization is regulated. Two nuclear localization signals located at position 76–81 (ARIRKK) and 99–125 (SPKKTPKILNKTPNKPPSAKKQKSPIK) were found in BmAly by using MultiLoc2 prediction (http://www-apb.informatik.uni-tuebingen.de/Services/MultiLoc); however, the result of cellular localization showed that BmAly was distributed uniformly both in the cytoplasm and nucleus of BmN cells, which were originally derived from silkworm ovaries. The result suggests that the localization of BmAly depends on the cell type and cell period. Other factors may alter BmAly activity by controlling its subcellular localization and in Drosophila was that Cookie monster (Comr) and Aly may mutually dependent on each other to nucleus. Achintya/Vismay (Achi/Vis) and Matotopetli (Topi) would then enter the nucleus independently, and then recruit Aly/Comr complex to these promoters.

Expression patterns of the *Bmaly* gene were in accordance with that of *Dmaly*, abundantly presented in testes (White-Cooper et al. 1998, 2000; Jiang et al. 2003). Expression of the *Bmaly* gene could be detected in the ovary but not in other tissues, suggesting that BmAly might play some role in ovary development. The relatively great expression in fifth instar may indicate that *Bmaly* was expressed from spermatogonial to primary spermatocytes but was highly expressed at primary spermatocyte stage, and this may be ascribed to the procession of meiosis I.

To understand the function of BmAly, the effects of injecting *Bmaly* siRNA into silkworm larvae were investigated on the development of sperm cells. The result showed that the sperm cells in the testes were almost arrested at the primary spermatocyte stage of fifth instar larvae that were injected with *Bmaly* siRNA-657 at third and fourth instar larval stages. Previous study showed that BmAly interacts with BmTGIF in the progression of spermatogenesis and may recruit other factors to form a complex to fully activate the progression of meiosis and spermatid differentiation in *B. mori* (Zhang et al. 2012). Therefore, we propose that the *Bmaly* gene is involved in meiotic arrest and is required for cell-cycle progression through the G2/M transition of meiosis I in males, like *Dmaly* functions in *Drosophila* spermatogenesis (White-Cooper et al. 1998, 2000).

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References Cited

Alphey, L., J. Jimenez, H. White-Cooper, I. Dawson, P. Nurse, and D. M. Glover. 1992. *twine*, a *cdc25* homologue that functions in the male and female germlines of *Drosophila*. Cell 69: 977–988.

Aoyagi, N., and D. Wassarman. 2001. Developmental and transcriptional consequences of mutations in *Drosophila* TAF(II)60. Mol. Cell. Biol. 21: 6808–6819.

Ayyar, S., J. Q. Jiang, A. Collu, H. White-Cooper, and R. A. White. 2003. *Drosophila* TGIF is essential for developmentally regulated transcription in spermatogenesis. Development 130: 2841–2852.

Beitel, G. J., E. J. Lambie, and H. R. Horvitz. 2000. The *C. elegans* gene *lin-9*, which acts in an Rb-related pathway, is required for gonadal sheath cell development and encodes a novel protein. Gene 254: 253–263.

Courtot, C., C. Frankhauser, V. Simanis, and C. F. Lehner. 1992. The *Drosophila cdc25* homolog *twine* is required for meiosis. Development 116: 405–416.

Edgar, B. A., and P. H. O'Farrell. 1990. The three postblastoderm cell cycles of *Drosophila* embryogenesis are regulated in G2 by *string*. Cell 62: 469–480.

Edgar, B. A., F. Sprenger, R. J. Duronio, P. Leopold, and P. H. O'Farrell. 1994. Distinct molecular mechanism regulate cell cycle timing at successive stages of Drosophila embryogenesis. Genes Dev. 8: 440–452.

Fuller, M. T. 1993. Spermatogenesis. In the development of *Drosophila*. Cold Spring Harb. Press 1: 71−147.

Gould-Somero, M., and L. Holland. 1974. The timing of RNA synthesis for spermiogenesis in organ cultures of *Drosophila melanogaster* testes. Wilhelm Roux' Arch. 174: 133–148.

Hiller, M., T. Y. Lin, C. Wood, and M. T. Fuller. 2001. Developmental regulation of transcription by a tissue-specific TAF homolog. Genes Dev. 15: 1021–1030.

Hsieh, J., J. Liu, S. A. Kostas, C. Chang, P. W. Sternberg, and A. Fire. 1999. The RING finger/B-Box factor TAM-1 and a retinoblastoma-like protein LIN-35 modulate context-dependent gene silencing in *Caenorhabditis elegans*. Genes Dev. 13: 2958–2970.

Hyman, C. A., L. Bartholin, S. J. Newfeld, and D. Wotton. 2003. *Drosophila* TGIF proteins are transcriptional activators. Mol. Cell. Biol. 23: 9262–9274.

Jiang, J. Q., and H. White-Cooper. 2003. Transcriptional activation in *Drosophila* spermatogenesis involves the mutually dependent function of aly and a novel meiotic arrest gene cookie monster. Development 130: 563–573.

Jiang, J. Q., E. Benson, N. Bausek, K. Doggett, and H. White-Cooper. 2007. *tombola*, a tesmin/TSO1 family protein, regulates transcriptional activation in the *Drosophila* male germline and physically interacts with Always early. Development 134: 1549–1559.

Kornfeld, K. 1997. Vulval development in *Caenorhabditis elegans*. Trends Genet. 13: 55–61.

Lin, T. Y., S. Viswanathan, C. Wood, P. Wilson, N. Wolf, and M. T. Fuller. 1996. Coordinate developmental control of the meiotic cell cycle and spermatid differentiation in *Drosophila* males. Development 122: 1331–1341.

Lu, X., and H. R. Horvitz. 1998. *lin-35* and *lin-53*, two genes that antagonize a *C-elegans* Ras pathway, encode proteins similar to Rb and its binding protein RbAp48. Cell 95: 981–991.

Olivieri, G., and A. Olivieri. 1965. Autoradiographic study of nucleic acid synthesis during spermatogenesis in *Drosophila melanogaster*. Mutat. Res. 2: 366–380.

Perezgazga, L., J. Q. Jiang, B. J. Bolival, M. Hiller, E. Benson, M. T. Fuller, and H. White-Cooper. 2004. Regulation of transcription of meiotic cell cycle and terminal differentiation genes by the testis-specific Zn finger protein *matotopetli*. Development 131: 1691–1702.

Quan, G. X., T. Kanda, and T. Tamura. 2002. Induction of the white egg 3 mutant phenotype by injection of the double-stranded RNA of the silkworm white gene. Insect Mol. Biol. 11: 217–222.

Rajagopal, R., S. Sivakumar, N. Agrawal, P. Malhotra, and R. K. Bhatnagar. 2002. Silencing of midgut aminopeptidase N of Spodoptera litura by double-stranded RNA establishes its role as *Bacillus thuringiensis* toxin receptor. J. Biol. Chem. 277: 46849–46851.

Schäfer, M., K. Nayernia, W. Engel, and U. Schäfer. 1995. Translational control in spermatogenesis. Dev. Biol. 172: 344–352.

Thompson, J. D., D. G. Higgins, and T. J. Gibson 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673–4680.

White-Cooper, H., M. A. Schafer, L. S. Alphey, and M. T. Fuller. 1998. Transcriptional and post-transcriptional control mechanisms coordinate the onset of spermatid differentiation with meiosis I in *Drosophila*. Development 125: 125–134.

White-Cooper, H., D. Leroy, A. MacQueen, and M. T. Fuller. 2000. Transcription of meiotic cell cycle and terminal differentiation genes depends on a conserved chromatin associated protein, whose nuclear localisation is regulated. Development 127: 5463–5473.

Xia, Q. Y., Z. Y. Zhou, C. Lu, D. J. Cheng, F. Y. Dai, B. Li, P. Zhao, X. F. Zha, T. C. Cheng, and C. L. Chaiet al. 2004. A draft sequence for the genome of the domesticated silkworm (*Bombyx mori*). Science 306: 1937–1940.

Xiang, Z. H., K. Mita, Q. Y. Xia, J. Wang, S. Morishita, and T. Shimada. 2008. The genome of a lepidopteran model insect, the silkworm *Bombyx mori*. Insect Biochem. Mol. Biol. 38: 1036–1045.

Zhang, P. J., G. L. Cao, R. Y. Xeu, and C. L. Gong. 2012. BmTGIF, a *Bombyx mori* homolog of *Drosophila* DmTGIF, regulates progression of spermatogenesis. PLoS One 7: e47861.

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