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Identification and functional analysis of a testis-biased gene encoding serine/arginine-rich protein in silkworm, *Bombyx mori*

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Abstract: Spermatogenesis is a fundamental process in sexual reproduction. In this study, we cloned a 716-bp cDNA of a testis-biased gene in *Bombyx mori*, named as *BmRS-TS*, which encodes a polypeptide of 164 amino acids, containing 26.7% arginine and serine residues. Sequence similarity analysis showed that *BmRS-TS* is a lepidopteran-specific gene. Results of RT-PCR and Western analysis revealed that *BmRS-TS* was expressed predominantly in the testis. Immunohistochemistry assay showed that the BmRS-TS protein was mostly located in primary spermatocytes. Moreover, knockdown of *BmRS-TS* by RNA interference (RNAi) showed that the morphology of the mature sperm was abnormal and that sperm bundles were broken up. Our results suggest that *BmRS-TS* plays an important role in silkworm spermatogenesis and provide some clues for understanding the mechanism that underlies spermatogenesis, which can be used as a reference for other lepidopterans.

Keywords: silkworm; spermatogenesis; serine/arginine-rich protein; lepidopteran-specific gene; RNAi

INTRODUCTION

The silkworm, *Bombyx mori* (*B. mori*), is not only of considerable economic value, but it also serves as a model insect for Lepidoptera. Understanding the process of spermatogenesis in *B. mori* is important for the control of lepidopteran pests by the sterile-male technique [1]. The gonads of male *B. mori* consist of two testes that are connected to each other by spermiducts. Both testes contain four compartments, each of which is surrounded by thin connective tissue. The testicular follicles have a long cavity and at the apex, germ cells develop into mature sperm [2].

Spermatogenesis is a continuous and precisely controlled process involving many genes that are expressed in male sperm cells [3-5]. Functional and morphological changes during spermatogenesis from primary spermatocytes to mature sperm are highly complex. They include meiotic division, axoneme elongation, nebenkern formation, mitochondrial modification and acrosome development, and have been examined extensively in *B. mori* [6-8]. Spermatogenesis in *B. mori* occurs throughout larval and adult life, from spermatogonia in newly hatched silkworms to mature sperm in the moth, which has been studied at the ultrastructural level [6,8]. However, the mechanisms that regulate cellular differentiation during testis development in the Ordo Lepidoptera are poorly understood [9,10]. To date, researchers have identified only a few genes that are involved in silkworm spermatogenesis; they include *β2 tubulin*, testis-specific *tektin*, *Aha1*, *BmTGIF*, *BmAly*, *Maelstrom* and two genes encoding for adenine nucleotide translocase [11-17]. Recently, the homolog of *Drosophila Sex-lethal* gene was reported to determine dimorphic sperm formation in the silkworm [18].

Herein, we cloned a testis-biased gene *BmRS-TS* encoding for a serine/arginine-rich protein and compared its predicted amino acid sequence with that of other organisms and we analyzed its mRNA expres-

sion levels in different tissues of *B. mori*. Additionally, a polyclonal antibody prepared against BmRS-TS protein was used to examine the distribution of BmRS-TS in testis and germline cells. Finally, we assessed the effects of *BmRS-TS* knockdown on the phenotype of the mature sperm by RNAi.

MATERIALS AND METHODS

Insect material

The silkworm strain Dazao was preserved at the Gene Resource Library of Domesticated Silkworm (Southwest University, Chongqing, China). Larvae were raised with fresh mulberry leaves at 25±2°C under a 12 h light-dark photoperiod.

Bioinformatics analysis and cloning of *BmRS-TS*

The genome sequence of *B. mori* has been completed and published [19,20]. Xia et al. [21] reported genome-wide gene expression profiles in multiple tissues of the domesticated silkworm based on microarray analysis. Using the microarray data, we found a gene (probe ID: sw07075) that showed testis-biased expression. Interestingly, the predicted protein sequence of the gene was enriched in Arg and Ser amino acid content. We called the gene *BmRS-TS*. Some properties of *BmRS-TS* were analyzed using online programs (http://www.expasy.org/), including open reading frame (ORF) search, nucleotide sequence translation and both isoelectric point and molecular weight prediction. Signal peptide and protein domain prediction were accomplished using the SMART program (http://smart.embl-heidelberg.de). Homologs in other species were screened by BLASTX search using the *BmRS-TS* nucleotide sequence as a query in the nr database of GenBank.

RT-PCR of *BmRS-TS* in different tissues

On the third of the fifth instar, total RNA isolated from the head, epidermis, midgut, fat body, Malpighian tubule, silk gland, testis and ovary tissues of *Dazao* was reverse transcribed into cDNA. TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen, China) was used to prepare cDNAs according to the manufacturer's instructions. Then, cDNAs were synthesized by reverse transcription using 2 μg total RNA as template. Total protein from various tissues of *Dazao* on the 3rd day of the fifth instar, including the fat body, midgut, gonad, head, silk gland and epidermis, was extracted and homogenized in 10 mM phosphate-buffered saline (PBS; pH 7.4) on ice. The homogenates were centrifuged at 10000×g at 4°C for 10 min and the supernatants were collected.

RT-PCR was performed to confirm the characteristics of gene expression in different tissues using customized primers. PCR was carried out according to the following program: one cycle at 95°C for 4 min; 30 cycles at 94°C for 40 s, 56°C for 45 s and 72°C for 30 s and one final cycle at 72°C for 10 min. Products were then analyzed by agarose gel electrophoresis. (All primers used in these experiments are listed in Supplementary Table S1). Relative amounts of *BmRS-TS* mRNA were normalized to those of *B. mori actin 3*, which was used as an internal control.

Western blotting

Each supernatant (0.8 mg/mL, 20 μL) was run on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel. Samples were boiled for 10 min in Tris-HCl buffer (pH 6.8) containing 10% SDS, 50% glycerin, 0.5% bromophenol blue and 5% dithiothreitol and were centrifuged at 12000×g for 3 min. Ten μL of supernatant was loaded onto a 12.5% polyacrylamide gel. Each sample was normalized to 2 μL on the basis of α-tubulin expression. After electrophoresis, separated proteins on each gel were transferred onto a polyvinylidene difluoride membrane with a semi-dry electron blotting system for 20 min in transfer buffer (39 mM glycine, 48 mM Tris, 0.037% SDS, 20% methanol). After transfer, the membrane was blocked in Tris-buffered saline, 0.1% Tween 20 (TBST) containing 5% non-fat powdered dry milk for 1 h at room temperature. The membrane was incubated with one of the rabbit antibodies (anti BmRS-TS or anti α-tubulin), diluted in TBST (1:2500 or 1:5000) containing 1% non-fat powdered dry milk and kept at 4°C overnight. The polyclonal antibody of BmRS-TS was obtained from ZeHeng Biotech, China. The tubulin antibody was purchased from Beyotime Biotechology, Shanghai, China. After removal of the primary antibodies, the blots were washed five times with TBST for 5 min per wash and then they were

incubated with secondary antibody, horseradish peroxidase conjugated goat anti-rabbit IgG (1:20,000; Beyotime, China), in TBST for 1 h at room temperature. Finally, the blots were washed five times with TBST. Immune activity was visualized using the Chemi Scope series with Clarity Western electrochemiluminescence (ECL) substrate (Bio-Rad, Hercules, CA, USA). The measured values for anti-BmRS-TS were normalized to those of α-tubulin.

Immunohistochemistry

PBS was administered for 5 min by *in vitro* methods to testes excised on the 3rd day of the 5th instar larvae, and fixed in 4% formaldehyde in PBS for 12 h at 4°C. Fixed samples were dehydrated by soaking in a series of 70%, 80%, 90% and 95% ethanol solutions at room temperature, followed by 100% xylene at room temperature, twice for 30 min. Samples were embedded in 50% paraffin in xylene at 60°C for 1 h, and then 60°C for 1 h in 100% xylene. Paraffin blocks were sectioned at a thickness of 5 μm using a microtome (RM2235C-CWUS, Leica, Germany), and sections were mounted on glass slides, then placed at 65°C for 1 h. Paraffin was removed by soaking in xylene. The slides were rehydrated in a series of 100%, 90%, 80% and 70% ddH_2O at room temperature for 10 min per step. To remove intrinsic peroxidase activity and for antigen retrieval, mounted sections were treated with 10% H_2O_2 in methanol for 10 min and slices were boiled in sodium citrate buffer solution (pH 6.0) for 5 min, then allowed to cool before washing with PBS. Specimens were incubated with anti-BmRS-TS (1:800) antibody at room temperature for 1.5 h. Excess antibodies were washed with PBS 5 times, followed by incubation with cyanine-3 (Cy3) dye-conjugated goat anti-rabbit IgG (1:500; Invitrogen, Carlsbad, CA, USA) at room temperature with the necessary PBS washes. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

RNAi and organism mashing

The *BmRS-TS*-specific double-stranded-RNA that was amplified by specific primers with a linker T7-TAATACGACTCACTATAGGGAGAT (Supplementary Table S1) was synthesized using a Ribo MAX Large Scale System-T7 (Promega, Madison, WI, USA). A total of 30 silkworm prepupa were injected with 40 μg *BmRS-TS*-specific double-stranded-RNA, *DsRed* and double distilled H_2O per 10 individuals at each prepupal stage. *DsRed* is the gene encoding for *Discosoma sp*. red fluorescent protein. *DsRed* dsRNA was used as the control group. The expression of *BmRS-TS* in the testes of injected prepupa was estimated using quantitative real-time PCR (qPCR) 48 h later. Additionally, the wild-type group and *DsRed* group were tested. The rest of the experimental and control group insects were mashed until the organisms split, after which tissues were immediately observed using a microscope (Olympus U-HGLGPS, Japan). For qPCR, total RNA was extracted using TRIzol® reagent (Introvigen, USA) following the manufacturer's instructions, and the first strand of the cDNA was synthesized using Moloney murine leukemia virus (MMLV) reverse transcriptase according to the manufacturer's instructions (Promega, USA). The qPCR reactions were run on an ABI7500 Real-Time PCR machine (Applied Biosystems, USA) with SYBR® Premix Ex Taq™II (TaKaRa, Japan). The eukaryotic translation initiation factor 4A (silkworm microarray probe ID sw22934) was used as the internal control [22]. (Primers used for qPCR are listed in Supplementary Table S1). We used the $2^{-\Delta\Delta Ct}$ method to analyze data.

RESULTS

Sequence analysis of *BmRS-TS*

The cDNA of *BmRS-TS* was cloned and sequenced. The full-length sequence was 716 bp, consisting of an ORF of 498 bp, a 5'-untranslated region (UTR) of 182 bp and a 36 bp 3'-UTR sequence (Fig. 1). The sequence of the *B. mori BmRS-TS* gene was submitted to GenBank (Acc. No.: KM884956). The ORF encoded a polypeptide of 164 amino acids with a calculated molecular weight of ~18.3 kDa. The predicted amino acid sequence of *BmRS-TS* showed that it was enriched in arginine and serine (26.8%). Interestingly, the protein contained a RSRSRS di-peptide repeat that is characteristic of RS protein family members.

Homology research, based on BLASTX in GenBank, indicated the presence of homology in the other eight species. Results indicated that *BmRS-TS* has the closest homology with *Bombyx mandarina* uncharacterized protein LOC114247851 (E value 6.00E-51),

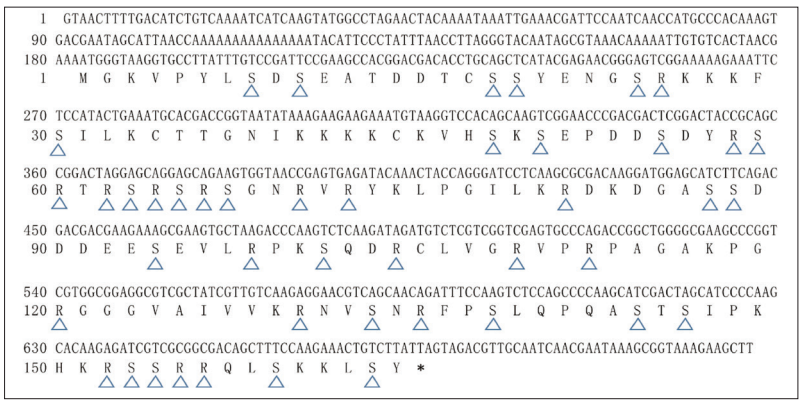

Fig. 1. Nucleotide and predicted amino acid sequence of *BmRS-TS*. The arginine and serine (RS) residues are marked with a triangle. The numerical positions of the nucleotide and amino acid sequences are shown on the left. ORF of *BmRS-TS* encodes a polypeptide of 164 amino acids, of which there are 44 serine/arginine residues (26.8%). The protein contains a RSRSRS triple di-peptide repeat that is characteristic of the RS protein family members.

Table 1. All homologs of the *BmRS-TS* gene in other species determined by BLASTX search in GenBank.

Acc. Number	E value[a]	Annotation	Organism	Order
XP_028036720.1	6.00E-51	uncharacterized protein LOC114247851	*Bombyx mandarina*	Lepidoptera
XP_026750535.1	2.00E-15	uncharacterized protein LOC113511134	*Galleria mellonella*	Lepidoptera
XP_028165190.1	9.00E-13	uncharacterized protein LOC114356302	*Ostrinia furnacalis*	Lepidoptera
XP_022818713.1	2.00E-11	uncharacterized protein LOC111351155	*Spodoptera litura*	Lepidoptera
PZC74609.1	4.00E-11	hypothetical protein B5X24_HaOG207627	*Helicoverpa armigera*	Lepidoptera
RVE41883.1	7.00E-10	hypothetical protein evm_013457	*Chilo suppressalis*	Lepidoptera
XP_026728482.1	8.00E-08	uncharacterized protein LOC113494369	*Trichoplusia ni*	Lepidoptera
PCG69041.1	4.00E-07	hypothetical protein B5V51_4589	*Heliothis virescens*	Lepidoptera

[a] E value is the number of distinct alignments that are expected to occur in a database search by chance.

and the farthest with *Heliothis virescens* hypothetical protein B5V51_4589 (E value 4.00E-7) (Table 1). All species belong to Lepidoptera, indicating that *BmRS-TS* is a lepidopteran-specific gene.

Expression profiles of *BmRS-TS*

We analyzed the expression profile of *BmRS-TS* in different silkworm tissues on the 3rd day of the 5th instar by RT-PCR. A PCR product with the expected 498-bp fragment size was detected in testis, but not amplified from the other tissues examined, including the epidermis, silk gland, head, fat body, midgut and female gonad (Fig. 2A).

Additionally, Western blotting revealed that testis extracts reacted with rabbit anti-BmRS-TS serum and formed a single band of about 18.3 kDa (Fig. 2B), which corresponded to the molecular mass predicted for *BmRS-TS* CDS. These findings suggested that the protein encoded by *BmRS-TS* was expressed predominantly in the testis of *B. mori*. Based on genome-wide microarray expression data of tissues [21], *BmRS-TS* was also highly and significantly expressed in the testis of *B. mori* (Fig. 2C).

Cellular localization of BmRS-TS

Testes of the 5th instar are about 2.5 mm in length, with a middle section of 1.5 mm and a thickness of 0.8-1.0 mm. Spermatocytes are located at the apical part of each compartment of the testes (Fig. 3). Cysts of primary spermatocytes are located at the most peripheral region of the middle zone. After division of primary spermatocytes by meiosis, each round spermatid of cyst began to elongate, after which the period of deformation began. Immunofluorescence analysis showed that BmRS-TS protein was most abundant in developing spermatogonia, as well as evenly abundant in the other types of cells. The BmRS-TS signal reached a peak in spermatogonia and primary spermatocytes, while its decrease was detected during meiosis (Fig. 3).

Fertilization rate after RNAi

To investigate the role of *BmRS-TS* in sperm development, RNAi assay was carried out to reduce *BmRS-TS* expression in male individuals. We found that *BmRS-*

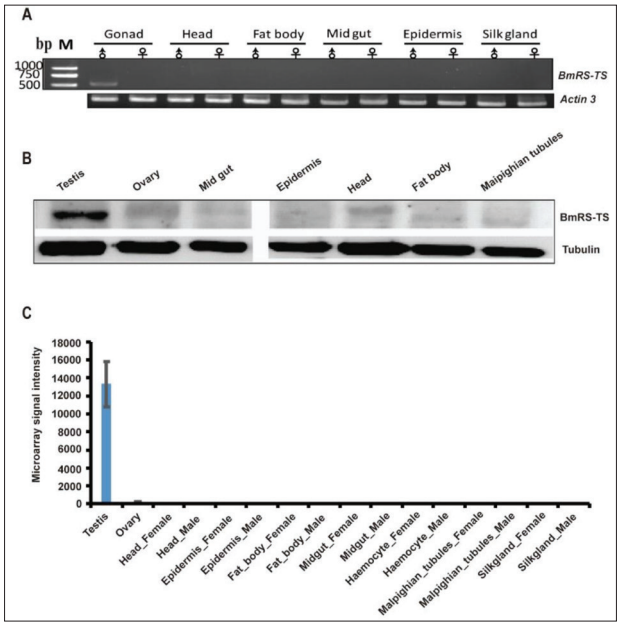

Fig. 2. (A) Semi-quantitative RT-PCR analyses were performed using cDNA from testis, ovary, head♂, head♀, fatbody♂, fatbody♀, midgut♂, midgut♀, epidermis♂, epidermis♀, silk gland♂, and silk gland♀ tissues of *Dazao* at the 3rd day of the 5th instar. (B) Detection of the protein levels of BmRS-TS. Western blot was performed to detect BmRS-TS with anti-BmRS-TS antibody (1:2500), with α-tubulin used for the internal control. (C) Microarray-based expression analysis of *BmRS-TS* in different tissues of the silkworm at the 3rd day of the fifth instar. Microarray data were downloaded from the previous study. The probe ID for *BmRS-TS* is sw07075.

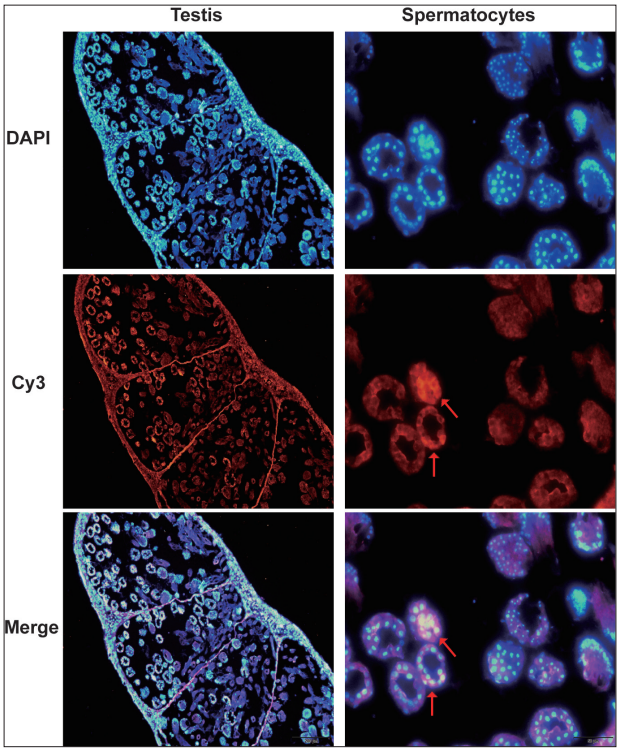

Fig. 3. Immunofluorescent images of BmRS-TS expression in testis at day 3 of the 5th instar. Immunostaining was performed with rabbit anti-BmRS-TS antibody, followed by treatment with Cy3-conjugated goat anti-rabbit IgG. Nuclei of testis were treated with DAPI (blue) and examined under an inverted fluorescence microscope. From top to bottom, DAPI-treated nuclei, red fluorescence for Cy3-treated BmRS-TS protein, and the merge images are shown. Primary spermatocytes showed prominent BmRS-TS expression with strong DAPI labeling, overlaid with Cy3 staining. The red arrow indicates the location of BmRS-TS.

TS expression level was dramatically reduced in the *BmRS-TS* RNAi group which was transfected with dsRNAs targeting *BmRS-TS* (Fig. 4D). After RNAi, testes of the silkworm were squashed on a slide and observed under a microscope. We noticed that the morphology of the sperms in the *BmRS-TS* RNAi group was different compared with that of the wild-type group and the *DsRed* RNAi group (Fig. 4A, B, C). The sperms were abnormal, and each sperm bundle seemed fragmentary (Fig. 4C). Additionally, we observed that there was an indication of expansion of the sperm acrosome. Also, autolysis of the acrosome membrane was observed (Fig. 4C). Because of the breakdown of the membrane of the sperm, we suspected that this gene might affect fertilization in silkworms. We carried out a survey of the fertilization rate. The results showed that in the control group, the fertilization rate of eggs reached 93.0%, while the fertilization rate of the *BmRS-TS* RNAi group was only 30.8% (Supplementary Table S2).

DISCUSSION

In this study, we identified the testis-biased gene *BmRS-TS* encoding a serine/arginine (RS)-rich polypeptide. Most RS-rich proteins belong to a highly conserved family of splicing factors that are present throughout metazoans and have diverse roles in both constitutive and regulated splicing [23-25]. These splicing factors have a similar bipartite structure that is composed of two functional domains: a N-terminal RNA-binding domain, composed of multiple RNA-recognition motifs (RRMs), and a C-terminal RS-rich domain. The RRM domain mediates the RNA interactions of the RS proteins, while the RS-rich domain regulates protein-protein interactions of RS proteins. Previous studies showed that the

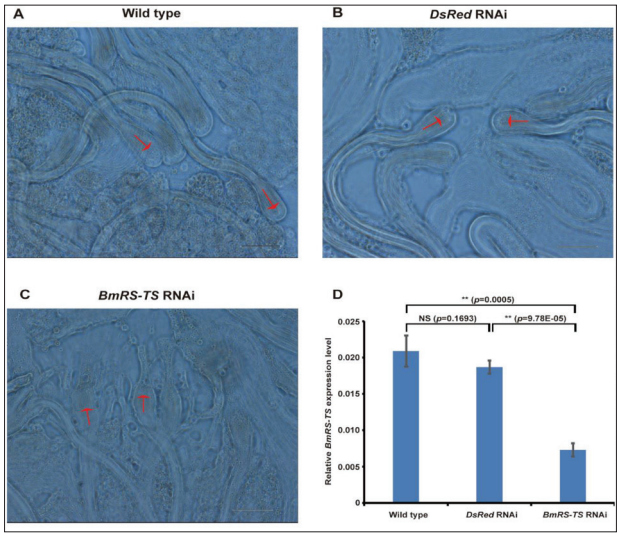

Fig. 4. Observation of the morphology after RNAi. **A** – Wild-type group. Normal sperm bundles were shown. The red arrow indicates the sperm head. **B** – Negative control group. This group was injected with DsRed dsRNA and showed normal sperm head. **C** – Experimental group. Injection of *BmRS-TS* dsRNA showed that the sperm bundle was disorganized and that the sperm had an acrosome that was not intact. The red arrow indicates a broken acrosome membrane. **D** – Expression levels of *BmRS-TS* were reduced after using dsRNA of *BmRS-TS*. The male silkworms on day 3 of the 5th instar larva were injected with dsRNA specific for *DsRed* (control) and *BmRS-TS*, respectively. After 48 h, the expression of *BmRS-TS* was detected by qPCR. Results showed that the expression of *BmRS-TS* was significantly reduced in the *BmRS-TS* RNAi group than that of the wild-type group and the *DsRed* RNAi group.

RS-rich domain plays an important role in mRNA export and translation initiation [26]. We found that BmRS-TS protein was mainly expressed in spermatogonia and primary spermatocytes, suggesting that it might regulate splicing or translation and thus affect sperm development.

Spermatogenesis for fertilizing sperm begins in the larval period and stops after pupation, whereas non-fertilizing sperm undergoes deformation. RNAi led to the abnormality in sperm morphology, including the intumescence and dissolution of the acrosome membrane and the breaking up of the sperm bundles. The break-up of the paired sperm of the opossum (*Didelphis virginiana*) *in vitro* was associated with the loss of motility by one of the participants, which is a critical indicator of the ability of a sperm to fertilize an egg [27]. In species where interactions on the cell surface are important for the formation or stabilization of conjugates, there is a great probability that death of participating sperm would lead to conjugate break-up [28]. Membrane disruption caused by conjugate break-up has been proposed as fitness cost associated with sperm conjugation [29]. However, the mechanism underlying conjugate dissociation remains poorly defined for many species [28]. In most diving beetles, the sperm remain conjugated until they are positioned for fertilization [30].

The aim of this study was to identify *BmRS-TS* and to characterize its transcript levels and predicted protein distribution in order to investigate how the protein might function. RNAi resulted in a reduction of the fertilization rate, mainly as a consequence of the abnormal morphology of the sperm and break up of sperm bundles. We propose that this sperm activation defect occurred because of failure in the breakdown of the sperm plasma membrane, i.e. at a step that normally occurs immediately after sperms enter the eggs. We believe that our study provides some important clues for understanding of the reproductive biology of the silkworm.

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/Zhang%20et%20al_4382_Supplementary%20Material.pdf