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Novel female-specific *trans*-spliced and alternative splice forms of *dsx* in the silkworm *Bombyx mori*

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ABSTRACT

The *Bombyx mori doublesex* gene (*Bmdsx*) plays an important role in somatic sexual development. Its pre-mRNA splices in a sex-specific manner to generate two female-specific and one male-specific splice forms. The present study investigated six novel *dsx* variants generated by *trans*-splicing between female *dsx* transcripts and two additional novel genes, *dsr1* and *dsr2*. Expression analysis indicated that *Bmdsx-dsr1* represented splicing noise, whereas *dsr2*, which *trans*-spliced with *dsx* to generate five variants, regulated the expression of the female-specific *B. mori dsx* transcript $Bmdsx^{F}$s. We unexpectedly found a novel exon 2n insertion during *Bmdsx* transcription, which did not influence the validity of the novel protein, $BmDSX^{F3}$. Ectopic expression of $BmDSX^{F3}$ repressed the pheromone-binding protein gene and the testis-specific gene *A2* in males, and activated of the storage protein 1 gene. Our findings suggest that *trans*-splicing is a novel regulatory function of *Bmdsx*, which participates in female sexual development by regulating the expression of three $BmDSX^{F}$ proteins.

1. Introduction

Sexual development involves a cascade of gene regulation. Despite differences among organisms in terms of the primary signal for sex determination, some key genes and functions lower down the cascade are relatively conserved [1,2], notably the *doublesex* gene. *dsx* in insects encodes a zinc-finger transcription factor of the *doublesex*/mab-3-related transcription factor (DMRT) family and is a homolog of the human divalent metal transporter 1 (*DMT1*) and mouse *Dmrt1* genes [3,4].

The sex determination mechanism in *Drosophila melanogaster* has been thoroughly examined, and a well-characterized genetic hierarchy (X: A > *Sxl* > *tra*/*tra2* > *dsx* and *fru*) has been shown to regulate somatic sexual differentiation and behavior [5–7]. *Dmdsx* alternatively splices into two sex-specific transcripts that encode two sex-specific proteins, $DmDSX^{F}$ in females and $DmDSX^{M}$ in males [8]. These two proteins have common amino termini but sex-specific carboxyl termini and act with other development-related genes to control somatic sexual differentiation and pattern dimorphisms, including the shape of sex-specific neurons [7], sex pheromone production [9], genital development [10,11], abdominal pigmentation [12,13], loss of the terminal male abdominal segment [14], and the origin and diversification of sex combs [15].

However, the hierarchy of sex determination in *Bombyx mori* has not been described in detail. *B. mori* demonstrates female heterogamety (ZW), which differs from that in female *Drosophila* (XX). Sex determination is initiated by the number of X chromosomes in *Drosophila* [16], whereas a putative feminizing factor (*Fem*) on the W chromosome is proposed to determine the femaleness of silkworms [17]. *Bmdsx*, the *B. mori* homolog of *Dmdsx*, was shown to splice in a sex-specific manner into two female forms ($Bmdsx^{F1}$ and $Bmdsx^{F2}$) and one male form ($Bmdsx^{M}$) [18,19]. The expression of $Bmdsx^{F1}$ in males or $Bmdsx^{M}$ in females can repress or activate the expression of three downstream genes (pheromone-binding protein, *PBP*; vitellogenin, *Vg*; and storage protein 1, *SP1*) [20,21]. However, limited knowledge of the regulatory mechanism of *Bmdsx* and the paucity of identified BmDSX downstream targets mean that our understanding of important aspects of sexual development in the silkworm is limited. In this study, we investigated the existence of *trans*-splicing forms of *Bmdsx* and their roles in the regulation of female-specific *dsx* mRNA translation.

2. Materials and methods

2.1. Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted from tissues (head, integument, hemocytes, midgut, fat body, trachea, Malpighian tubule, silk gland, gonads)

Abbreviations: *Bmdsx*, *Bombyx mori doublesex*; $Bmdsx^{F}$, female-specific splicing form of *Bmdsx*; $Bmdsx^{M}$, male-specific splicing form of *Bmdsx*; *dsr1* and *dsr2*, *Bmdsx*-related gene 1 and 2; ncRNA, non-coding RNA; pA, SV40 polyadenylation signal.

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from both sexes, and first-chain cDNA synthesis and RT-PCR analysis were performed as described previously [22]. PCR amplifications were conducted using the primer sets (Table S1) P9/P2 for *Bmdsx-dsr1*, P5/P6 for *Bmdsx-dsr2*, and P7/P8 for the novel exon 2n. P5, P7, and P9 bound specifically to the exon 1 common to both sex *Bmdsx* transcripts. P2 bound to *dsr1*, P6 bound to *dsr2*, and P8 bound to exon 2n.

2.2. Embryo injection and screening of transformed animals

The helper plasmid pHA3PIG [23] was mixed with the transgenic construct (Supporting Information). About 10–15 nl of a 1:1 mixture of construct and helper plasmids (400 ng/μl total DNA concentration) in Buffer EB (Qiagen, Germany) were micro-injected into each non-diapause egg (Table S2), which was prepared using the acid-treatment method after chilling. The embryos were maintained at 25 °C in moist petri dishes until hatching. G0 adults were backcrossed and the G1 progeny were screened for enhanced green fluorescent protein (EGFP) fluorescence in the embryo using an Olympus MVX10 fluorescence stereomicroscope (Olympus, Japan).

2.3. Inverse PCR analysis (iPCR)

Genomic DNA was extracted from G1 adults as described previously [23], digested with *Hae*III and circularized by ligation overnight at 16 °C. PCR was performed on the circularized fragments using the primer sets (Table S1) P16/P17 for the left-arm region and P18/P19 for the right-arm region. PCR fragments were cloned, and compared by BLAST analysis against the BGI assembly (http://www.silkdb.org/silkdb/genome/index_png.html) [24,25].

2.4. Southern blot analysis

Genomic DNA was digested with *Xba*I and the digested DNA (50 μg per lane) was separated on a 0.8% (wt/vol) agarose gel and transferred onto a nylon membrane (Roche, Germany) under a vacuum. The membrane was hybridized at 46 °C with a 720-bp digoxigenin-labeled EGFP probe and immersed in a solution containing alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche). The EGFP probe was synthesized using a PCR DIG Probe Synthesis Kit (Roche) and the hybridization signal was visualized using a chemiluminescent method with a Clinx Chemiscope Series Chemiluminescence imaging system (Clinx Science Instruments, China).

2.5. W chromosome-specific PCR

Transgenic animals were sexed by assaying for the presence of a W chromosome, using the W chromosome-specific primer set P20/P21 (Table S1). Genomic DNA was extracted as above and used as a template.

2.6. Real-time quantitative PCR (Q-PCR)

RNA was extracted from female fat bodies and male testes from G2 day-3 fifth-instar larvae and male antennae from G2 adults. cDNAs were produced as above. The primer sets P22/P23, P24/25 and P26/P27 (Table S1) were used to compare the expression levels of *SP1*, *PBP* and *tsA2*, respectively [20,27,28] between transgenic and wild-type silkworms. Q-PCR was carried out using SYBR® Premix Ex Taq™ II (TaKaRa), and amplifications were detected with the StepOnePlus™ Real-Time PCR System (Applied Biosystems). Relative expression levels were calculated after correction for expression of the translation initiation factor 4A (*eIF-4A*), which was chosen as a reference gene [29] and assayed in triplicate, in parallel with *SP1*, *PBP* and *tsA2*.

Before Q-PCR analysis, RNA from the same transgenic individuals was used to analyze the expression of the $Bmdsx^{F3}$ transgene by 3′ rapid amplification of cDNA ends (RACE), as described (Supporting Information), and RT-PCR was performed using the primer set P9/P28 (Table S1). Primer P28 was specific for the transgenic construct (Fig. S4).

3. Results

3.1. Trans-spliced variants between Bmdsx and two dsx-related genes, Bmdsr1 and Bmdsr2

Cloning of the 3′ end of *Bmdsx* produced two novel *trans*-spliced variants, *Bmdsx-dsr1* and *Bmdsx-dsr2* (Fig. S1). Structural analysis of the two variants suggested that *Bmdsr1* and *Bmdsr2* participate in the regulation of silkworm sexual development by *trans*-splicing with the female splice form $Bmdsx^{F1}$. We therefore investigated these potential *trans*-spliced variants by PCR amplification to determine whether the two genes also *trans*-spliced with another female splice form, $Bmdsx^{F2}$, and with the male-splicing form, $Bmdsx^{M}$. *Trans*-spliced variants of *Bmdsx* and *dsr2* were expressed only in the female integument, fat bodies, trachea, silk gland, ovary, and testis, whereas *Bmdsx-dsr1* expression was undetected (Fig. 1A). Cloning and sequencing all RT-PCR products demonstrated that the putative *Bmdsr2* gene was able to *trans*-splice with $Bmdsx^{F2}$ in several ways, but was unable to *trans*-splice with $Bmdsx^{M}$ (Figs. 1B and S7). Interestingly, two *trans*-spliced variants occurred in female-specific tissues: *Bmdsx-dsr2*a in the female fat

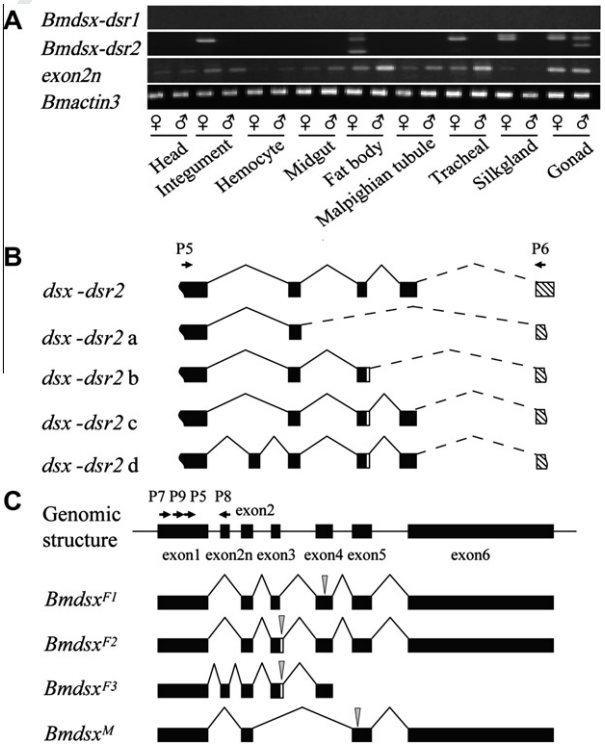

Fig. 1. *Trans*-spliced and alternative-spliced forms of *Bmdsx*. (A) expression patterns of *Bmdsx-dsr1*, *Bmdsx-dsr2*, and the novel exon 2n in nine tissues from female and male silkworms. *Bmactin3* was used as control. (B) structures of the five *trans*-spliced variants between *Bmdsx* and *Bmdsr2*. Solid bar represents *Bmdsx* exons, and the "V" line indicates introns. *Bmdsr2* exon is indicated by the slash box. (C) pre-mRNA of *Bmdsx* splices to produce four outcomes. Primers are indicated by horizontal arrows. Grey arrowhead shows the stop codon. Exon 2n represents the novel 81-bp exon.