

RESEARCH PAPER



Alternative splicing regulation of *doublesex* gene by RNA-binding proteins in the silkworm *Bombyx mori*

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ABSTRACT

Doublesex is highly conserved and sex-specifically spliced in insect sex-determination pathways, and its alternative splicing (AS) is regulated by Transformer, an exonic splicing activator, in the model system of *Drosophila melanogaster*. However, due to the lack of a *transformer* gene, AS regulation of *doublesex* remains unclear in Lepidoptera, which contain the economically important silkworm *Bombyx mori* and thousands of agricultural pests. Here, we use yeast three-hybrid system to screen for RNA-binding proteins that recognize sex-specific exons 3 and 4 of silkworm *doublesex* (*Bm-dsx*); this approach identified BxRBP1/Lark binding to the exon 3, and BxRBP2/TBPH and BxRBP3/Aret binding to the exon 4. Investigation of tissues shows that *BxRBP1* and *BxRBP2* have no sex specificity, but *BxRBP3* has – three of its four isoforms are expressed with a sex-bias. Using novel sex-specific silkworm cell lines, we find that BxRBP1 and BxRBP3 directly interact with each other, and cooperatively function as splicing repressors. Over-expression of BxRBP1 and BxRBP3 isoforms efficiently inhibits splicing of the exons 3 and 4 in the female-specific cells and generates the male-specific isoform of *Bm-dsx*. We also demonstrate that the sex-determination upstream gene *Masc* regulates alternatively transcribed BxRBP3 isoforms. Thus, we identify a new regulatory mechanism of *doublesex* AS in the silkworm, revealing an evolutionary divergence in insect sex-determination.

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Introduction

Sex determination in animals is determined by both the composition of sex-chromosomes and the environment [1]. Pathways of sex determination in insects, the most abundant and diverse animals on the earth, can be divided into three stages: the primary signal on sex-chromosomes, a cascade of alternatively spliced genes, and the expression of sex-specific developmental genes [2,3].

Many types of sex-chromosome composition have been identified in insects, such as the XY type in the fruit fly, mosquito, and housefly, the XO type in the grasshopper, and the ZW type in silkworm, and no sex chromosome in the honeybee [4,5]. Primary sex-determining signals are different, even in insects with the same type of sex-chromosome composition. For example, the initial signal is believed to be the dosage of XSE (X chromosome-encoded signal element) in *Drosophila melanogaster* [6,7], Nix (a dominant male-determining factor) in mosquito *Aedes aegypti* on Y chromosome [8], Mdmd/CWC22 (*Musca domestica* male determiner) in housefly [9], CSD (complementary sex determiner) in the honeybee *Apis mellifera* [10], and piRNA in the silkworm *Bombyx mori* on W chromosome [11].

Alternative splicing (AS) cascades have been well characterized in *Drosophila melanogaster*, in which XSE triggers a cascade of genes that are sex-specifically spliced. These

include *sex-lethal* (*Sxl*) [12], *transformer* (*tra*) [13], and *doublesex* (*dsx*) [14]. Most insects use *tra* as the first regulator in the alternatively spliced cascade instead of *Sxl*, such as medfly (*Ceratitis capitata*) and red flour beetle (*Tribolium castaneum*), although the protein sequence of Tra homologues is not well conserved among insects [15,16]. In honeybee, the AS cascade contains a *tra* homologous gene *feminizer* (*fem*) and *dsx*, while the initial signal CSD encodes a tra-like protein and is a potential splicing regulator for *fem* [17]. Tra/tra-2 complex binds to the exon 4 of *dsx* and acts as a splicing activator in *Drosophila* [14], while the detail regulation mechanism still unknown in other insects. So far, the only conserved gene in the insect sex-determination pathway is the downstream gene *dsx*, which is sex-specifically spliced and encodes transcription factors in both female and male insects for regulation of downstream sexual development genes [2,4,18].

Lepidoptera is the second largest order in insects, comprised of hundreds of thousands of species, including the economically important silkworm *Bombyx mori* and thousands of agricultural pests. For silk production, the male produces better quality silk and is more efficient due to less consumption of mulberry leaves. A female-specific lethal system was previously used to control insect pests based on the sex-specific alternative splicing of genes from insect sex-determination pathway [19,20]. Thus, sex-determination

genes could be applied as targets for optimized silk production as well as targets for pest control. The female silkworm contains a Z and a W sex-chromosome, whereas the male silkworm has two Z chromosomes. Despite a long history of silkworm domestication, the sex-determination pathway has not been well understood except for a predicted *Feminizer* (*Fem*) gene on the W chromosome [21,22] and the downstream *Bm-dsx* gene [23,24]. A few years ago, a PSI was identified to facilitate the male-specific AS of *dsx*, but it is not sex specific [25,26]. Recently, the *Fem* gene on the W chromosome was revealed to be a piRNA [11,27], which inhibits a downstream gene *Masculinizer* (*Masc*) on the Z chromosome in the female silkworm, whereas in the males, *Masc* expression results in the male-specific AS of *dsx* [28–30].

The *Bm-dsx* gene consists of 6 exons, of which exons 3 and 4 are sex specific through alternative splicing, retained in females but skipped in males [23,31]. This is different from *Drosophila*, in which only exon 4 is sex specific [32]. It remains unclear whether *Masc* directly regulates AS of *Bm-dsx*; if not, the additional factors are unknown. Three regulatory *cis*-acting RNA elements have been found in *Bm-dsx* exon 4 [25], and our previous studies found that the exon 3 in the silkworm *dsx* also contains conserved elements required for its splicing regulation [33]. Based on the presence of these

cis-acting RNA elements, we performed yeast three-hybrid screens using sex-specific silkworm cDNA libraries to search for proteins that bind to exon 3 or exon 4 of *dsx*, investigated their regulatory functions in the alternative splicing of *dsx*, and demonstrated that expression of one of the RNA-binding proteins is controlled by *Masc*. Thus, here we reveal a novel AS regulatory mechanism in the sex-determination pathway of Lepidoptera.

Results

Screen for RNA-binding proteins that bind to sex-specific exons of *Bm-dsx*

We previously found that the two sex-specific exons (3 and 4, Fig. 1A) in Lepidoptera *dsx* mRNAs are more conserved than the four common exons and have three *cis*-RNA elements, mutations in which affect AS of *Bm-dsx* [33]. Thus, we used exons 3 and 4 in a yeast three-hybrid system [34] to screen for binding proteins that would regulate AS of *Bm-dsx*. We constructed two RNA bait plasmids, in which an RNA-binding sequence of MS2 was linked with either *Bm-dsx* exon 3 or 4; and we prepared three sex-specific libraries of cDNA linked to an activation domain (Fig. 1B), including two libraries from male and female embryos of a sex-limited *B. mori* strain R01

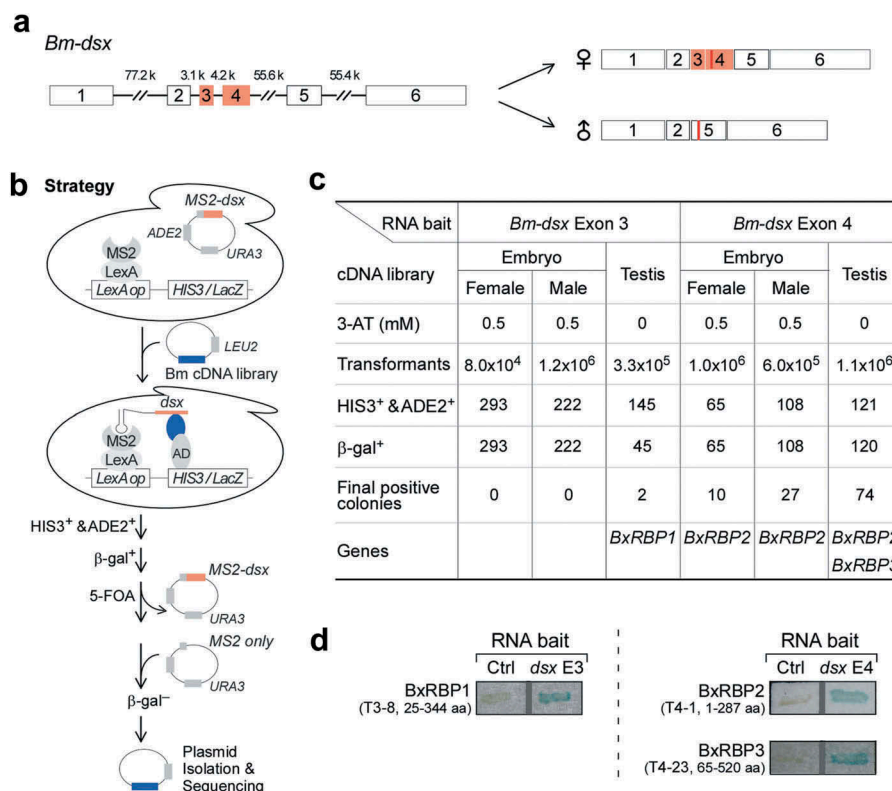


Figure 1. Identification of RNA-binding proteins of *Bm-dsx* sex-specific exons by yeast three-hybrid screens.

(A) Schematic diagram of alternative splicing of *Bm-dsx* in the female and male silkworm. Introns are in lines and exons are in boxes (orange: female specific, white: common). Lengths of each intron are indicated. (B) Strategy to identify RNA-binding proteins of *Bm-dsx* by yeast three-hybrid screening. Sex-specific exons 3 and 4 of *Bm-dsx* were fused with the MS2 RNA stem-loop to form an RNA bridge between factors that bind to the *LexA* operator and the *HIS3/LacZ* site. MS2 coat protein (MCP)-LexA fusion protein was used to recruit RNA to the *LexA* operator, and the cDNA library would express *Bm-dsx* RNA-binding proteins that fused to a transcription activation domain (AD) to induce expression of β -galactosidase (*LacZ*) and *HIS3*. (C) Summary of the six yeast three-hybrid screens. Final positive clones were sequenced and are listed in Table S2. (D) *LacZ* assays of representative clones that interact with exons 3 and 4 of *Bm-dsx*. Plasmids from the positive colonies were isolated and re-tested in a clean yeast three-hybrid strain. Clone numbers and CDS coverage are indicated. Ctrl: MS2-binding sites only.

[35] and one from testis of the WT silkworm p50 strain. After selection for HIS3+/ β -gal+ colonies, there were 113 positive clones eventually obtained in the 6 screens (2 plasmids x 3 libraries). All clones contained full or partial coding sequences (CDS) from three silkworm genes. We designated these as *BxRBP1*, *BxRBP2*, and *BxRBP3* (*Bombyx mori dsx* RNA-binding proteins). *BxRBP2* was identified using exon 4 as RNA bait and was frequently obtained from all three screened cDNA libraries, whereas *BxRBP1* and *BxRBP3* were obtained only from the testis cDNA library and were identified from exon 3 and exon 4 RNA baits, respectively (Fig. 1C and Table S2). These RNA–protein interactions were further confirmed by additional yeast three-hybrids tests using the isolated plasmids (Fig. 1D).

In *B. mori*, *BxRBP1* is located on chromosome 3 and is a homologue of *Drosophila lark* and mammalian *RBM4* (Fig. S1). *BxRBP2* is located on chromosome 11 and is a homologue of *Drosophila TBPH* and mammalian *TDP-43* (Fig. S2). *BxRBP3* is located on chromosome 12 and is a homologue of *Drosophila aret/bruno* and mammalian *ETR-3/CELF-2* (Fig. S3). All three proteins contain multiple RNA recognition motifs (RRMs), consistent with a role in RNA binding.

Identification of mRNA isoforms and sex specificity of *BxRBP* genes

To obtain full-length transcripts and all isoforms of the three *BxRBP* genes, we performed 5'- and 3'-RACE using tissues from both male and female silkworms. We found three alternatively spliced isoforms of *BxRBP1*, of which two were previously reported [36]. The two long *BxRBP1* isoforms are slightly different in their 5'-UTR regions and encode the same protein with two RRM and one Zinc-Finger (ZnF) domain, while the short isoform would encode a small protein without any potential RNA-binding domain (Fig. 2A). As previously described [37], only one transcript was obtained by RACE for *BxRBP2*, which is an intron-less gene and encodes a protein with two RRM (Fig. 2B). We identified four isoforms of *BxRBP3*, designated as *BxRBP3-A*, *-B*, *-C* and *-D*; none of these have been annotated or reported. They share most downstream exons but vary in the upstream transcription start sites, which are scattered across a 406-kb locus according to the genome sequence in SilkDB [38]. Isoforms A, B, and C of *BxRBP3* have the same three RRM with 15~45 aa variations at their N-termini, while the D isoform, the shortest one, lacks the first RRM (Fig. 2C).

We then tested the RNA-binding abilities of these identified isoforms to *Bm-dsx* exons. Consistent with the partial sequences found in the three-hybrid screens, the full-length *BxRBP1* bound efficiently to exon 3, but not 4; while the full-length *BxRBP2* bound efficiently to exon 4 but not 3 (Fig. 2D). The full-length A, B, and C isoforms of *BxRBP3* bound to exon 4 but not exon 3, while the D isoform could not bind to exon 4, indicating that the first RRM of *BxRBP3* is required for binding. We further quantified binding activities of these four isoforms by β -galactosidase assay [34] and found that *BxRBP3-A* and *-B* had similar binding activities to the exon 4, whereas the *BxRBP3-C*'s binding was ~40% lower and *BxRBP3-D*'s was totally abolished (Fig. 2E).

We then investigated sex specificities of the *BxRBP1-3* transcripts in different developmental stages and tissues from female and male silkworms. Expression of *BxRBP1* isoforms and *BxRBP2* were at similar levels in female and male samples (Fig. 3A upper). However, three isoforms of *BxRBP3* (*-A*, *-B*, and *-C*) were expressed in sex-biased patterns in gonad-containing samples (Fig. 3A middle). Isoform A was significantly higher in males (testis, pupa, and adult) than in females, whereas the isoform C exhibited an opposite pattern, higher in females than males. Isoform B was slightly higher in male samples and isoform D showed no observable sex-bias.

To reveal the function and regulatory roles of the three RBPs on AS of *Bm-dsx*, we established two sex-specific cell lines from embryos of the above-described R01 silkworm, BmF cells from the female and BmM cells from the male (Fig. S4A). Sex specificities of these two cell lines were confirmed by detection of three random amplified polymorphic DNA (RAPD) markers (*Musashi*, *Sasuke*, *Bonsai*) on the female-specific W chromosome locus [11,21] and by AS patterns of the *Bm-dsx* (Fig. S4B). Expression patterns of *BxRBP*s in the two sex-specific cell lines were similar to gonadal tissues, except for a lower level of *BxRBP3-C* isoform (Fig. 3B).

BxRBP1 and *BxRBP3* together efficiently inhibit female AS of *Bm-dsx*

We then tested the effect of expression of the three RBPs on AS of *Bm-dsx* in the BmF and BmM cell lines. In comparison to over-expression (OE) of the upstream gene *Masc*, which significantly inhibited splicing of exons 3 and 4 in BmF cells (Fig. 4A lane 9), individual OE of the four *BxRBP3* isoforms only slightly inhibited splicing of exons 3 and 4, generating the male-specific AS product of *Bm-dsx* in BmF cells (Fig. 4A, cf. lanes 5–8 to 9). OE of *BxRBP1* or *BxRBP2* had no detectable effects in BmF cells; as well, none of the OE of RBPs had detectable effects on the AS of *Bm-dsx* in BmM cells (Fig. 4A lanes 10–18).

Since the inhibition of female-specific splicing of *Bm-dsx* by single-RBP-OE was subtle, we then tested all possible combinations of double-RBP-OEs (Figs. 4B and S5A). We found that *BxRBP1* strongly enhanced the splicing inhibition by *BxRBP3* isoforms and resulted in more male-specific products in BmF cells (Fig. 4B lanes 3–6). The most significant combination was *BxRBP1* with *BxRBP3-B* (lane 4), which shifted ~65% of the female-specific *Bm-dsx* isoforms to the male-specific isoform, similar to the effect of OE of *Masc*. This *BxRBP1* enhancement was also observed in BmN cells (Fig. S5B), a frequently used female silkworm cell line [39]. In contrast, OE of *BxRBP2* did not show any enhancement of exon-skipping of *Bm-dsx* in combination with *BxRBP3* isoforms, nor did any combination of two *BxRBP3* isoforms (Fig. S5A).

BxRBP2 lacks a nuclear localization signal

To further understand the function of the three RBPs in regulation of *Bm-dsx* alternative splicing, we analysed their phylogenetic conservation. Their homologues in other species have been reported as splicing regulators. For example, *RBM4* regulates alternative splicing of *alpha-tropomyosin*, *PTB*, *pyruvate kinase M* and *Disabled-1* genes in human [40–43];

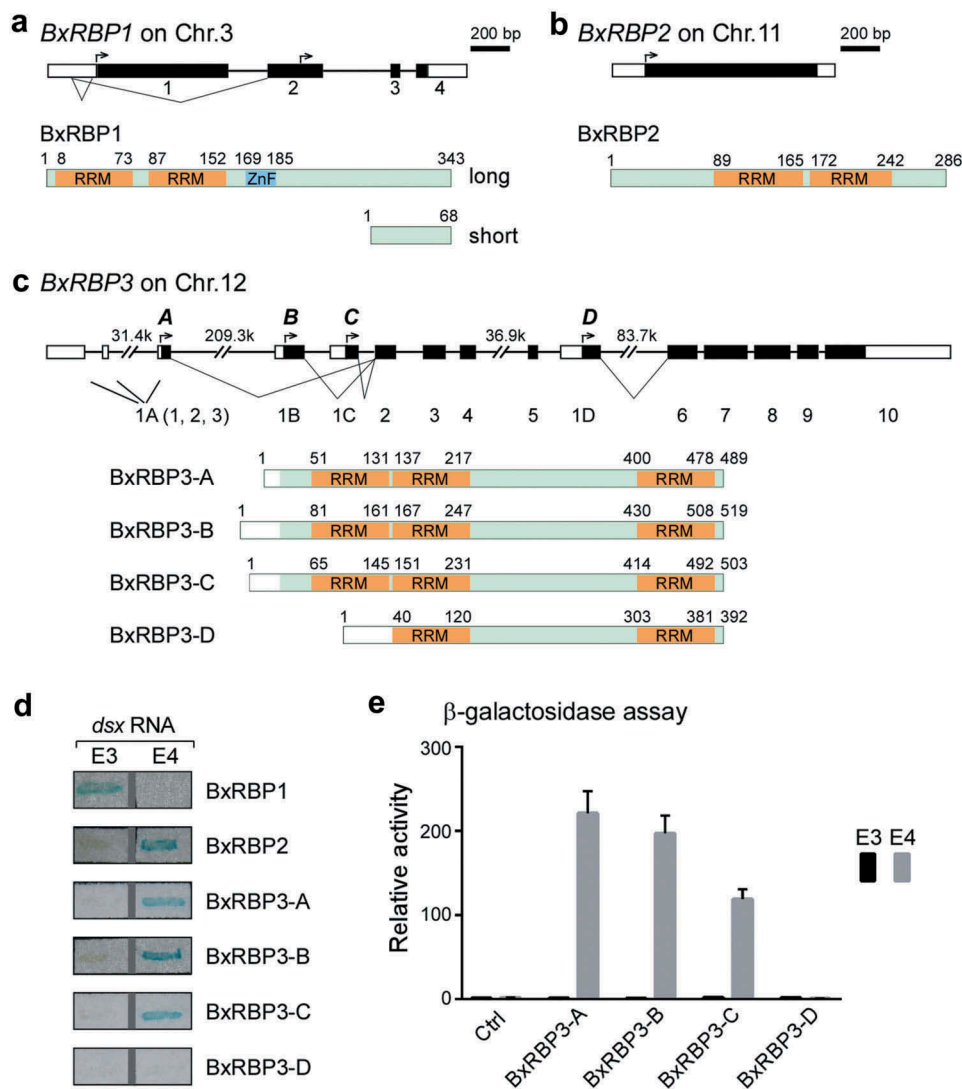


Figure 2. Gene structures and transcripts of the three identified RNA-binding proteins in *Bombyx mori*.

Exons and introns of the *BxRBP1* (A), *BxRBP2* (B) and *BxRBP3* (C) transcripts are defined according to results from 5'- and 3'-RACE. Functional protein domains of each transcript are generated according to NCBI information based on their coding sequences (black boxes), in which RRM s are highlighted in orange. Translation start sites (black arrows) and alternative splicing isoforms (V-shape lines) are also indicated. (D) Interaction between full-length of the three BxRBPs with *Bm-dsx* sex-specific exons by *Lac-Z* assay. All the four isoforms of BxRBP3 are tested. (E) Quantitation of interactions by β -galactosidase assay. The luminescent signal output from *LacZ* gene was normalized to cell number. Mean values \pm s.d. of three technical replicates from one of three independent experiments are shown.

skipping of exon 9 of the *CFTR* gene was regulated by both TDP-43 and ETR-3/CELF-2 [44–48]; and Aret regulates alternative splicing of various genes including *Stretchin* and *wupa* in *Drosophila* flight muscles [49,50].

In general, these three RBPs are highly conserved from insects to mammals; however, one notable feature is that a nuclear localization signal (NLS), present in the N-terminus of fly and mammalian homologues, is absent in BxRBP2 (Fig. S2B). Therefore, we tested the cellular location of all three RBPs in silkworm cell lines using EGFP-fusion proteins. As indicated by EGFP fluorescence, BxRBP1, BxRBP3-B, and BxRBP3-D are mostly located in the nucleus of BmF cells, whereas BxRBP3-A and -C preferentially locate in the cytoplasm. In contrast, BxRBP2 is exclusively located in the cytoplasm (Fig. 4C). These results are consistent with the above findings that BxRBP1 and BxRBP3 isoforms facilitate the inhibition of splicing of

Bm-dsx exons 3 and 4 in BmF cells, whereas BxRBP2 has no function on splicing of *Bm-dsx*.

We also knocked down the three BxRBPs in the silkworm sex-specific cells using various sets of siRNAs. In comparison to the upstream gene *Masc* knockdown, knockdown of these BxRBPs did not change AS of the *Bm-dsx* in BmM cells, in contrast to our expectation (Fig. 5A). Two possibilities could explain these results. First, the remaining of BxRBPs after RNAi knockdown (about 20–30%) could still be sufficient to inhibit splicing of exons 3 and 4 of *Bm-dsx*. Second, the role of BxRBPs in the AS regulation of *Bm-dsx* could be taken over by other RBPs, which we don't know.

***BxRBP1* directly interacts with *BxRBP3* isoforms**

Above, we demonstrated that BxRBP1 binds to the *Bm-dsx* exon 3 and BxRBP3 isoforms bind to exon 4, and they coordinately

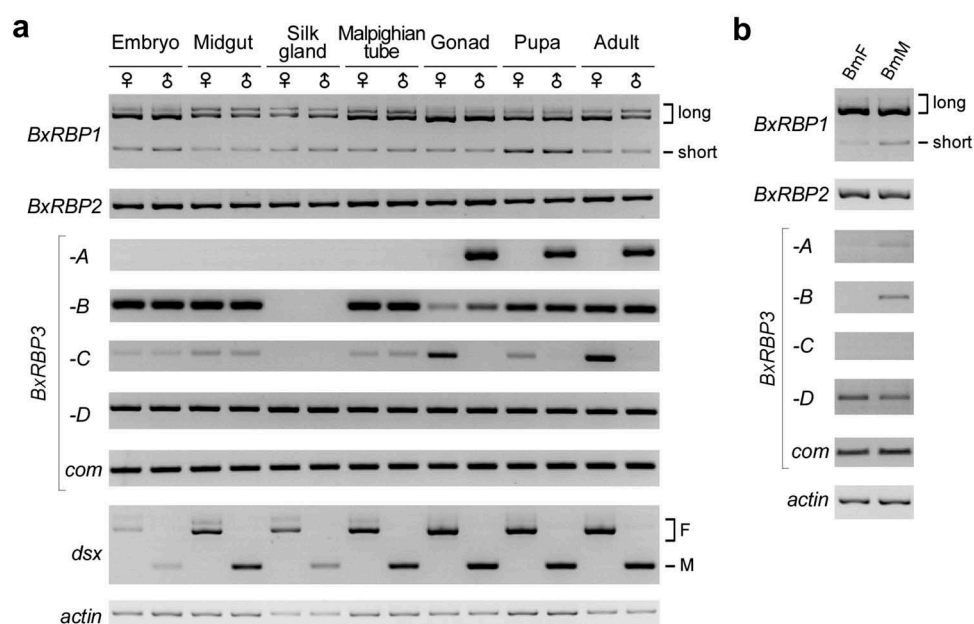


Figure 3. Expression profiles of all the *BxRBP* transcripts in stages and tissues of the female and male *Bombyx mori*.

(A) RT-PCR of *BxRBP* transcripts in the silkworm stages and tissues. Tissues were dissected from day 3 of the 5th larva stage. Transcripts of *Bm-dsx* are used as sex-specific marker, and *actin* is used as loading control. (B) Expression profiles of the *BxRBP*s in the new generated sex-specific cell lines.

facilitate the skipping of exons 3 and 4 in *B. mori* female-specific cells, resulting in the male-specific *Bm-dsx* isoform. Therefore, we asked whether *BxRBP1* and *BxRBP3* isoforms have direct interaction by two kinds of assays, the yeast two-hybrid and *in vitro* protein–protein interaction. In the yeast two-hybrid assay, we found that *BxRBP1* (bait) with *BxRBP3-B* (prey) effectively induced expression of reporter genes (*His3* and *Ade2*), whereas the bait with binding domain (BD) alone could not, showing the interaction between *BxRBP1* and *BxRBP3-B* (Fig. 5B). Interactions of *BxRBP1* with the other three isoforms of *BxRBP3* are weak, especially the -A and -D isoforms. Similarly, using purified recombinant proteins, all four GST-tagged *BxRBP3* isoforms could pull down His-tagged *BxRBP1*, in which *BxRBP3-B* was the most efficient, whereas GST alone could not (Fig. 5C). Together, these results demonstrate that *BxRBP1* directly interacts with *BxRBP3* isoforms, and imply a cooperative mechanism for skipping of exons 3 and 4 in the male-specific splicing of *Bm-dsx* that is ensured by protein–protein interactions between *BxRBP1* and *BxRBP3-B*.

It was previously identified two proteins, PSI and IMP, that bind with exon 4 of *Bm-dsx* [25,26]. Therefore, we asked whether these two proteins directly interact with our identified *BxRBP*s. Using yeast two-hybrid and *in vitro* pulldown assays, we found that PSI interacts with *BxRBP3*, but not *BxRBP1* or *BxRBP2*. No interaction was found between IMP and the three *BxRBP*s (Fig. S6).

Masc regulates transcription of *BxRBP3* isoforms

When either *Masc* or *BxRBP1*+ *BxRBP3-B* were over-expressed in the female-specific BmF cells, we observed the same AS changes of *Bm-dsx*: efficient skipping of exons 3 and 4 generating the male *dsx* isoform in female-specific silkworm

cells. *Masc* is a zinc-finger motif-containing protein and regulates transcription of a number of genes [11]. To test whether *Masc* regulates transcription of the three identified *BxRBP*s, we measured expression levels of the RBPs in both BmF and BmM cells, with or without over-expression of *Masc*. We found that *Masc*-OE resulted in significantly increased *BxRBP3-A* (up to more than 25 folds) and a mild increase of *BxRBP3-B*, but no significant change in *BxRBP3-C* and -D isoforms (Figs. 6A & 6B). Expression of total *BxRBP3* was also significantly increased. However, *Masc*-OE had no detectable effect on the other two *BxRBP*s, *BxRBP1* and *BxRBP2* (Figs. 6A & 6B). These results suggested that *Masc* regulates alternative transcription of *BxRBP3*, activating transcription of the -A and -B isoforms of *BxRBP3*.

To further distinguish whether *Masc* and *BxRBP*s directly regulate AS of *Bm-dsx*, we constructed a plasmid-borne *Bm-dsx* mini-gene that contains all exon sequences but shorter introns sequences (Fig. 6C), and tested its AS in both human HEK293T cells and silkworm BmF cells with expression of *Masc* or *BxRBP*s. In silkworm BmF cells, the *Bm-dsx* mini-gene was spliced in a female-specific pattern, recapitulating the effects on the endogenous *Bm-dsx* gene previously observed (Fig. 6D lane 2, compare to Fig. 3A lane 1). Expression of *Masc* efficiently inhibited splicing of exons 3 and 4 and resulted in male-specific splicing, whereas expression of *BxRBP2* had no effect. However, unlike the effects on the endogenous *Bm-dsx* gene, expression of *BxRBP1* alone or *BxRBP3-B* alone was sufficient to inhibit splicing of exon 3 or 4 of *Bm-dsx* mini-gene, resulting in F(-3) isoform (female isoform without exon 3) or F(-4) isoform (female isoform without exon 4), respectively (Fig. 6D, lanes 3 and 5). In human HEK293T cells, the *Bm-dsx* mini-gene was spliced into two groups of female-isoform-like products: one was the silkworm female-specific product (F isoform), the other

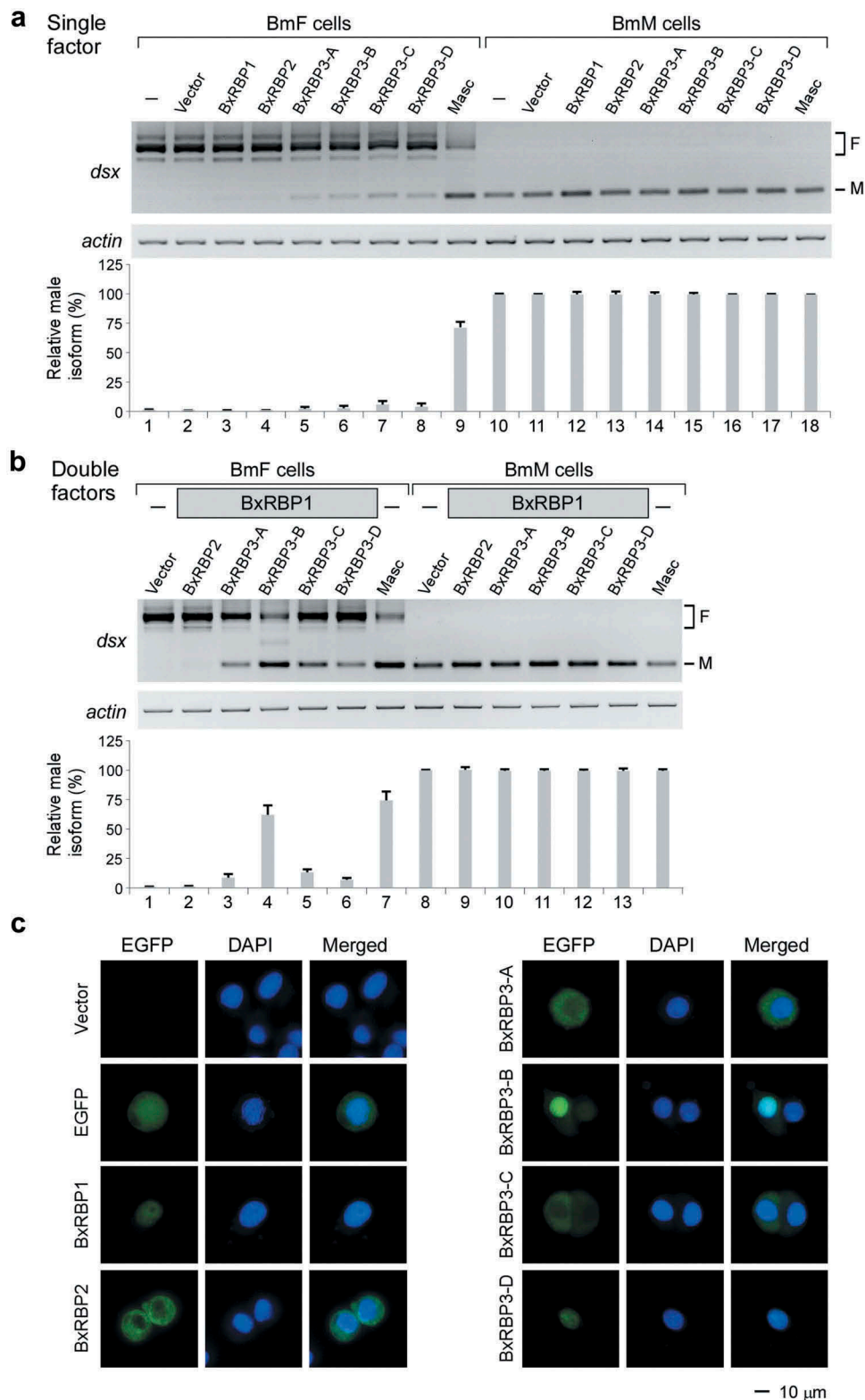


Figure 4. BxRBP1 and BxRBP3-B together efficiently inhibit splicing of the sex-specific exons of *Bm-dsx*.

(A) Individual over-expression of BxRBP3 isoforms in BmF cells slightly inhibits splicing of *Bm-dsx* exons 3 and 4. (B) Co-OE of BxRBP1 with BxRBP3 isoforms significantly inhibits splicing of *Bm-dsx* exons 3 and 4, resulted in male-specific isoform of *Bm-dsx* in the BmF cells. Empty vector and Masc-OE are tested as negative and positive controls, respectively. Semi-quantitative data shown are mean \pm s.d. of three independent experiments. (C) Fluorescence assay of BxRBP cellular localizations. BxRBPs are fused with EGFP and expressed in BmF cells. Nuclei are visualized by DAPI.

was products of F(-3) isoform and F(-3)' isoform which is F(-3) isoform with an extra 59-nt exon from intron 4 (Fig. 6E lane 2). In contrast to the silkworm BmF cells, expression of *Masc* in human HEK293T cells did not inhibit splicing of

exon 4 in the *Bm-dsx* mini-gene, neither BxRBP1 nor BxRBP2, implying that *Masc* is a direct factor to regulate AS of *Bm-dsx*. However, expression of BxRBP3-B alone was sufficient to inhibit splicing of exon 4 in the human cells

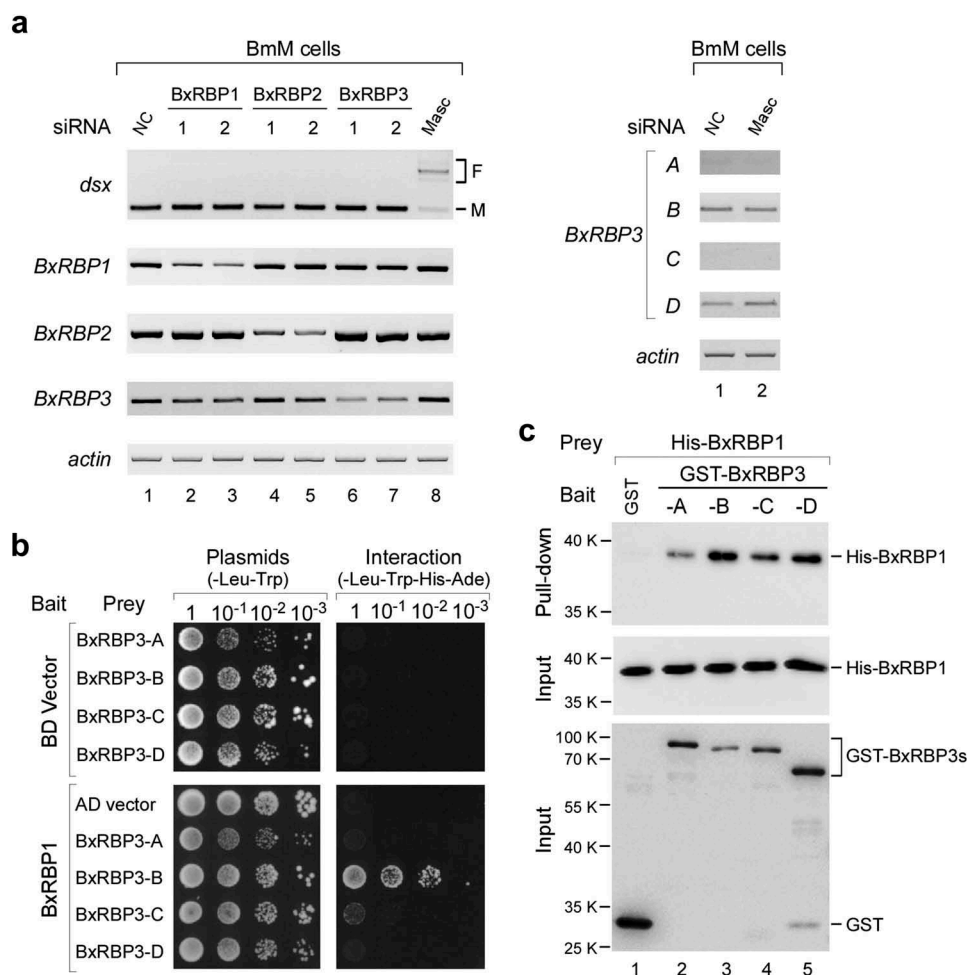


Figure 5. BxRBP1 strongly interacts with BxRBP3-B.

(A) Down-regulation of *BxRBPs* do not facilitate female splicing of *Bm-dsx* in the male cells, and down-regulation of *Masc* does not affect the mRNA level of *BxRBP3* isoforms in male cells. Both yeast two-hybrid (B) and in vitro protein interactions (C) show that BxRBP1 directly interacts with BxRBP3 isoforms, in which the BxRBP3-B is the strongest. In the yeast two-hybrid, BxRBP1 is used as bait and BxRBP3 isoforms are used as preys, interactions are visualized by reporter of HIS3 and ADE2. In the in vitro protein interaction assay, GST-BxRBP3s and His-BxRBP1 are incubated together and pulled down by glutathione beads. GST alone protein is used as negative control.

(Fig. 6E lane 5). Taken together, these results demonstrate that in the sex-determination pathway of silkworm, *Masc* is an upstream gene, which does not directly regulate AS of *Bm-dsx*, whereas *BxRBP3-B* is a direct regulator of AS.

Discussion

Regulation of alternative splicing is determined by various *cis*-acting RNA elements in pre-mRNA and many *trans*-acting protein factors [51]. To elucidate the sex-specific AS regulation of *doublesex* gene in the silkworm and in important agricultural pests of Lepidoptera, it is necessary to identify *Bm-dsx* RNA binding proteins that would function as splicing *trans*-acting factors. In this study, we identified three BxRBPs that bind to exon 3 or exon 4 RNA of the silkworm *doublesex* and found two of them are important for AS regulation. Based on our data, we propose a cooperative model for AS regulation of *Bm-dsx* in the silkworm sex-determination pathway (Fig. 7). BxRBP1 and BxRBP3 bind to sex-specific exons 3 and 4 of *Bm-dsx*, respectively, and cooperatively function as *trans*-acting repressors to inhibit the spliceosomal recognition of

these two exons, resulting in male-specific splicing of *Bm-dsx*. In addition, in the absence of the primary sex-determination signal piRNA that is located on the female W chromosome, the upstream *Masc* gene is expressed in the male silkworm and stimulates transcription of *BxRBP3-A* and *B* isoforms, resulting in inhibition of splicing of *Bm-dsx* exons 3 and 4 in males.

In the well-known sex-determination pathway of *Drosophila melanogaster*, AS of *doublesex* (*Dm-dsx*) is regulated by Tra and Tra-2, which bind to exon 4 of *Dm-dsx* in the female fruit fly and function as *trans*-acting splicing activators [52,53]. However, homologues of *tra* have not been found in silkworm and other Lepidoptera; and it has been shown that *tra-2* homolog in silkworm is not involved in the regulation of AS of *Bm-dsx* [54]. Therefore, AS regulation of *doublesex* genes in the fruit fly and the silkworm (Lepidoptera) is different [31]. The model supported by our data suggests a distinctly different mechanism of regulation of the sex-specific exon(s) of *doublesex* genes in the two insects, implying an evolutionary divergence between these two species.

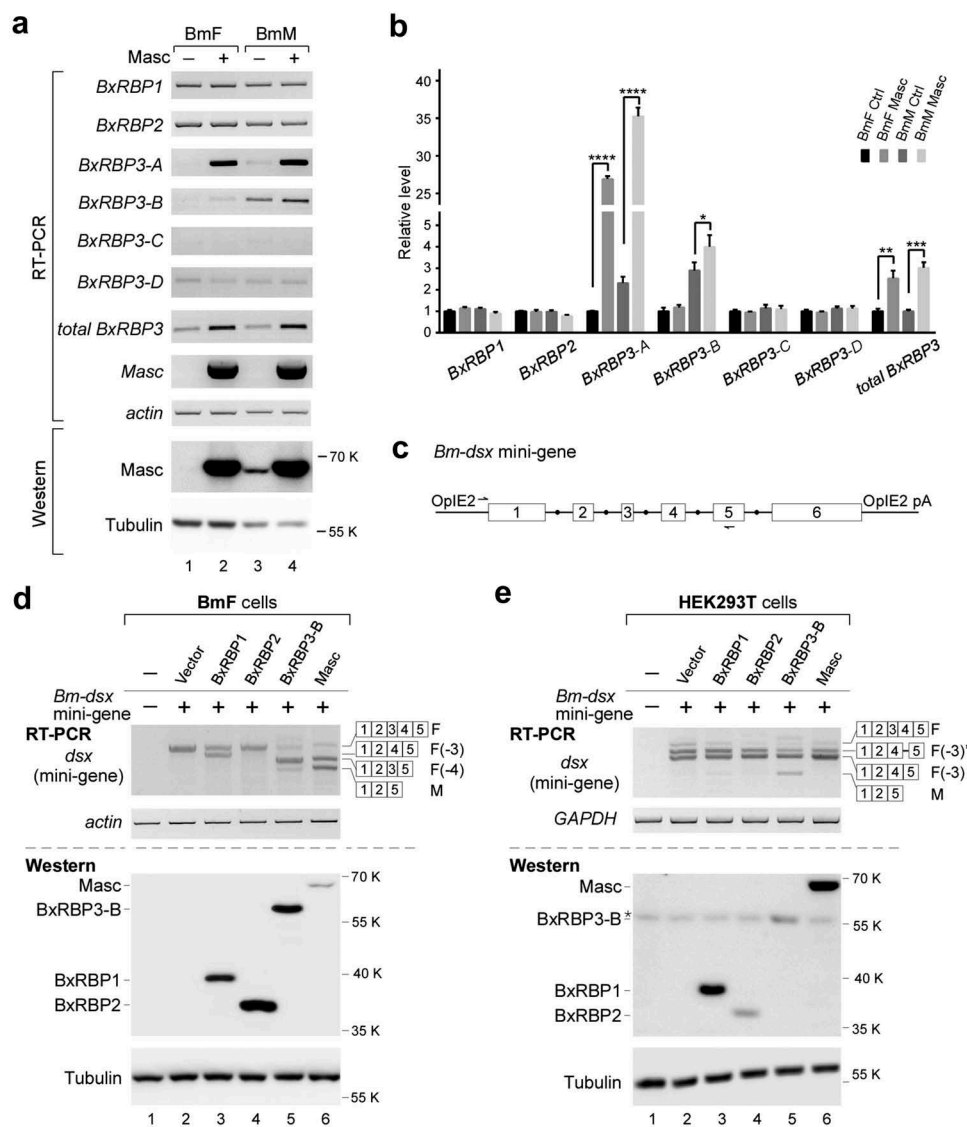


Figure 6. *Masc* regulates expression of *BxRBP3* isoforms but not *Bm-dsx*.

(A) OE of *Masc* in the silkworm cell lines stimulates expression of *BxRBP3-A* and *-B*. Empty vector is tested as a negative control. Expression levels of *Masc* were shown by RT-PCR and western blotting. *Actin* and *Tubulin* are used as loading controls. (B) Semi-quantitative detection of RT-PCR products in panel (A), which are mean \pm s. d. of three independent experiments. (C) Schematic of *Bm-dsx* mini-gene. The arrows indicate locations of primers used for amplification. OpIE2, *Orgyia pseudotsugata* immediate early 2 promoter sequence; OpIE2 pA, *Orgyia pseudotsugata* immediate early 2 polyadenylation sequence. (D) Alternative splicing of *Bm-dsx* mini-gene in BmF cells with OE of BxRBPs or *Masc*. (E) Alternative splicing of *Bm-dsx* mini-gene in human HEK293T cells with OE of BxRBPs or *Masc*. *Actin* and *GAPDH* are used as loading controls of total RNA in BmF and HEK293T, respectively, and *Tubulin* is used as loading control of total protein for western blotting. Isoforms of *Bm-dsx* mini-gene are schematically indicated. Asterisk: non-specific signal.

The three RNA-binding proteins of *Bm-dsx* identified here are highly conserved from insects to mammals. As described above, their homologous proteins have been characterized as splicing *trans*-acting activators or repressors in many species [40,41,44,45,49,50]. In comparison to their homologues, BxRBP2 is special in having lost its NLS and thus cannot be imported into the nucleus. Therefore, it has no effective function as a splicing regulator. We hypothesize that BxRBP2 might regulate translation or other events of the female *Bm-dsx* mRNA in the cytoplasm, but not the splicing of exon 4 in the *Bm-dsx* (Fig. 7). Homologues of BxRBP2 without an NLS can only be found in the Lepidoptera, not other insects or mammals (Fig. S7A upper). In the silkworm, we found two BxRBP2-like proteins, BxRBP2-L1 and BxRBP2-L2, which have NLS

sequences (Fig. S7A lower) and can be localized in the nucleus (Fig. S7B). We over-expressed them in the BmF cells, and found that they did not change AS of *Bm-dsx* in the BmF cells with or without OE of BxRBP1 (Fig. S7C), unlike the previously described effects of BxRBP3-B. Taken together, these data implied that lack of an NLS, BxRBP2 might have a different or additional regulatory function for *doublesex* gene in the Lepidoptera.

In the BmM cells, knockdown of BxRBPs and *Masc* did not change AS of the *Bm-dsx* and the expression of BxRBP3, respectively (Fig. 5A). These results imply that other factors may be involved in the AS regulation of *Bm-dsx*, although our insufficient efficiency of RNAi silencing could be another possibility. In addition, individual over-expression of BxRBP1 or BxRBP3 in the BmF cells is sufficient to skip

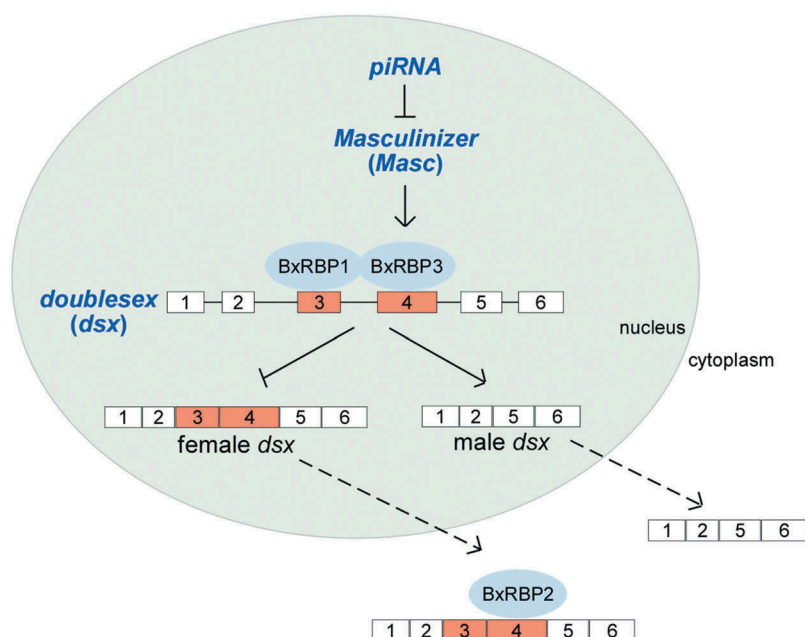


Figure 7. A proposed model for sex-determination regulation in *B. mori*.

In the absence of the primary sex-determination signal *piRNA* on W chromosome, *Masc* facilitates the expression of BxRBP3 isoforms, which can bind exon 4 of *Bm-dsx*. BxRBP3 isoforms function together with BxRBP1 that bind exon 3 of *Bm-dsx*, efficiently inhibit the female splicing of *Bm-dsx*, and induce male-specific splicing. BxRBP2, lacking an NLS, binds exon 4 of *Bm-dsx* in the cytoplasm.

exon 3 or 4 of the *Bm-dsx* mini-gene, which has intact exons but shorter introns (Figs. 6C & 6D), suggesting that the cooperative function between BxRBP1 and BxRBP3 is no-longer necessary. Taken together, there may be other factor(s), which could interact with intron sequence of *Bm-dsx* and play a key role in the regulation of *Bm-dsx* AS.

Materials and methods

Silkworm culture and sample collection

R01 and P50 strains were cultured by standard methods [35]. Embryonic samples were collected from R01 strain. Larval, pupal, and adult samples were collected from P50 strain. Silkworm individuals and tissues were separated and collected by gender, frozen in liquid nitrogen, and stored at -70°C .

Cell line culture

To establish sex-specific embryonic cell lines, primary cell cultures were initiated from silkworm embryos after 5 days of the termination of diapause using an R01 strain, in which the egg shells of females are dark blue and those of males are pink (Fig. S4A). Approximately 25 embryos without an eggshell of each gender were cultured at 27°C in a 25 cm^2 flask with TNM-FH medium (Sigma) plus 10% fetal bovine serum (GIBCO) and antibiotics penicillin-streptomycin (GIBCO). To obtain enough cells for subculture, half of the primary culture medium was changed every two weeks. Cells for subcultures were dissociated by treatment with 0.25% Trypsin-EDTA solution (GIBCO) and transferred to a new flask at a split ratio of 1:2. This process was continued ~ 3 years to establish two stable cell

lines, which were named BmF cells from the female embryos and BmM cells from the males. The doubling time of both cell lines is approximately 3 days. The BmN cell line was cultured by standard methods [33], and the HEK293T cell line was obtained from ATCC and cultured in DMEM (GIBCO) with 10% fetal bovine serum at 37°C with 5% CO_2 .

Yeast three-hybrid screens and yeast two-hybrid (Y2H) assay

The yeast three-hybrid system, including YBZ-1 strain and a pIII/MS2-2 plasmid, was provided by Prof. Marvin Wickens at University of Wisconsin–Madison. cDNA libraries from the silkworm testis and R01 strain embryos were prepared in pDEST32 (Invitrogen) and pGADT7 vector (Oebiotech), respectively. All six yeast three-hybrid screens were performed according to the previously described protocol [34] with the following modifications. The first-step selection was carried out on $-\text{His}$ media with 0.5 mM 3-aminotriazole (AT) for embryo cDNA libraries and without 3-AT for testis cDNA library. After the second-step selection by assays for β -galactosidase activity, plasmids with MS2-ligated *dsx* exon 3 or 4 was replaced by control MS2-only plasmid using 0.1% 5-FOA media to exclude false-positive clones. Quantitative β -galactosidase assays were performed as described [34]. For Y2H assay, coding sequences of RBPs were individually cloned into pGADT7 prey vector or pGBKT7 bait vector and then transformed into Y2HGold yeast strain. The Y2H assay was performed according to the manufacturer's instructions (Matchmaker[®] Gold Yeast Two-Hybrid System, Clontech).

Total RNA isolation and RT-PCR

Total RNA was extracted using Trizol (ThermoFisher) and reverse transcriptions were performed using RevertAid First Strand cDNA Synthesis kit (ThermoFisher). PCRs were performed using 2× Hieff™ PCR Master Mix (Yeasen) with programs containing 21–35 cycles (58°C for annealing). Primers used for RT-PCR are listed in Table S1.

5'- and 3'-RACE

The 5' and 3' terminal sequences of *BxRBPs* in silkworm samples were obtained using 5'-Full RACE Kit and 3'-Full RACE Core Set Ver.2.0 (TaKaRa), respectively. Primers used are listed in Table S1. Nested PCR programs for RACE were performed in 25 cycles with outer primers, followed by 32 cycles with inner primers using KOD-Plus-Neo DNA polymerase (TOYOBO) with additional 5% dimethyl sulfoxide. PCR products were then purified by DNA gel extraction kit (Axygen) and cloned into pMD18-T simple vector (TaKaRa) for sequencing. Obtained sequences were compared by BLAST against the SilkDB (Xia et al, 2009) to confirm their novelties. Encoded proteins and homologs were aligned and analyzed by DNAMAN (LynnonBiosoft). Novel sequences in this study have been deposited to GenBank under accession numbers from MH745573 to MH745580.

Over-expression and RNAi knockdown

For over-expression of proteins containing V5 and 6xHis C-terminal tags, full-length coding sequences of *BxRBP1*, *BxRBP2*, *BxRBP3*, and *Fem* piRNA-resistant *Masc* [11] were cloned into the pIZT/V5-his vector (Invitrogen). For RNAi knockdown of each target gene, two different short interfering RNAs (siRNAs) were used (sequences listed in Table S1). siRNAs were synthesized by GenePharma Corp and dissolved in RNase-free water (GIBCO) for storage at –80°C. Transfections were performed using TransIT-Insect Transfection Reagent (Mirus) according to the manufacturer's instructions. Cells were then collected at 72 hr after transfection for further analyses. Protein signals were then detected by western blotting using anti-*Masc* polyclonal antibody (Angobiotech).

Subcellular localization

For detecting the subcellular localization of the RBPs, EGFP was fused to the C-terminus of RBPs in a GFP-minus pIZT/V5-his vector. BmF cells were transferred onto coverslips after 2 days of transfection and fixed for 15 min with 4% paraformaldehyde in PBS, and permeabilized for 20 min in 0.1% Triton X-100 in PBS. Nuclei then were counterstained with DAPI (ThermoFisher). Images were captured at room temperature using a Nikon ECLIPSE Ni-U microscope with NIS-Elements Documentation software.

Recombinant protein purification

Coding sequences were individually cloned into pGEX-4T-1 (GST tag) or pET-28a vector (6xHis tag). Recombinant proteins were induced by 1.0 mM isopropyl β-D-1-thiogalactopyranoside

(IPTG) and expressed in *E. coli* BL21 for 24 hr at 16°C and then purified by either glutathione-Sepharose (GE Healthcare) or Ni agarose (Qiagen) chromatography under standard conditions followed by dialysis against buffer D (20 mM Tris-HCl at pH 7.9, 0.2 mM EDTA, 100 mM KCl, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20% glycerol).

In vitro protein–protein interaction

Purified 6xHis-tagged protein (10 pmol) and GST-tagged protein (40 pmol) were incubated together in 600 μL with binding buffer (20 mM Tris-HCl at pH 7.9, 150 mM NaCl, 1 mM EDTA, 0.2% Triton X-100, 0.5 mM PMSF) for 4 hr at 4°C with glutathione-Sepharose. Bead pellets were washed five times with wash buffer (50 mM Tris-HCl at pH 7.9, 140 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) and then resuspended in 50 μL of sample loading buffer for SDS-PAGE. For detecting the interaction between *BxRBP3-B* and *PSI*, 1×10^7 BmM cells were treated by lysis buffer (10 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM PMSF, 1% Triton X-100, 0.5% NP-40), then incubate with 10 μg GST-tagged *BxRBP3-B* protein for 4 hr at 4°C with glutathione-Sepharose. Protein signals were then detected by western blotting using anti-GST (GST-2) monoclonal antibody (Sigma), anti-*BxRBP1* and anti-*PSI* polyclonal antibody (Angobiotech).

Mini-gene assay

The *Bm-dsx* mini-gene, the same structure as previous described [33], was cloned into pIZT/V5-his vector. For a higher expression level in HEK293T cell line, *Masc* was cloned into pcDNA3 vector. Mini-gene and RBPs or *Masc* were co-transfected into HEK293T cells by using Attractene Transfection Reagent (Qiagen) according to the manufacturer's instructions. Cells were then collected at 48 hr after transfection. Protein signals were then detected by western blotting using anti-V5-HRP antibody or anti-tubulin (Sigma).

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Disclosure of potential conflicts of interest

No potential conflict of interest was reported by the authors.

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