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Conserved RNA *cis*-elements regulate alternative splicing of *Lepidopteran doublesex*

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ABSTRACT

Doublesex (*dsx*) is a downstream key regulator in insect sex determination pathway. In *Drosophila*, alternative splicing of *Dm-dsx* gene is sex-specifically regulated by *transformer* (*tra*), in which the functional TRA promotes female-specific *Dm-dsx*. However, the sex determination pathway in *Lepidoptera* is not well understood; here we focused on alternative splicing of *doublesex* (*dsx*) in two agricultural pests, Asian corn borer (*Ostrinia furnacalis*) and cotton bollworm (*Helicoverpa armigera*), as well as the silkworm (*Bombyx mori*). More than a dozen new alternative splicing isoforms of *dsx* were found in the *Lepidopteran* females, which exist in all tested developmental stages and differentiated tissues. Alignment of mRNA and protein sequences of *doublesex* revealed high conservation of this gene in *Lepidoptera*. Strength analysis of splice sites revealed a weak 5′ splice site at intron 3 in *Lepidopteran dsx*, which was experimentally confirmed. Furthermore, we identified highly conserved RNA sequences in the *Lepidopteran dsx*, including RNA elements I (14 nt), II (11 nt), III (26 nt), IV (17 nt), 3E-1 (8 nt) and 3E-2 (8 nt). The RNA elements III and IV were previously found in exon 4 of *B. mori dsx* and bound with Bm-PSI, which suppressed the inclusion of exons 3 & 4 into the male-specific *Bm-dsx*. Then we identified and analyzed the homologous genes of *Bm-psi* in the two *Lepidopteran* pests, which expressed at similar levels and exhibited a unique isoform in the males and females from each *Lepidoptera*. Importantly, mutagenesis of *Bm-dsx* mini-genes and their expression in BmN cell line demonstrated that three RNA elements are involved in the female-specific alternative splicing of *Bm-dsx*. Mutations in the RNA *cis*-elements 3E-1 and 3E-2 resulted in decreased inclusion of exon 3 into the female-specific *dsx* mRNA, suggesting that these two elements would be exonic splicing enhancers that facilitate the recognition of the weak 5′ splice site at intron 3 of *Lepidopteran dsx*. We propose that the 5′ splice sites at intron 3 are weak, resulting in multiple alternative splicing events in intron 3 of female *Lepidoptera dsx*. Activation of the 5′ splice site requires regulatory *cis*-elements in exons 3 for female-specific splicing of *Lepidoptera dsx*.

1. Introduction

The sex determination pathway in insects is composed of a cascade of alternative splicing (AS) events, which has been well characterized in *Drosophila melanogaster* (Black, 2003; Graveley, 2011) and the honeybee *Apis mellifera* (Beye et al., 2003; Hasselmann et al., 2008; Gempe et al., 2009), and partly in other insects, such as *Ceratitis capitata* (Pane et al., 2002; Saccone et al., 2011), *Musca domestica* (Dubendorfer and Hediger, 1998; Meise et al., 1998) *Nasonia vitripennis* (Oliveira et al., 2009; Verhulst et al., 2010) and *Tribolium castaneum* (Shukla and Palli, 2012). In *Drosophila*, the initial signal for sex determination is dependent upon the dosage of XSE (X chromosome-encoded signal element); double doses of XSE triggers AS of *sex-lethal* (*Sxl*) gene to produce a female-specific isoform and a functional SXL protein, whereas single dose of XSE allows an exon inclusion with a pre-termination codon (PTC) into the *Sxl* mRNA in the male (Erickson and Quintero, 2007; Salz and Erickson, 2010). Similarly, the female SXL then activates AS of *transformer* (*tra*), generating a female-specific and functional TRA protein, whereas only non-sex-specific mRNA containing a PTC of *tra* is produced in the absence of functional SXL in males (Inoue et al., 1990; Bopp et al., 1991). Together with constitutive factors TRA2 and RBP1, TRA then promotes AS of *doublesex*

Abbreviations: AS, alternative splicing; *Of*, *Ostrinia furnacalis*; *Ha*, *Helicoverpa armigera*; *Bm*, *Bombyx mori*; SS, splice site.

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(*dsx*) to produce a female-specific *Dm-dsx* isoform with exons 1–4 (Lynch and Maniatis, 1996). However, exon 4 is skipped in males due to lack of a functional TRA, and the male-specific *Dm-dsx* isoform contains exons 1–3 and 5–6 (Nagoshi et al., 1988; Hoshijima et al., 1991; Inoue et al., 1992). Both the female and male DSX are transcription factors and key regulators for sexual development in insects (Burtis and Baker, 1989; Coschigano and Wensink, 1993; An and Wensink, 1995). AS of *male specific lethal-2* (*msl2*) and *fruitless*, two other genes involved in fly sexual development, are also regulated by sex-specific *Sxl* and *tra*, respectively (Bashaw and Baker, 1995; Gebauer et al., 1998; Heinrichs et al., 1998; Salvemini et al., 2010). In fact, the autoregulation to maintain female sex determination and dosage compensation by the master regulator *Drosophila Sxl* are only unique to *Drosophilidae*. In contrast, a novel autoregulation discovered in *Ceratitis transformer* (Pane et al., 2002; Graham et al., 2003; Salvemini et al., 2009; Saccone et al., 2011) is more ancient and widely conserved in *Dipteran*, *Hymenopteran* and *Coleopteran* species (Pane et al., 2002; Gempe et al., 2009), but was lost in *Drosophilidae*. In *A. mellifera*, the sex-determining cascade is initiated by a complementary sex-determining (*csd*) gene (Beye et al., 2003) and followed by a *tra* homologous gene *feminizer* (*fem*), which regulates the sex-specific splicing of *Am-dsx* gene (Hasselmann et al., 2008; Gempe et al., 2009). AS cascade from *tra* to *dsx* were also found in *C. capitata*, *N. vitripennis* and *T. castaneum* (Oliveira et al., 2009; Beukeboom and van de Zande, 2010; Saccone et al., 2011; Shukla and Palli, 2012). Although the sex-specific splicing of *dsx* is widely conserved in insects, and the TRA/TRA-2 binding elements are highly conserved and involved in splicing of *dsx* in *Dipteran* (Shearman and Frommer, 1998; Pane et al., 2002), it is still unclear how CSD regulates sex-specific splicing of *fem* and how FEM regulates splicing of *dsx* in *Apis*.

Lepidoptera, comprised of thousands of agricultural pests and the economic insect silkworm, is the second largest order in insects. However, key regulators for sex determination pathway in *Lepidoptera* remain unclear except that homologous genes of downstream *dsx* were found to be sex-specifically spliced in silkworm *Bombyx mori* (Ohbayashi et al., 2001; Shukla et al., 2010), wild silkmoths *Antheraea assama* and *Antheraea mylitta* (Shukla and Nagaraju, 2010), and Adzuki bean borer *Ostrinia scapulalis* (Sugimoto et al., 2010). Identified male-specific *Lepidopteran dsx* isoform (*dsxM*) lacks exons 3 & 4, while the two female-specific isoforms (*dsxF1* and *dsxF2*) contain all six exons but are slightly different in 15-nt due to two alternative 5′ splice sites (SS) in intron 3, which generate two female-specific DSX proteins with 20 aa differences at the C-terminus (Shukla et al., 2010). It has been demonstrated that AS regulation of *Lepidopteran dsx* is different from that of *Dm-dsx* (Suzuki et al., 2001). Recently, two proteins, PSI and IMP, were found to be involved in the regulation of *dsx* AS in *B. mori*, which bound to splicing silencer element in exon 4 and suppressed the inclusion of exons 3 & 4 into the male-specific *Bm-dsx* (Suzuki et al., 2008, 2010). IMP is a male-specific protein, while no evidence showed that PSI is a sex-specific factor. Co-overexpression of IMP and PSI in female silkworm cell line slightly increased the male-specific splicing, suggesting that PSI and IMP play important roles in the AS of *Bm-dsx*, but the mechanism of female-specific splicing of *Bm-dsx* is still unclear. In addition, the homologous gene of *Dm-tra* has not been found in silkworm (Mita et al., 2004; Xia et al., 2004); and *Bm-Sxl* and *Bm-tra2* genes were identified, but not either sex-specifically expressed or involved in the AS splicing of *Bm-dsx* (Niu et al., 2005; Niimi et al., 2006; Suzuki et al., 2013), implying that upstream genes for sex determination in *Lepidoptera* are different from genes in fruit fly.

To investigate the AS mechanism of *Lepidopteran dsx* gene, we first focused on two *Lepidopteran* pests, Asian corn borer (*Ostrinia furnacalis, Of*) and cotton bollworm (*Helicoverpa armigera, Ha*), and identified *Of-dsx* and *Ha-dsx* genes by degenerate primers and RACEs. Extensive *dsx* mRNA analysis showed that multiple *dsx* AS isoforms exist in females of the two pests as well as in the female silkworm. Alignment of *dsx* mRNA sequences revealed several invariable RNA sequences from six *Lepidoptera*. Further mutagenesis in BmN cells demonstrated that three of the RNA sequences are critical *cis*-elements and enhancers for the regulation of AS of *dsx* in the female *Lepidoptera*.

2. Materials and methods

2.1. Insect sample

Silkworm strain P50 (*B. mori*), Asian corn borer (*O. furnacalis*) and cotton bollworm (*H. armigera*) were raised by standard methods (Zhou et al., 2010; Wang et al., 2011; Wan et al., 2012). Insect individuals and tissues were separated and collected by gender, and instantly frozen in liquid nitrogen and stored at −80 °C.

2.2. mRNA isolation and RT-PCR

Total RNAs were extracted using Trizol (Invitrogen) and mRNAs were purified by mRNA Isolation Systems III (Promega). Reverse transcription (RT) was performed by ReverTra Ace-α-(FSK-100CH) (TOYOBO). Sequences of *Of-dsx* and *Ha-dsx* were amplified by Ex Taq DNA polymerase under GC I buffer (TaKaRa) with program containing 14 cycles of temperature decreased gradient (from 63 °C to 56 °C) and 24 regular cycles (56 °C). *Lepidopteran psi* sequences were amplified by KOD-plus-neo DNA polymerase (TOYOBO) with 5% DMSO under regular PCR program. Primers used for RT-PCR are listed in Table S1.

2.3. 5′ and 3′ RACE

The 5′ and 3′ terminal sequences of *dsx* and *psi* in *O. furnacalis* and *H. armigera* were obtained by 5′-Full RACE Kit and 3′-Full RACE Core Set Ver.2.0 (TaKaRa) based on information from above amplified mRNA fragments, respectively. Used primers are listed in Table S1. Nested PCR programs for RACEs were performed by outer primers (26 cycles) followed by inner primers (32 cycles). PCR products were then purified by DNA gel extraction kit (Axygen) and cloned into pMD18-T vector for sequencing by Biosun Company.

2.4. Inverse PCR and determination of exon/intron boundaries

Genomic DNA was extracted from pupa of *O. furnac*alis and *H. armigera*, and then digested by restriction enzymes Dra I, EcoR I, EcoR V and Hind III for *O. furnac*alis, and Dra I, Eco47 III, and Nco I for *H. armigera*. After purification, digested genomic DNAs were self-ligated by T4 DNA ligase (Sugimoto et al., 2010), and then amplified by primers in exons of *Of-dsx* or *Ha-dsx* to obtain exon flanking sequences using Kod-plus-neo DNA polymerase (TOYOBO). The exon-intron boundaries of *Of-dsx* and *Ha-dsx* were determined based on sequencing results from inverse-PCR and the identities of splice sites in introns.

2.5. Analysis of splice site strength and construction of phylogenetic tree

The strength of 5′ and 3′ splice sites from *Bm-dsx, Of-dsx*, *Ha-dsx* and *Dm-dsx* were scored by online software “Analyzer Splice Tool” (http://ibis.tau.ac.il/ssat/SpliceSiteFrame.htm) (Koren et al., 2007). Sequences of splice sites were entered according to the request, 9 nt (position −3 to +6) for 5′ splice site, 15 nt (position −14 to +1) for 3′ splice sites. The phylogenetic tree for six *Lepidoptera* was

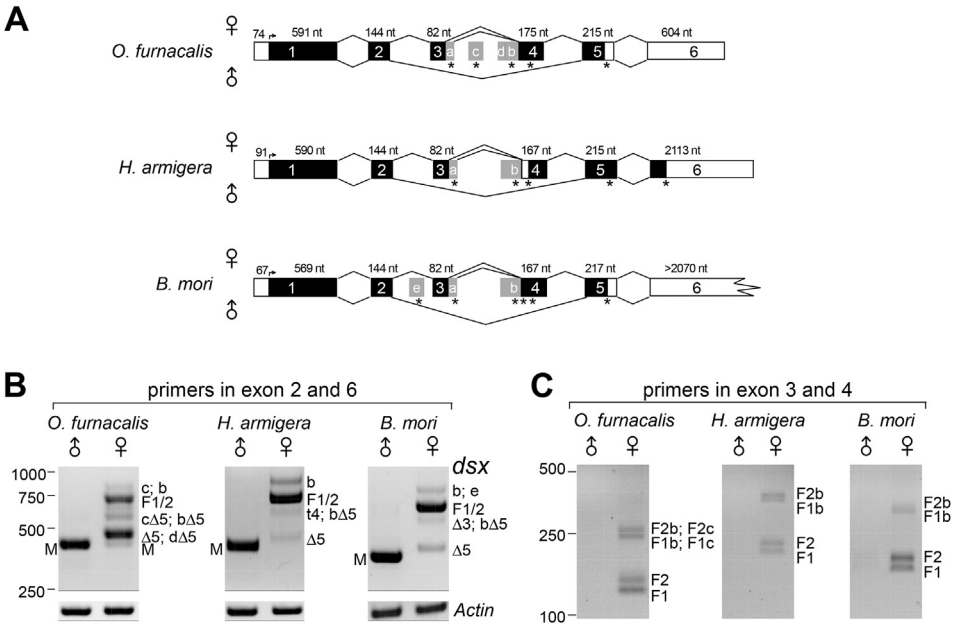

Fig. 1. Alternative splicing of *doublesex* genes in *O. furnacalis*, *H. armigera* and *B. mori*. (A) Schematic of exons (boxes) and introns (lines) in the three *Lepidopteran dsx*. Full-length mRNA of *Of-dsx* and *Ha-dsx* were obtained from RACEs, and the exon–exon boundaries were determined by inverse PCR; numbering of all exons is as in previously reported canonical *Bm-dsx*. Splicing of male-specific and major female (F1 and F2) isoforms are indicated; other female-specific isoforms are listed in Table 1. Black boxes: canonical exons; blank boxes: 5′- and 3′-UTRs; gray boxes: new identified alternative exons in females; arrows: start codons; asterisks: stop codons. (B) Multiple isoforms of *dsx* were identified in the three female *Lepidoptera*, whereas only one isoform in the males. Isoforms of *dsx* were amplified from pupa using primers at exon 2 and 6 by RT-PCR; amplification of actin was used as loading control. Visible bands were gel purified and sequenced. M: male-specific isoform; a–e: new included exons; F1: canonical female-specific isoform; F2: canonical female-specific isoform plus exon 'a'; Δ3 and Δ5: skipping of exon 3 or 5; t4: truncated exon 4. Most visible single bands contain at least two isoforms, which differ in inclusion of the 15-nt exon 'a' and are not indicated here for clarity (for details see Table 1). (C) Usages of the two alternative 5′ splice sites in intron 3. Selection the two alternative 5′ splice sites resulted in the production of paired isoforms, such as F1/F2 and F1b/F2b, which are similarly present in all the three *Lepidoptera*. Shorter female-specific *dsx* fragments were amplified from the three *Lepidoptera* using primers in exon 3 and 4, and separated by 3% agarose gel and identified by sequencing. Each set of the two close bands has 15-nt (exon 'a') differences.

constructed based on their *dsx* mRNA sequences, carried by Clustal W and MEGA 5.1 software using Maximum Likelihood method (Toyota et al., 2013).

2.6. Construction of Bm-dsx mini-genes and in vivo assay in BmN cell line

Bm-dsx mini-gene was constructed by insertion of over-lapping PCR products into pBac-A3-EYFP vector, which contains exons 1–5 and part of exon 6, and shortened intron flanking sequences (200 bp after 5′SS and 300 bp before 3′SS) of *Bm-dsx*. Mini-gene mutants, including mutations in the conserved RNA elements and a mock region for specific amplification, were generated based on the WT construct. *Bm-dsx* mini-genes were transfected into BmN cells using Effectene Transfection Reagent (QIAGEN) when the cell confluence reached 80%. Cells were then cultured in TC 100 Insect cell Medium (PAA) with antibiotics penicillin-streptomycin (Gibco) at 27 °C. Total RNAs were isolated 48 h after transfection, followed by DNase I (TaKaRa) digestion and phenol extraction. mRNA products from the *Bm-dsx* mini-genes were detected by RT-PCR using primers from the pBac-A3-EYFP vector and the mock region in *Bm-dsx* to distinguish products from the endogenous *Bm-dsx* gene.

3. Results

3.1. Sex-specific isoforms of dsx in O. furnacalis and H. armigera

Since genome sequences of *O. furnacalis* and *H. armigera* have not been released, we used degenerate primers to amplify cDNA sequences of *Of-dsx* and *Ha-dsx*. The degenerate primers were designed based on the conserved sequences of OD1 region in DSX proteins from *B. mori*, *T. castaneum*, *D. melanogaster*, *A. mellifera* and *N. vitripennis* (Fig. S1 and Table S1). This selected region is transcribed from exon 1, a common exon in both the male and female *dsx* of all insects. Using degenerate primers, RT-PCR amplified 114-bp products from *O. furnacalis* and *H. armigera*, respectively. Then full-length mRNA sequences of *Of-dsx* and *Ha-dsx* were subsequently obtained by 5′ and 3′ RACEs based on sequences from the two 114-bp products (Fig. 1A and S2). Furthermore, exon/intron boundaries and flanking intron sequences in *Of-dsx* and *Ha-dsx* were amplified by inverse PCR (Fig. S2).

To identify possible AS isoforms of *dsx* in the two *Lepidopteran* pests, we next amplified mRNA products from the male and female pupae using primers located in the common exons 2 and 6 (Fig. 1B). For clarity, we referred to previously numbered *Bm-dsx* exons and introns as the canonical exons and introns (Ohbayashi et al., 2001), and named our new identified exons as 'a', 'b', 'c' etc. In the males, only one isoform of *dsx* was obtained from each pest, which contains exons 1, 2, 5, and 6, but lack exons 3 and 4 (Table 1 and Fig. 1), consistent with reported male *dsx* isoforms from other *Lepidoptera* (Ohbayashi et al., 2001; Shukla and Nagaraju, 2010; Sugimoto et al., 2010). The male-specific isoforms will encode DSX proteins with 283 aa and 277 aa in *O. furnacalis* and *H. armigera*, respectively (Table 1). Unexpectedly, in the females, we identified 14 *dsx* isoforms from *O. furnacalis* and 10 isoforms from *H. armigera* (Fig. 1 and Table 1). These multiple *dsx* isoforms in females can be classified into four groups based on their AS types: i) Alternative 5′SS of intron 3. There are two alternative 5′ splice sites for intron 3 in both *Of-* and *Ha-dsx* mRNA, which are 15-nt apart and generate two female-specific *dsx* isoforms, F1 and F2, named after the similar previously identified isoforms from silkworm (Ohbayashi et al.,

Table 1
Alternative splicing isoforms of *doublesex* in three *Lepidoptera*.

Species	Source	Transcript	Exons included	Encoding protein (aa)
O. furnacalis	Female	*Of-dsxF1*	1, 2, 3, **4**, 5, 6	271
		Of-dsxF2	1, 2, 3+**a**, 4, 5, 6	251
		Of-dsxF1Δ5	1, 2, 3, **4**, 6	271
		Of-dsxF2Δ5	1, 2, 3+**a**, 4, 6	251
		Of-dsxF1b	1, 2, 3, **b**+4, 5, 6	258
		Of-dsxF2b	1, 2, 3+**a**, b+4, 5, 6	251
		Of-dsxF1bΔ5	1, 2, 3, **b**+4, 6	258
		Of-dsxF2bΔ5	1, 2, 3+**a**, b+4, 6	251
		Of-dsxF1c	1, 2, 3, **c**, 4, 5, 6	263
		Of-dsxF2c	1, 2, 3+**a**, c, 4, 5, 6	251
		Of-dsxF1cΔ5	1, 2, 3, **c**, 4, 6	263
		Of-dsxF2cΔ5	1, 2, 3+**a**, c, 4, 6	251
		Of-dsxF2dΔ5	1, 2, 3+**a**, d, 4, 6	251
		Of-dsxM	1, 2, **5**, 6	283
	Male	*Of-dsxM*	1, 2, **5**, 6	283
H. armigera	Female	*Ha-dsxF1*	1, 2, 3, **4**, 5, 6	263
		Ha-dsxF2	1, 2, 3+**a**, 4, 5, 6	245
		Ha-dsxF1Δ5	1, 2, 3, **4**, 6	263
		Ha-dsxF2Δ5	1, 2, 3+**a**, 4, 6	245
		Ha-dsxF1b	1, 2, 3, **b**+4, 5, 6	255
		Ha-dsxF2b	1, 2, 3+**a**, b+4, 5, 6	245
		Ha-dsxF1bΔ5	1, 2, 3, **b**+4, 6	255
		Ha-dsxF2bΔ5	1, 2, 3+**a**, b+4, 6	245
		Ha-dsxF1t4	1, 2, 3, t4, 5, **6**	407
		Ha-dsxF2t4	1, 2, 3+**a**, t4, 5, 6	245
	Male	*Ha-dsxM*	1, 2, **5**, 6	277
B. mori	Female	*BmdsxF1*	1, 2, 3, **4**, 5, 6	264
		BmdsxF2	1, 2, 3+**a**, 4, 5, 6	246
		Bm-dsxF1Δ5	1, 2, 3, **4**, 6	264
		Bm-dsxF2Δ5	1, 2, 3+**a**, 4, 6	246
		Bm-dsxF1b	1, 2, 3, **b**+4, 5, 6	253
		Bm-dsxF2b	1, 2, 3+**a**, b+4, 5, 6	246
		Bm-dsxF1bΔ5	1, 2, 3, **b**+4, 6	253
		Bm-dsxF2bΔ5	1, 2, 3+**a**, b+4, 6	246
		Bm-dsxF1e	1, 2, **e**, 3, 4, 5, 6	225
		Bm-dsxF2e	1, 2, **e**, 3+a, 4, 5, 6	225
		Bm-dsxFΔ3	1, 2, **4**, 5, 6	223
	Male	*BmdsxM*	1, 2, **5**, 6	279[#]

3+**a:** elongated exon 3 with the 15-nt exon 'a' due to an alternative 5′ splice site; Bold exons: stop codon containing exons; *Δ5* or *Δ3*: skipped exon 5 or exon 3; t4: a truncated exon 4 due to an alternative 3′ splice site; Shadows: new identified *dsx* isoforms from *Bombyx mori*; #: with modified sequence.

2001). ii) Exon inclusions. Three included exons ('b', 'c', and 'd') in *O. furnacalis* and one ('b') in *H. armigera* were detected from intron 3. iii) Exon 5 skipping. The skipping isoforms (Δ5) were found in both pests. iv) Combination of alternative 5′SS events with other two AS events. For example, the isoforms F1b/F2b and F1Δ5/F2Δ5 exist in both female pests. In addition, a unique *dsx* isoform in the female *H. armigera* was identified, in which an alternative 3′SS is selected inside exon 4 and would encode a much larger DSX protein with 407 aa (Fig. 1 and Table 1).

3.2. The multiple dsx isoforms are conserved in the female silkworm

Existence of the multiple *dsx* isoforms in female *Lepidopteran* pests of *O. furnacalis* and *H. armigera* raised the question whether the female *B. mori* also has such *dsx* isoforms. To address this, further RT-PCR from the female pupae of *B. mori* revealed eleven *dsx* isoforms, which follow conserved patterns as in the two pests (Fig. 1A and Table 1), including the alternative 5′SS in intron 3, exon 'b' inclusion, exon 5 skipping, and their combinations (Fig. 1 and Table 1). Among these, nine *Bm-dsx* mRNA products are novel and would encode three new BmDSX protein isoforms that have different C-terminals (Table 1). Again, only one isoform could be identified in the male silkworm. Remarkably, a thymidine in the exon 5 of *Bm-dsx*, which was overlooked in previously reported *Bm-dsx* mRNA (Ohbayashi et al., 2001), is found in our identified sequences and consistent with the sequence in silkworm genome (Mita et al., 2004; Xia et al., 2004). This change resulted in the amino acid sequence at the C-terminal of male-specific BmDSX from $_{249}$LRDTCWRPRSRRVWCPSS$_{266}$ to $_{249}$SPRYVLAPEVPPRLVPFQLTTGRHSPRPAPL$_{279}$, the latter peptide has much higher identity with other *Lepidopteran* DSX proteins (see details below and Fig. 2).

Exon 'a' is a highly conserved 15-nt region between the two alternative 5′SS of intron 3 in all the three *Lepidoptera dsx*, which contains a PTC and allows isoform F2 to encode a smaller female DSX protein than the isoform F1, 20 aa shorter in *O. furnacalis* and 18 aa shorter in *H. armigera*, respectively (Table 1). Interestingly, selection of these two alternative 5′ splice sites is combined with other types of *dsx* splicing and resulted in the production of paired isoforms, such as F1/F2 and F1b/F2b, which are similarly present in all the three female *Lepidoptera* (Fig. 1C).

We aligned DSX proteins from six *Lepidopteran* species, including those previously reported from *B. mori*, *A. assama*, *A. mylitta* and *O. scapulalis*, and the new ones identified from *O. furnacalis* and *H. armigera*. The *Lepidopteran* DSX proteins are highly conserved in both the male and female-specific forms. Encoded by the common exons 1 and 2, N-terminal regions of DSX proteins are 92% identical across the six species, which contain OD1 domain and exist in both males and females (Fig. 2 upper). Alternatively spliced exons 3 to 6 of *Lepidopteran dsx* generate multiple C-terminal regions of DSX proteins: regions that are encoded by the canonical exons 3 + 4 (*dsxF1*) in females are 96% identical; while those that are encoded by female-specific exons 3+'a' (*dsxF2*) are 97% identical (Fig. 2 middle); and that encoded by male-specific exon 5 are 87% identical (Fig. 2 bottom). Conservation of the male-specific region in *Lepidopteran* DSX proteins shows higher identity than previously reported (Shukla and Nagaraju, 2010; Sugimoto et al., 2010) and this is due to a nucleotide sequence modification in *Bm-dsx* exon 5 (see above).

3.3. Multiple dsx isoforms are expressed during Lepidopteran development

To investigate the expression profiles of these multiple *dsx* isoforms, we thoroughly analyzed *dsx* isoforms in various developmental stages, tissues and organs of *O. furnacalis*, including larva, pupa and moth; head, fatbody, silkgland, midgut and gonads. In general, multiple *dsx* isoforms were expressed in all the tested female *O. furnacalis* samples with variable expression levels and mild changes in ratios between the isoforms (Fig. 3). In addition, the male *O. furnacalis* always has only one male-specific *dsx* isoform, regardless of the developmental stages or tissues. This result suggests that expression of the multiple *dsx* isoforms always exist during the developmental stages or in differentiated tissues in *Lepidoptera*.

3.4. Lepidopteran dsx has weak 5′ splice sites in intron 3

Occurrence of AS is dependent on *cis*-elements in pre-mRNA and upstream regulatory proteins. By interacting with protein regulators, RNA *cis*-elements in exons and introns could function as silencers to inhibit recognition of regular splice sites, or as enhancers to activate recognition of weak splice sites, and thus determine the final AS products (Keren et al., 2010). To address the possible mechanism for AS of *Lepidopteran dsx*, we analyzed the strength of all the 5′ and 3′ splice sites in *Of-dsx, Ha-dsx and Bm-dsx*, as well as in *Drosophila dsx* by Analyzer Splice Tool (Koren et al., 2007). Our genome-wide analysis of consensus sequences of splice sites in *B. mori*, *D. melanogaster* and human

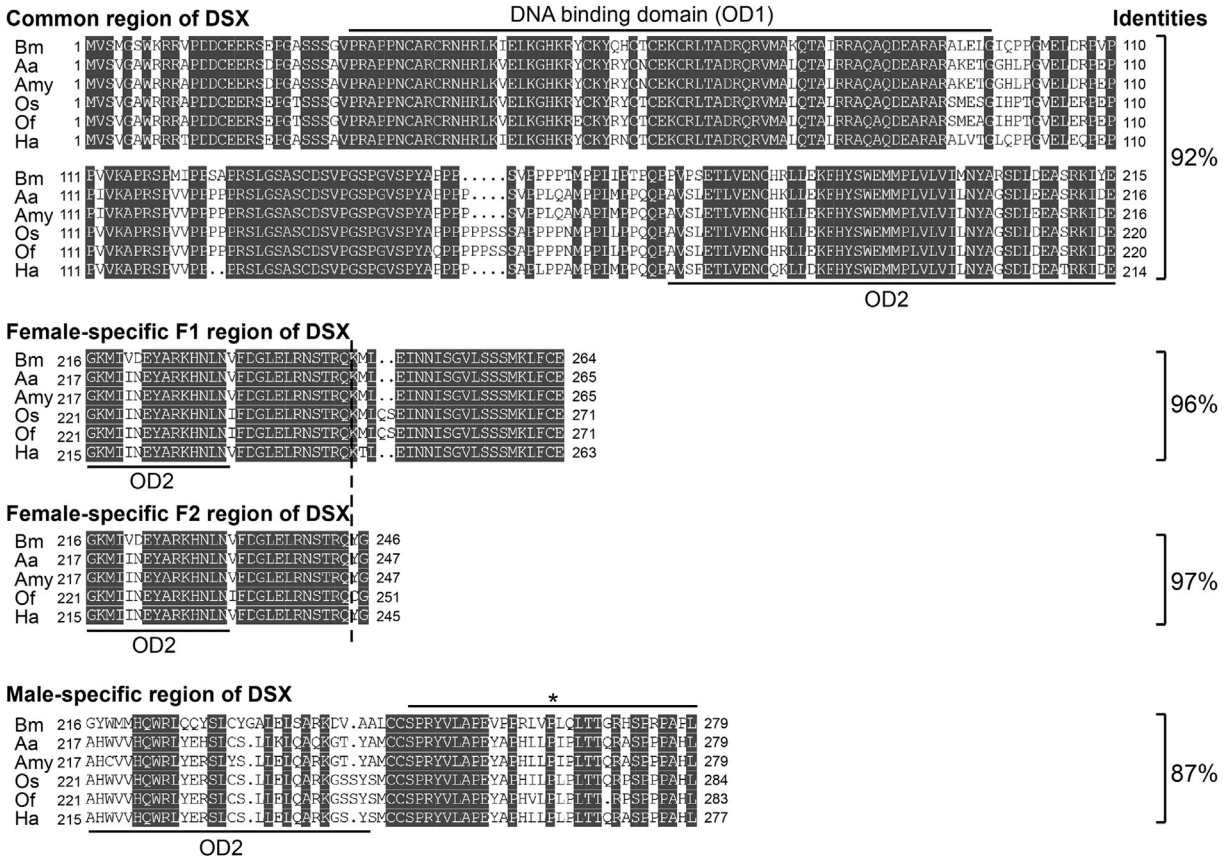

Fig. 2. Conservation of the *Lepidopteran* DSX proteins. DSX proteins were aligned from six *Lepidoptera*, including previously reported sequences from *Bombyx mori*, *A. assama*, *A. mylitta* and *O. scapulalis*, and our new identified sequences from *O. furnacalis* and *H. armigera*. Alignments are presented separately for the common, the female-specific (F1 and F2) and the male-specific regions. Conservation of the C-terminal region in the male-specific *Lepidopteran* DSX has increased due to an updated sequence (indicated by asterisk). DNA binding domain OD1 is boxed and OD2 is underlined. Region identities calculated by DNAman software are indicated.

indicated that the 5′ and 3′ SS consensus sequences are similar in these three species (Table S3); therefore, we believe this software is suitable for analyzing the splice site strength in *B. mori*. The strength of all detected splice sites in *Lepidopteran dsx* introns are at similar levels as their counterparts in *Dm-dsx* except the 5′ and 3′SS of intron 3 (Fig. 4A). The 3′SS of intron 3 in *Dm-dsx* (CUGAUCUAAACCAG/G) scored at a lower level, consistent with its previous characterization as a weak splice site (Baker and Wolfner, 1988; Burtis and Baker, 1989; Hoshijima et al., 1991), but its counterpart in *Lepidopteran dsx* (UUUCUUUUUUGUAG/G) scored at the relatively normal level (Fig. 4A). In contrast, the 5′SS of intron 3 in *Lepidopteran dsx* (CCA/GUGCGG for *Of-dsx*; CCA/GUACGG for *Ha-* and *Bm-dsx*) are obviously weak, showing scores much lower than the 5′SS of *Dm-dsx* intron 3 (AGG/GUAAGU) (Fig. 4B). Similarly, the strength of alternative 5′SS of intron 3a in *Lepidopteran dsx* are also in low levels (Fig. 4B). To demonstrate that the 5′SS of intron 3 in *Lepidopteran dsx* are weak in vivo, we replaced the 5′SS of other introns in *Bm-dsx* with the 5′SS from intron 3 using a *Bm-dsx* mini-gene system in BmN cell line (for details see below) and tested their splicing products. Indeed, the replacement of 5′SS at intron 1 totally abolished splicing (Fig. 4C lane 3), replacement at intron 2 resulted in selection of the other splice site in intron 2 and significantly decreased the normal splicing (Fig. 4C, lane 4), replacement at the intron 4 caused skipping of exon 4 (Fig. 4C, lane 5). These results demonstrated that the 5′SS of intron 3 in *Lepidopteran dsx* is weak in vivo and would be activated for the inclusion of exon 3 in the females, whereas the recognition of 3′SS of intron 3 would be inhibited for

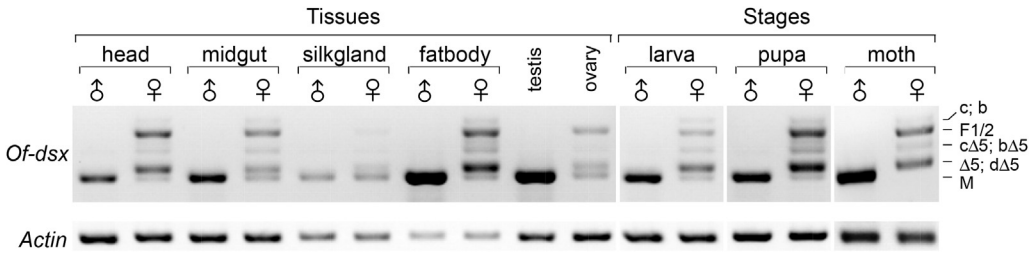

Fig. 3. Multiple mRNA isoforms of *dsx* are expressed during the developmental stages and differentiated tissues in the female *O. furnacalis*. *dsx* isoforms in the male and female *O. furnacalis* were identified by RT-PCR using primers in exons 2 and 6. All the identified isoforms of female *Of-dsx* are detectable in various developmental stages and tissues. Tissue samples were dissected from larva at the 5th instar.

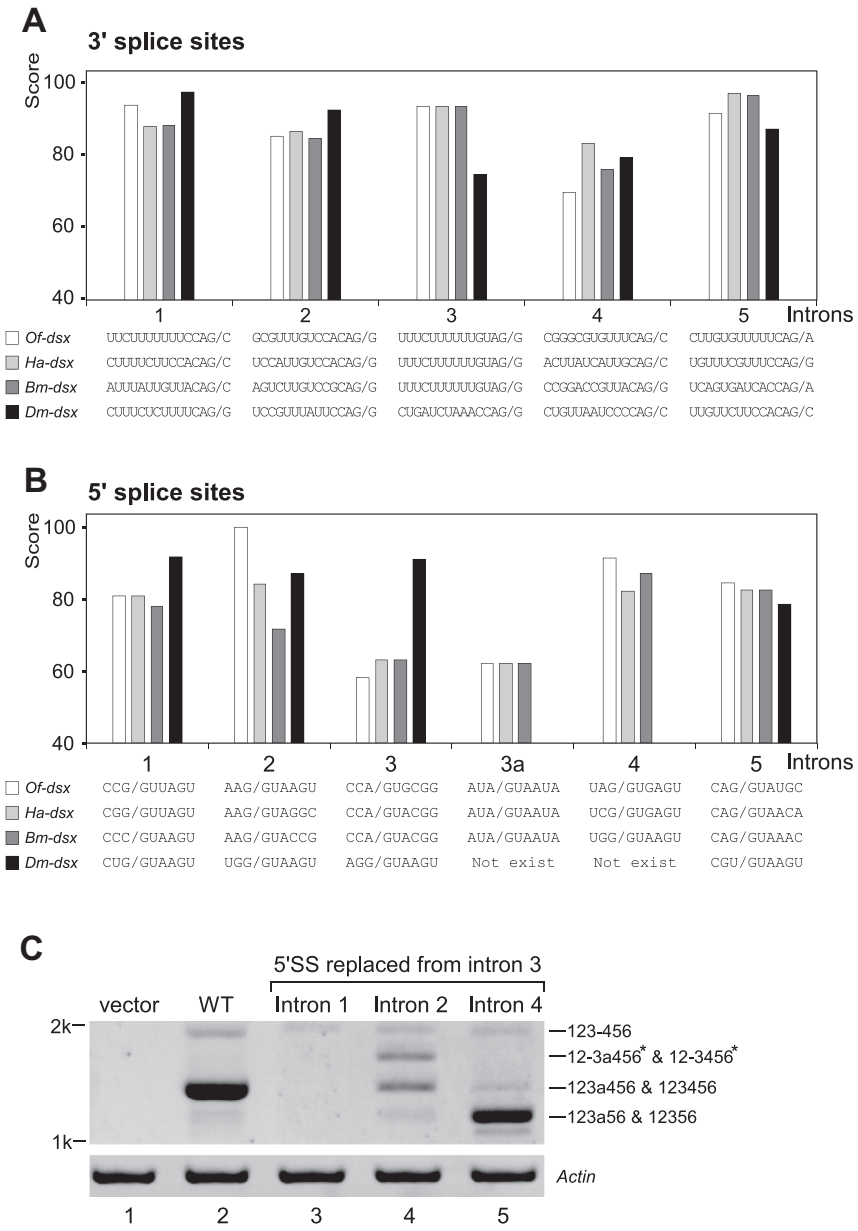

Fig. 4. The 5′ splice sites of intron 3 in *Lepidopteran dsx* are relatively weak. Strength of 3′ splice sites (A) and 5′ splice sites (B) in intron 3 of *Lepidopteran dsx* is opposite to the *Drosophila dsx*. All splice sites of *dsx* introns from *O. furnacalis*, *H. armigera*, *B. mori* and *D. melanogaster* were analyzed by Analyzer Splice Tool software to evaluate their relative strength for pre-mRNA splicing. Two alternative 5′SS in intron 3, 3 and 3a, were separately analyzed. In comparison with the *Drosophila dsx* introns, strength of splice sites in intron 3 of *Lepidopteran dsx* exhibited an obvious opposite pattern, which suggested that there would be a different regulatory mechanism for AS of *dsx* in *Lepidoptera*. Sequences of splice site regions are listed, and the exon-intron boundaries are indicated by slashes. (C) The weakness of 5′SS of intron 3 in *Lepidopteran dsx* is experimentally confirmed. The 5′SS region of intron 1 (CCC/GUAAGU), 2 (AAG/GUACCG) and 4 (UGG/GUAAGU) were replaced by the alternative 5′SS region of intron 3 (CCA/GUACGGACUUUAAUAGUAAUA) in the *Bm-dsx* mini-gene, respectively. These replacements resulted in abnormal intron selections and disorder of splicing, demonstrating that the 5′SS of *Lepidopteran dsx* intron 3 is weak in vivo. Asterisk: partial region of intron 2 was retained in the isoforms 12-3456 and 12-3a456.

exon 4 skipping of in males. Therefore, putative RNA *cis*-elements in *Lepidopteran dsx* would exist to regulate such activation and inhibition.

3.5. Conserved RNA fragments in Lepidopteran dsx

To search for conserved RNA *cis*-elements that would potentially regulate AS of *Lepidopteran dsx*, we aligned canonical full-length *dsx* mRNA sequences from the six *Lepidoptera* species (Fig. 5A and S2). For function at protein level, conserved genes normally have conserved sequences of amino acids; however, the third nucleotides of codons are not highly conserved due to degeneracy. In contrast, for the function at DNA or RNA level, conserved genes would have highly conserved nucleotides at the third positions of codons if they are important regulatory elements. The binding sites of RNA-binding proteins are typically around 6–12 nt (Long and Caceres, 2009). Therefore, we searched for putative RNA *cis*-elements that contain at least 10 continuous invariable ribonucleotides from mRNAs of all the six *Lepidopteran dsx*. At this level, four highly conserved RNA fragments (I, II, III and IV) in *Lepidopteran dsx* were obtained (Fig. 5B and S2). Due to lack of genome information in most *Lepidoptera*, searching for conserved intronic RNA fragments in *dsx* is not feasible. In addition, based on these six mRNA sequences, the evolutionary

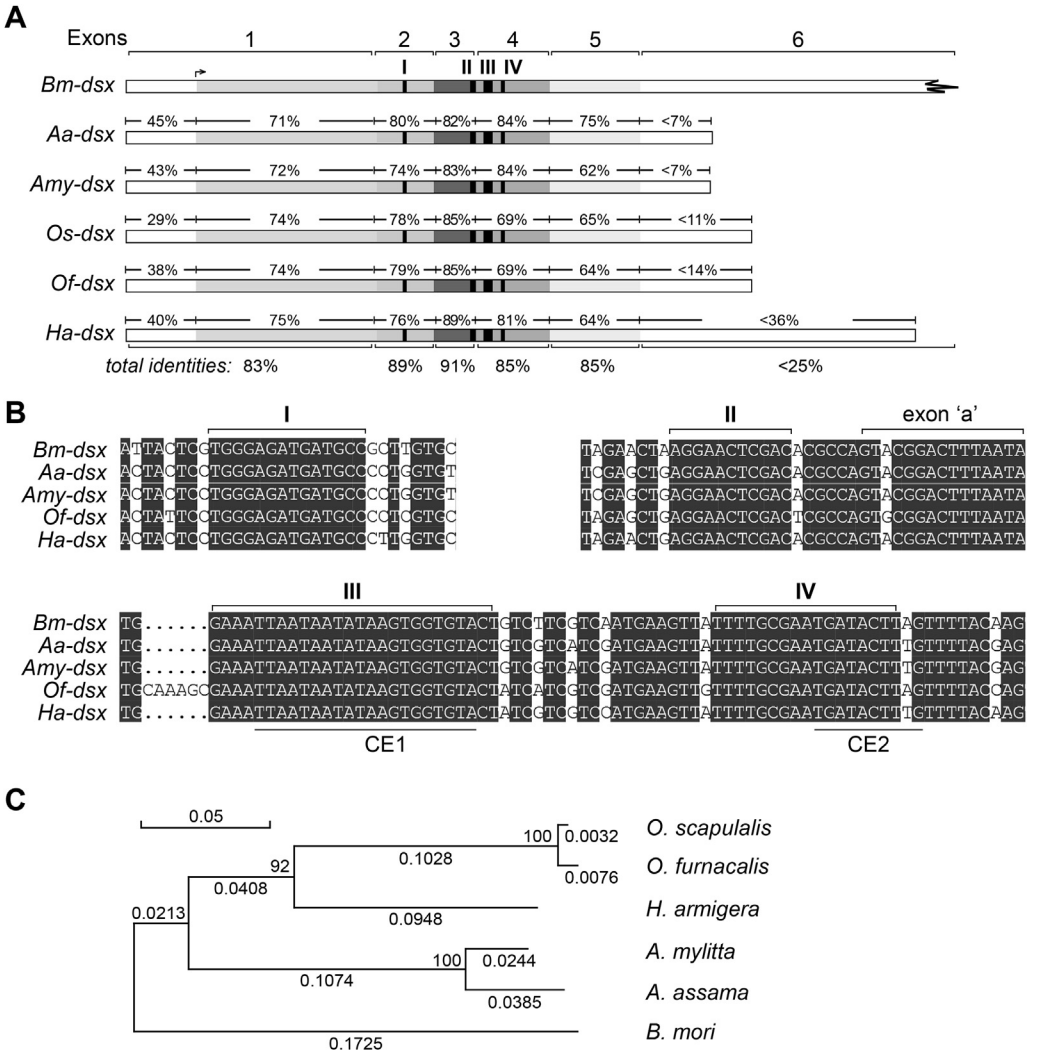

Fig. 5. Analysis of *Lepidopteran dsx* mRNAs reveals highly conserved RNA *cis*-elements. (A) Alignment of six *Lepidopteran dsx* mRNAs. Identities were calculated for each exon with the counterpart in *Bm-dsx*, and presented in gradients for the degree of conservation. For detailed sequences and alignment see Supplementary Fig. 2. (B) Four highly conserved RNA fragments exist in *Lepidopteran dsx* mRNAs. The criteria for a highly conserved RNA fragments are that each region contains at least 10 continuous invariable nucleotides. RNA fragment I is located in exon 2, II in exon 3; III and IV are located in exon 4. (C) Phylogenetic tree of six *Lepidoptera* based on their *dsx* mRNA sequences. It is constructed by MEGA 5.1 software using Maximum Likelihood method (Toyota et al., 2013). Horizontal distances are proportional to the number of nucleotide changes calculated by Tamura-Nei model. The reliability of the dendrogram was evaluated by 500 bootstrap replicates, and presented as percentages at the branch points.

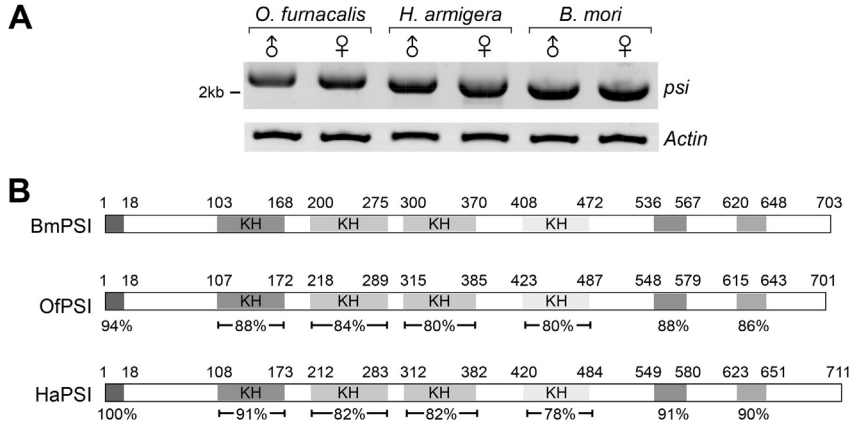

Fig. 6. Identification of *psi* genes in *O. furnacalis* and *H. armigera*. (A) Transcripts of *psi* in the male and female from each *Lepidoptera* are unique and identical. Sequences of *psi* mRNAs in *O. furnacalis* and *H. armigera* were obtained by RT-PCR and RACEs. (B) Identities between three *Lepidopteran* PSI proteins. The four conserved KH domains and other motifs are indicated in dark region. Identities were calculated to the corresponding region in BmPSI.

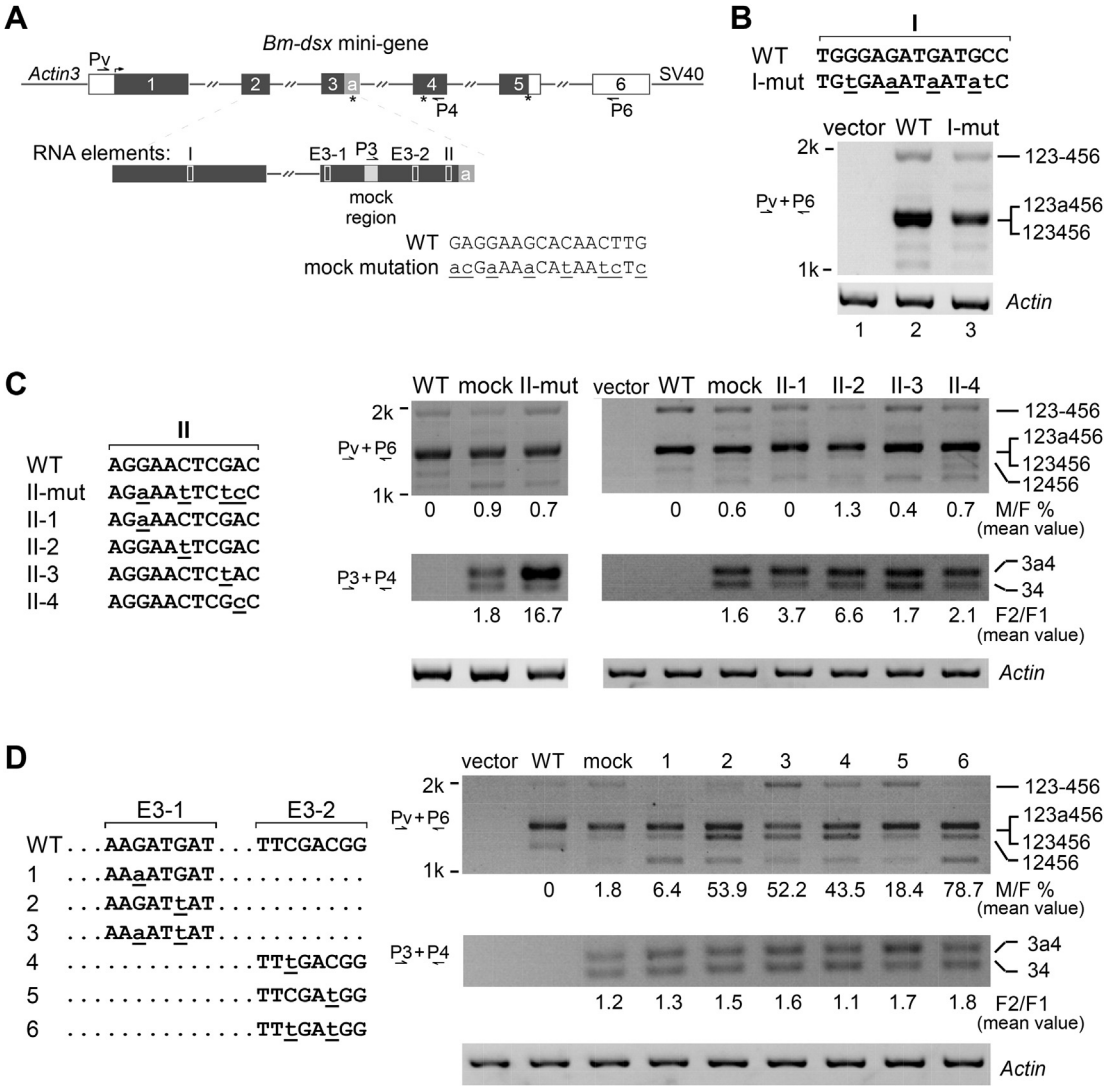

Fig. 7. Conserved RNA *cis*-elements regulate the alternative splicing of *Lepidopteran dsx*. (A) Schematic of *Bm-dsx* mini-genes. The mini-gene constructs were transfected into silkworm ovary BmN cell line. Investigated RNA elements and mock mutation region are indicated in a larger view. (B) RNA fragment I in exon 2 contributes to the overall splicing efficiency of *Bm-dsx*, but is not involved in the sex-specific AS of *Bm-dsx*. Mutated sites in the mini-gene are indicated. (C) RNA fragment II is a *cis*-element for regulation of the alternative 5′SS at intron 3. Multiple-sites and single-site mutations in RNA fragment II were generated, which significantly altered the ratio between two female specific isoforms F2 and F1. (D) Two RNA *cis*-elements are critical for female-specific AS of *Lepidopteran dsx*. Both E3-1 and E3-2 contain an 8-nt continuously invariable sequence in *Lepidopteran dsx*. Mutations in these two RNA fragments significantly increased the skipping of exon 3, generating more male-specific AS products of *Bm-dsx*. Used amplification primers were indicated on left side of each gel. Mutations in the mock region were designed on the non-conserved nucleotides in the exon 3 of *Lepidopteran dsx*, and primer P3 from the mock region was used to distinguish the cDNA products from exogenous *Bm-dsx* mini-genes and the endogenous *dsx* gene. The mock mutant did not alter the splicing of exon 3, and was considered as the WT mini-gene for controls to other mutants. M/F%=(Isoform 12456)/(isoforms 123456 + 123a456) × 100, stands for the percentage of male-specific isoform to the female-specific isoforms. Mean values are average from triplicate samples.

relationship between the six *Lepidoptera dsx* is also addressed. As shown in the phylogenetic tree, *O. furnacalis* has the closest relationship with *O. scapulalis*, and *H. armigera* has diverged from *B. mori* much before it diverged from *O. furnacalis* (Fig. 5C).

RNA fragment I (14 nt) is in the exon 2; fragment II (11 nt) in the exon 3, and fragments III (26 nt) and IV (17 nt) are in the exon 4 (Fig. 5A and B). In *B. mori*, the RNA fragments III and IV contain previously reported Bm-PSI binding site CE1 and CE2, respectively. Bm-PSI is critical for the production of male-specific *Bm-dsx*, which binds to the CE1 region in exon 4 and prevents the inclusion of exons 3 & 4 (Suzuki et al., 2008). High conservation of the RNA *cis*-element III and IV in *Lepidopteran dsx* suggests that *psi* genes would also exist in other *Lepidoptera*. Therefore, similar strategies were used to amplify *Bm-psi* homologous mRNA in *O. furnacalis* and *H. armigera* by degenerate primers, and 5′ and 3′ RACEs. Obtained coding sequences of *Of-psi* and *Ha-psi* are 2106 and 2136 bp, respectively. Extensive RT-PCRs and sequencings of mRNA products from *psi* genes resulted in only one mRNA in each of the three *Lepidoptera*, irrespective of male or female samples, suggesting that *psi* genes in *Lepidoptera* are not alternative spliced (Fig. 6A). The three *Lepidopteran* PSI proteins from silkworm, Asian corn borer and cotton bollworm are highly conserved, especially in their four RNA-binding KH domains and being overall 73% identical (Fig. 6B and S3).

3.6. Conserved RNA cis-elements regulate AS of Bm-dsx

To further investigate the role of the identified conserved RNA fragments, we constructed *Bm-dsx* mini-genes, including the intact wild type (WT) or mutant exons with shortened intron sequences

(Fig. 7A), and transfected them into BmN cell line. Since the BmN cell line is derived from the ovary of *B. mori* (Maeda, 1984), therefore AS of the WT *Bm-dsx* mini-gene are carried out in the female-specific way. We considered several criteria for generating *Bm-dsx* mutants: 1) prefer to mutate the third nucleotides of codons so that it does not change the coding potential; 2) prefer to mutate the middle sites of RNA elements due to their importance for regulator binding; 3) multiple mutations were made for more significant effects, and 4) avoid generating new potential splice sites or PTC. Indeed, WT *Bm-dsx* mini-gene produced the female-specific *dsx* isoforms containing exons 1–6 in BmN cell line (Fig. 7B, lane 2), while mutations in the RNA fragment I resulted in significantly decreased mRNA levels of all the multiple isoforms. However, the ratios among these isoforms did not obviously change (Fig. 7B, cf. lanes 3 to 2), suggesting that RNA fragment I only contributes to the overall splicing efficiency of *Bm-dsx* and is consistent with its location – in the common exon 2. However, mutations in the RNA fragment II, including multiple-sites and single-site mutations, significantly altered production of the two female specific isoforms F1 and F2, generating more isoform F2 but less isoform F1 (Fig. 7C middle). The ratio of F2/F1 increased from 1.6 by the WT RNA fragment II (AGGAACTCGAC) to 6.6 by the single-site mutant II-2 (AGGAAtTCGAC), and to 16.7 by the multiple-sites mutant II-mut (AGaAAtTCtcC). This result demonstrate that the RNA fragment II is an important *cis*-element for regulation of the alternative 5′SS of intron 3. Meanwhile, these mutations did not alter the selection of the full exon 3. Exon 3 inclusion represents the female-specific AS of *Bm-dsx* and exon 3 skipping represents the male-specific AS. All the fragment II mutants did not generate detectable male-specific products (Fig. 7C upper). To search for more potential RNA *cis*-elements in exon 3, we constructed *Bm-dsx* mini-genes with mutations in other two conserved regions E3-1 and E3-2, each containing an 8-nt invariable sequence in all the six *Lepidopteran dsx* (Fig. 7D and S2). In comparison to the WT, *Bm-dsx* mini-genes containing single mutations in the E3-1 (AAGATGAT mutated to AAGATtAT) and E3-2 (TTCGACGG mutated to TTtGACGG) significantly increased the skipping of exon 3, generating more male-specific AS products of *Bm-dsx*, and mini-genes with multiple-sites mutations exhibited stronger effects (Fig. 7D, cf. lanes 2–4 and 6 to WT). This result strongly suggests that E3-1 and E3-2 regions are critical *cis*-elements for the inclusion of exon 3, which would function as exonic splicing enhancers for recruiting regulatory splicing factors to activate the weak 5′SS of intron 3, thus, resulting in female-specific splicing of *Bm-dsx*.

4. Discussion

Gene conservation across species is characterized at different levels. Conservation at the protein and peptide levels reflect the common and important function of a gene, while additional conservation at nucleotide level reveals the essential regulatory *cis*-elements for transcription or pre-mRNA splicing during the expression of this gene. In this study, we obtained *dsx* mRNA sequences from two agricultural pests, *O. furnacalis* and *H. armigera* and analyzed the sex-specific isoforms generated by AS. Together with *dsx* mRNA sequences from other reported *Lepidoptera* species, we identified several highly conserved RNA sequences and found that three RNA *cis*-elements are important for the regulation of female-specific AS of *Lepidopteran dsx* by the assay in BmN cell lines.

4.1. Multiple alternative splicing of dsx is conserved in female Lepidoptera

Alternative splicing of *dsx* gene is critical for sex determination and differentiation in insects. In the beginning of this study, we found that more than a dozen AS products of *dsx* genes were generated in the female *O. furnacalis* and *H. armigera* although only one isoform was produced in each male. Further investigation revealed that similar multiple mRNA products also exist in the female of well-studied economic insect *B. mori*, in which nine *Bm-dsx* isoforms were not reported before. Recently, several *trans*-spliced and one *cis*-spliced isoforms of *dsx* were also identified in the female silkworm (Duan et al., 2013), suggesting that production of multiple *dsx* isoforms in females is a conserved and common feature of *Lepidoptera*. We classified those isoforms into four groups based on their AS types; most of the AS events occurred at various splice sites in the intron 3 between the canonical *dsx* exon 3 and 4, consistent with the presence of these two exons in female *Lepidoptera*. Therefore, intron 3 of *Lepidopteran dsx* is the hot spot for AS, which contains several regions ('b', 'c' and 'd') that are included in the female-specific products. Another important AS event is the alternative 5′SS of intron 3, which was first observed in *B. mori* (Shukla et al., 2010). Further studies in *A. assama* by RNAi methods demonstrated that the two alternative 5′SS products (dsxF1 and dsxF2) are both required for the survival of wild silkmoth. Knockdown of either dsxF1 or dsxF2 completely abolished the expression of *dsx* downstream genes vitellogenin and hexamerin, and resulted in deformation of female gonads and complete lethality of eggs (Shukla and Nagaraju, 2010). We found that this alternative 5′SS event is also conserved in the females of *O. furnacalis* and *H. armigera*. Moreover, this event is always coupled with other AS events in female *Lepidopteran dsx*, generating many mRNA products in pairs with 15-nt difference. However, due to the presence of a stop codon in the exon 'a', combination of the alternative 5′SS event with other AS events would not generate more putative proteins (Table 1).

4.2. Splice sites in intron 3 of Lepidopteran dsx

Our analysis further showed that the strength of the 5′ and 3′SS of intron 3 in *Lepidopteran dsx* are opposite to their counterparts in *Diptera Drosophila dsx*, consistent with the previous finding that default splicing of *dsx* are opposite in *B. mori* and *Drosophila* (Suzuki et al., 2001). The weak 5′SS in intron 3 of *Lepidoptera dsx* suggested that inclusion of exon 3 in the females must be activated by exonic splicing enhancers (ESE) and/or intronic splicing enhancers (ISE). Through mutagenesis assays in the silkworm ovary BmN cell line, we found two highly conserved RNA *cis*-elements in exon 3 (E3-1 and E3-2) and demonstrated that these two elements function as ESE to enhance the inclusion of exon 3. Mutations in these two *cis*-elements caused significant skipping of exon 3, implying that these two *cis*-elements could be potentially bound with splicing activators to overcome the weakness of the 5′SS of intron 3 and recruit core spliceosomal components for catalysis. Actually, the RNA *cis*-element E3-2 (TTCGACGG) contains an enhancer motif 'CGACGG', which was characterized as a binding motif of SR protein SF2/ASF (Cartegni et al., 2003). Further investigation is important and necessary to address which activator(s) bind to these RNA *cis*-elements and how to regulate AS of *dsx*.

4.3. Sex determination pathway in Lepidoptera

In *Drosophila*, the sex determination pathway occurs mainly through a cascade of AS events from *sxl* to *tra*, then to *dsx*. However, the upstream genes of sex determination cascade in *Lepidoptera* have not been identified. The regulation of *Bm-dsx* AS in males was identified to be achieved through the inhibition of exon 4 inclusion by PSI and IMP, but how exon 3 is included in the *Lepidopteran* females presently remains unclear. Since the initial sex determination signal in the silkworm was demonstrated to be located on

the female specific chromosome W, and our results in this study reveal that exon 3 in *Lepidopteran dsx* contains highly conserved RNA *cis*-elements, we propose that the identified three RNA *cis*-elements, including 3E-1, 3E-2 and II, would function coordinately to recruit *trans*-activators for the selection of the weak 5′ SS of intron 3 in the female *Lepidoptera*.

It has been demonstrated that specific RNA *cis*-elements in the alternatively spliced genes from the sex determination pathway can be engineered for the construction of sex-specific lethal system to control the population of *Diptera* pests based on sterile insect technique (SIT), a species-specific and environmentally nonpolluting method (Bushland et al., 1955). Sex-specific sequences, such as transcription enhancer (Heinrich and Scott, 2000), promoter (Thomas et al., 2000), and Tra intron (Fu et al., 2007), were engineered with the tetracycline-repressible transcriptional activator (tTA) protein. Sex-specific expression of tTA leads to expression of either a toxin or proapoptotic gene, which is driven by a tetO promoter in the absence of tetracycline and finally causes the sex-specific lethality of insects (Heinrich and Scott, 2000; Thomas et al., 2000; Alphey, 2002; Rendon et al., 2004; Marec et al., 2005; Wimmer, 2005; Fu et al., 2007). Recently, specific sequences of *dsx* were also used for generating female-specific lethal system in silkworm and pink bollworm (Morrison et al., 2012; Jin et al., 2013; Tan et al., 2013). Therefore, identified RNA *cis*-elements in this study, the female-specific exonic splicing enhancers from *Lepidopteran dsx*, could be used as female-specific signal in the SIT technique to develop a more effective and specific method for *Lepidopteran* pest control.

5. Accession numbers

Sequences obtained in this study were deposited in NCBI and the GenBank accession numbers are listed in Table S2.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2013.10.009.

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