Female-specific doublesex dsRNA interrupts yolk protein gene expression and reproductive ability in oriental fruit fly, Bactrocera dorsalis (Hendel)

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Abstract

A homologue of the doublesex gene (Bddsx) has been cloned from the oriental fruit fly, Bactrocera dorsalis (Hendel). Northern analysis indicates a differential expression of Bddsx in male and female flies, as reported for other dsx genes. A structural conservation of DNA binding domain/oligomerization domain 1 and oligomerization domain 2 suggests that the doublesex protein (BdDSX) of this fruit fly serves as a transcriptional factor for downstream sex-specific gene expression. The putative transformer/transformer-2 protein binding sequence in female-specific transcript suggests that a preserved alternative splicing process found in other flies mediates the synthesis of Bddsx transcript. RNA interference (RNAi) data from adult abdominal dsRNA injection assays indicate that female-specific dsx dsRNA reduces specifically its own transcript, inhibits selectively expression of the yolk protein gene (Bdyp1), and delays ovary development. The number of matured eggs is significant reduced after RNAi treatment, but the sex ratio of offspring is not biased. Moreover, 27\% of female progeny with RNAi show deformed ovipositor, but male flies are not affected. Although this is a transient treatment, the specific Bddsx\textsuperscript{f} interference offers a promising and novel approach to oriental fruit fly control in the future.

Keywords: Doublesex gene; Bactrocera dorsalis (Hendel); Alternative splicing; RNA interference

1. Introduction

The oriental fruit fly, Bactrocera dorsalis (Hendel), one of the most destructive pests in several Asian countries, can cause severe economic loss to more than 150 fruit crops. During the past several decades, neither methyl eugenol/protein baiting nor large-scale eradication based on sterile insect technique has kept this insect pest effectively controlled in Taiwan. Since the number of female flies in the field is directly correlated with the degree of damage to the fruits, a comprehensive understanding of sexual differentiation may consequently help the development of novel control techniques.

Genes involved in the sex-determination of Bactrocera pests have been proposed as the potential targets for developing new genetic control strategies (Raphael et al., 2004). Doublesex (dsx) gene, first identified in Drosophila melanogaster, is a double switch gene at the bottom of the sexual-determination hierarchy (Wilkins, 1995). Although the sex-determination system is highly diversified among dipteran insects, dsx genes are unique: all dsx genes are evolutionarily conserved with structural and functional likenesses (Hediger et al., 2004). Dsx gene acts as a linker between the upstream sexual-determination hierarchy and downstream genes that perform various sex-specific functions. The primary transcript of dsx gene, through alternative splicing, is processed into male-specific (DSXM) or female-specific (DSXF) proteins. Both DSX proteins are transcriptional regulators controlling somatic sexual differentiation (Burtis and Baker, 1989). Up to now, dsx homologues have been identified in many organisms,
including Bactrocera tryoni (Shearman and Frommer, 1998), Megaselis scalaris (Kuhn et al., 2000), Ceratitis capitata (Saccone et al., 1998), Musca domestica (Hediger et al., 2004), Bombyx mori (Ohbayashi et al., 2001), Anopheles gambiae (Seal et al., 2005), Bactrocera oleae (Lagos et al., 2005), Anastrepha obliqua (Ruiz et al., 2005), Caenorhabditis elegans (Raymond et al., 1998), human (Raymond et al., 1999a), mouse and chicken (Raymond et al., 1999b). Structural analyses have shown that these dsx genes share several characteristics, such as non-sex-specific DBD/OD1 domain and sex-specific OD2 domain, which are important to DNA-binding, oligomerization, and transcriptional regulation properties (Erdman and Burts, 1993; Erdman et al., 1996; An et al., 1996).

The yolk protein (YP) of insects is a glycoprotein with extreme importance to reproduction; its precursor is synthesized in the fat body of females, released into the circulatory system, and transported into growing oocytes through receptor-mediated endocytosis (Haunerland, 1996). The regulation of sex-specific and stage-specific expression of yolk protein gene has been well characterized in Drosophila. DSX M and DSX X proteins with different C-terminal domains can either activate or inactivate yolk protein gene (yp) through their binding to the promoter (An and Wensink, 1995a, b).

RNA interference (RNAi), first reported in C. elegans, utilizes introduced dsRNA to trigger sequence-dependent cleavages and subsequently lead to the silencing of target gene expression (Fire et al., 1998). This phenomenon has been observed in insects, e.g., Drosophila, Tribolium, Apis mellifera, and later in mice (Hutvagner and Zamore, 2002). In view of its specificity and simplicity, this post-transcriptional gene silencing technique has quickly become a powerful tool for function studies (Elbashir et al., 2001; Hannon, 2002). There are a number of ways to introduce dsRNA into organisms, such as stable transgenic expression (Kennerdell and Carthew, 2000), viral delivery (Travanty et al., 2004), soaking, oral feeding (Timmons et al., 2006), dsRNA delivery into growing oocytes of transgenic embryos (An and Wensink, 1995a, b), dsRNA delivery into growing oocytes of transgenic embryos (An and Wensink, 1995a, b) and dsRNA delivery into growing oocytes of transgenic embryos (An and Wensink, 1995a, b). Subsequently, RNA interference silencing complex (RISC) coupled with these small interfering RNAs accomplish the knockdown reaction to the complementary transcripts. Taking the advantages of this RNAi technique, we used it for functional analyses of dsx gene.

In the present study, we have isolated full-length dsx cDNAs of B. dorsalis, named Bddsx, based on the conserved sequences of known dsx genes, and confirmed the presence of alternative splicing in male and female oriental fruit flies. RNAi has been used to characterize the putative Bddsx X gene in vivo. Administration of Bddsx X dsRNA into the female abdomen leads to ovary underdevelopment and a reduced number of matured eggs. The signals of Bddsx X transcript, and yolk protein1 gene of B. dorsalis (Bdyp1, GenBank accession number AF368053) repression has been observed. The effect of RNAi can even extend to the progeny as deformed ovipositors were observed in some F1 females.

2. Material and methods

2.1. Origin of insects

A laboratory strain of B. dorsalis that has been raised in the laboratory for over 6 years was used as experimental material. The fly strain was kept at 28°C in a 12 h:12 h light:dark photoperiod. Adult flies were housed in cages for mating and egg laying; hatched larvae were cultured by periodic transfer to fresh food.

2.2. Cloning of the oriental fruit fly dsx cDNA

To isolate cDNA fragments containing the coding region of dsx, reverse transcription and polymerase chain reaction (RT-PCR) was applied using degenerated primers, dsx-B: 5’-TATCCTNGGAATGTGCACTATM-3’ and dsx-G: 5’-AATTATCATCCACATTGCC-3’, which were based on the female dsx consensus sequences of B. tryoni, D. melanogaster, and M. scalaris (Bddsx, GenBank accession number AF029675, nt 898–924 and nt 1082–1066; dsx, GenBank accession number AY060257, nt 1979–2005 and nt 2163–2145; Msdsx, GenBank accession number AF0283695, nt 814–840 and nt 995–977). Preparations of male and female RNAs of B. dorsalis were performed separately using TRI Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer’s protocol. Subsequently, the first-strand cDNA was synthesized from 1 µg of total RNA with oligo-dT containing primer and PowerScript™ reverse transcriptase (Clontech, Palo Alto, CA, USA). PCR amplification with primers dsx-B and dsx-G was performed as follows: 94°C for 2 min; 35 cycles of 94°C for 45 s, 56°C for 1 min, and 72°C for 2 min; and 72°C for 10 min at the end. The amplified BG fragment with the expected size of 185 bp was purified using GeneClean III (Promega, Madison, WI, USA) and cloned into pGEMR-T Easy vector (Promega, Madison, WI, USA) and sequenced. Each cloned cDNA fragment was sequenced by dideoxy-chain termination using ABI PRISM 377 DNA autosequencer (DNA Sequencing Core Facility, Biotechnology Center, National Chung-Hsing University).

For full-length cDNA determination, the 5’-end of the dsx cDNA fragment was produced using the 5’-RACE system (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. Bddsx-specific primers, dsx-com (nucleotides 1024–1006; 5’-CAATGCGTCTTGAGGCCT-3’) and dsx-R (nucleotides 500–478; 5’-GCGTGTGCTCATGACCTGG-3’), were designed based on the partial Bddsx cDNA mentioned above. After PCR...
amplification, the amplified DNA fragments were purified, cloned and sequenced by standard protocols.

For 3′-RACE, Bddsx-specific primer, dsx-H (nucleotides 1009–1030; 5′-GCTTCAAGGCAGCTGGAAG-3′), was designed based on BG fragment sequence. Sex-specific 3′ regions were obtained after PCR amplification with RT products as templates. The resulting DNA fragments were purified, cloned and sequenced as described above.

2.3. Northern blot analysis

Total RNA was extracted from the whole body of adult flies using TRI reagent, and poly(A)+ RNA samples were prepared from 150 µg of total RNA using Dynabeads mRNA Purification Kit (Dynal Biotech ASA, Oslo, Norway) following manufacturer’s instruction. Individual poly(A)+ RNA (2.5 µg) was separated on 1% denaturing formaldehyde agarose gel containing 0.7 M formaldehyde and 1 × MOPS at 80 V for 90 min, and visualized by ethidium-bromide. The gel was washed in 10 × SSC before the RNA was transferred to a positively charged nylon membrane (Hybond XL, Amersham Pharmacia, Piscataway, NJ, USA) with 10 × SSC. Prehybridization was performed in Ultrasyph hybridization solution (Ambion, Austin, TX, USA) at 65 °C for 4 h. The 32P labeled Bddsx probe (male nucleotides 467–1189M) was prepared by PCR with dsx-W primer (nucleotides 467–493; 5′-ACGAGG-TACCGCCAGTGGTAC-3′) and dsx-M primer (nucleotides 1189M–1166M; 5′-GCCGAAGTGCAGAGGTCAGTGA-3′), and a32P labeled dCTP (specific activity 3000 Ci/mmol, Perkin-Elmer Life and Analytical Sciences, Shelton, CT, USA). For hybridization, the RNA-containing membrane was incubated with the labeled Bddsx probe of 1 × 106 cpm per ml of hybridization solution. Following overnight hybridization at 65 °C, the membrane was washed twice for 5 min at low stringency (2 × SSC, 0.1% SDS) at 42 °C and twice for 15 min at high stringency (0.1 × SSC, 0.1% SDS) at 65 °C. The membrane was exposed to X-ray film for 3 days and developed according to the standard procedures.

2.4. Preparation of dsRNA for RNA interference

To prepare dsRNA from the female-specific region of Bddsx gene, PCR with dsx-S primer (nucleotides 1031F–1048F; 5′-GCCTCAAGAGCGCATTGAGGAAG-3′) and dsx-P (nucleotides 1300F–1277F; 5′-CCAAACATGTAATGAGCTTTCC-3′) primer produced a 270 bp DNA fragment which was cloned into pGEM®-T Easy Vector (Promega). The two DNA plasmids with Bddsx genomic fragment inserted in opposite orientation were digested separately with Sac I or Apa I (New England Biolabs, Inc., Beverly, MA, USA) restriction enzymes. Subsequently, the linear plasmids precipitated with ethanol were used as templates for in vitro transcription. Two Bddsx RNAs with opposite polarities were synthesized using T7 RNA polymerases MEGAscript™ kit (Ambion), treated with DNase I to remove DNA templates, and then precipitated with LiCl. The in vitro-made sense and antisense RNAs were mixed in DEPC-treated water, incubated at 75 °C for 5 min, and allowed to anneal at room temperature for a 4-h period. The prepared Bddsx dsRNA was stored as aliquots at −20 °C until used. We also prepared a 631 bp egfp dsRNA from the pEGFP vector (Clontech) as ambiguous control. Primer EGFP-F (5′-CAGCAACTCCAGAGCCAT-3′) and primer EGFP-R (5′-CTGGTGAGCTGGAC-GGCCGAC-3′) were used for DNA template synthesis, and then subcloned into pSPT19 vector (Roche Diagnostics, Penzberg, Germany) for in vitro transcription using T7 RNA polymerase. All preparations of dsRNAs were diluted in injection buffer (5 mM KCl, 0.1 mM sodium phosphate, pH 6.8) before microinjection.

2.5. Adult abdominal microinjection

A total of 187 newly emerged female flies (day 0) were used for each experiment, and three independent experiments were performed. The flies were immobilized on ice, and 1 µl (2 µg/µl injection buffer) of dsRNA solution (either ds-BddsxF or ds-egfp) was injected into the ventral abdomen of each fly. Throughout the 10 days after eclosion, three individuals were collected at each sampling time for ovary size measurements and fat body RNA preparation. The dsRNA-treated flies were immobilized on ice, fixed and cut in cold saline (5 mM KCl, 0.1 mM sodium phosphate, pH 6.8). The fat bodies adhering with the abdominal wall were collected after ovaries and other alimentary canal had been discarded. These fat bodies were soaked in liquid nitrogen immediately and stored at −80 °C until use. Ovary size was measured with maximum diameter under microscope and statistic analyses were performed by one-way ANOVA assay.

2.6. Real-time PCR

Fat bodies dissected from abdomens of B. dorsalis were homogenized using TRI reagent following the manufacturer’s instruction. Five micrograms of total RNA was reverse transcribed by SuperScript reverse transcriptase II supplied by SuperScript™ Preamplification System for first-strand cDNA Synthesis Kit (Invitrogen). Real-time PCR was performed using the iCycler iQ system (Bio-Rad Laboratories Inc., Hercules, CA, USA); primers were designed with Beacon Designer 7 (PRIMER Biosoft International, Palo Alto, CA, USA), and reactions were performed in 96-well plates with a SYBR® GreenER™ qPCR SuperMix for iCycler® (Invitrogen). The protocol for cdNA amplification was one cycle of 50 °C for 2 min, one cycle of 95 °C for 8 min 30 s, and 50 cycles of 95 °C for 15 s, then 57.5 °C for 1 min, followed by melting curve analysis to detect-specific product amplification. Real-time PCR primers are as follows: Bdyp1 forward primer: 5′-TCAACATGCAATACCCAGGAC-3′ (position
516–530 of \textit{Bdyp1}); reverse primer, 5’-GCATCACCAGAGCCAAACC-3’ (position 919–930 of \textit{Bdyp1}); \textit{BddsxF} forward primer: 5’-TTCTCAATCAACTATCCACTCG-3’ (position 1464–1487 of \textit{BddsxF}; GenBank accession number AY669318); reverse primer: 5’-GTGCCCTTTAACTCCATGC-3’ (position 1599–1618 of \textit{BddsxF}); \textit{Bdactin} forward primer: 5’-ATCTGGCATCACACTTTCTAC-3’ (position 2236–2256 of \textit{Bdactin}; GenBank accession number L12254; He and Haymer, 1994); and reverse primer: 5’-ATCTGGCATCACACTTCTAC-3’ (position 2323–2342 of \textit{Bdactin}). Each sample was analyzed in triplicate and normalized to the \textit{actin} expression of \textit{B. dorsalis} as internal control. Real-time data were collected by the Bio-Rad iQ5 2.0 Standard Edition Optical System Software V2.0.

2.7. Western blot analysis

Protein was extracted from \textit{B. dorsalis} at different developmental stages by PBS buffer containing 0.5 mM phenylmethylsulfonyl fluoride. Cell lysates were cleared by centrifugation at 14,000 rpm (16,000 \textit{g}) for 20 min at 4°C. Proteins of ca. 10 \textmu g in the supernatant, determined by detergent compatible Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA), were separated by SDS–PAGE on mini-gels and electro-transferred to a nitrocellulose membrane (Schleicher and Shuell, Keene, NH, USA). Yolk protein was identified with a rabbit anti-rabbit IgG antibody linked with alkaline phosphatase.

2.8. Reproductive ability analyses

Newly emerged female flies (day 0) were treated with either \textit{BddsxF} or \textit{egfp} dsRNAs through abdominal injection. Each individual female was moved into a separate box on day 8, and supplied three male flies for mating. On day 14 after eclosion, eggs were collected and counted from each female, and the hatched larvae were maintained with fresh food. The emergence rate, sex ratio, and morphological changes were checked and recorded periodically.

3. Results

3.1. Characteristics of sex-specific \textit{BddsxF} transcripts

RT-PCR using first-strand cDNA of female oriental fruit fly and degenerate primers designed according to the alignment results of three known female-specific \textit{dsx} sequences gave a 185 bp product (BG fragment) with 99% nucleotide identity to \textit{dsx} of \textit{B. tryoni} (\textit{BddsxF}) (Shearman and Frommer, 1998) (Fig. 1A; 944C–1128F). Based on this partial cDNA sequence of \textit{BddsxF}, full-length cDNAs of \textit{BddsxF} and \textit{Bddsxm} were subsequently obtained using RT-PCR and 5’- and 3’-RACE; their nucleotide sequences and deduced amino acid sequences are listed in Fig. 1A. \textit{Bddsxm} cDNA (GenBank accession number AY669317) had a 1203 bp open reading frame (ORF) encoding 400 amino acid residues, and the shorter \textit{BddsxF} cDNA (GenBank accession number AY669318) had a 966 bp ORF encoding 321 amino acid residues.

Domain structure analysis shows that DSX proteins of \textit{B. dorsalis} (\textit{BddsxF}) consist of a common N-terminal region (amino acids 1–291) and a sex-specific C-terminal region (amino acids 292–321 for the female and 291–400 for the male) (Fig. 1A). The common region contains a DDBD/OD1 domain (amino acids 39–104) known to be involved in DNA binding and protein oligomerization. The OD2 domain, having a function to enhance protein dimer formation, is sex-specific and composed of two parts. The first part is the same for both male and female flies, amino acids 244–291 in the common region; the second part covers either the entire female-specific portion or the 36 amino acids in the male-specific region (Fig. 1A). A comparison of the female DSX protein sequences of the four known tephritids, \textit{B. dorsalis}, \textit{A. obliqua}, \textit{B. tryoni}, and \textit{B. oleae}, is shown in Fig. 1B. The alignment data indicate the female \textit{BddsxF} has 94%, 98%, and 98% amino acid identity to \textit{AoSxX}, \textit{BtDSX}, and \textit{BoDSX}, respectively. Furthermore, we would like to point out that the amino acid sequences of DBD/OD1 and OD2 domains are completely identical between \textit{BddsxF} and \textit{BtDSX}; and the \textit{BddsxF} and \textit{BoDSX} also share identical amino acid sequences in OD2 domain.

Another structural characteristic of \textit{BddsxF} is the presence of four 13-nucleotide repeats and a purine rich enhancer element (PRE)-like fragment (nucleotides 1610–1624), which are putative transformer protein and transformer-2 protein (TRA/TRA2) binding motifs (Fig. 1).

3.2. Sex-specific expression of \textit{BddsxF} gene

Northern blot analyses using a 32P-labeled probe covering part of the common region and part of the male-specific region (nt 467–1189M) (Fig. 1A) detected signals of both \textit{BddsxF} and \textit{Bddsxm}, with two transcripts of 1.3 and 2.6 kb for the female, and two transcripts of 1.3 and 2.8 kb for the male (Fig. 2). The 2.8/2.6 kb transcripts of \textit{BddsxF} and \textit{Bddsxm} are longer than the corresponding lengths of \textit{BddsxF} cDNA.

3.3. Repressive effects of \textit{BddsxF} dsRNA on ovary development

After dsRNA injection performed within 1 h of eclosion (day 0), ovary size was measured and expressed as maximum diameter during days 0–15. Comparing the collected data from both ds-\textit{BddsxF}− and ds-\textit{egfp}-treated groups (Fig. 3A), the average ovary size shows significant differences from days 2 to 15. Under normal conditions, the ovarioles enlarge and produce mature eggs on day 6.
However, no mature eggs could be detected in ds-Bddsxf-treated flies at the time point. In contrast to the ds-egfp-treated control group, the number of developed ovarioles was variously reduced in ds-Bddsxf-treated flies on day 10 (Fig. 3B and C). The above results suggest that ds-Bddsxf treatment leads to repressive effects on the female reproductive system of *B. dorsalis*.

3.4. Specific inhibition of Bddsxf transcripts and repression of Bdyp1 expression by female abdominal RNAi

Real-time PCR is a highly sensitive tool used to detect small changes in targeted gene expression. To quantitatively evaluate the direct effects on *Bddsxf* transcripts, we extracted RNAs from the fat body and performed...
real-time PCR analyses (Fig. 4A). Our data indicate that the \( Bddsxf \) gene was expressed in the fat body right after eclosion. Relative to the expression of the \( actin \) gene, the amount of \( Bddsxf \) transcript increased with time. Significant RNAi effects to \( Bddsxf \) transcripts occurred within 1 h after injection. The \( Bddsxf \) transcript in \( Bddsxf \) dsRNA-treated females was obviously inhibited during days 0–6. Though the effect of \( Bddsxf \) dsRNA became slightly less significant on day 10, inhibition on \( Bddsxf \) expression could still be detected (Fig. 4A). Three independent experiments showed similar results, providing direct evidence that the RNAi could mediate the sequence-dependent repression of \( Bddsxf \) transcript in vivo.

For \( Bdyp1 \) expression, \( Bdyp1 \) was undetectable on day 0. The transcript of \( Bdyp1 \) appeared 6 days after eclosion in our real-time PCR experiments, and its signal quickly accumulated in the fat body of both groups until day 10 (Fig. 4B). Under \( Bddsxf \) RNAi, the repressive effect on \( Bdyp1 \) expression was significant on day 6 after dsRNA treatment (Fig. 4B), and this gene silencing process continued until the end of the experiment (day 10). These observations indicate that the repression of \( Bddsxf \) precedes the silencing of \( Bdyp1 \) gene. The results of western blotting
also supported that the Bddsxf RNAi can lead to the repression of Bdyp1 gene expression. YP protein in Bddsxf-treated females started to appear on day 6, and remained at lower levels compared with that in egfp control flies (Fig. 5).

3.5. Egg reduction and deformed organ formation by transient RNAi

To examine the RNAi on productive activity, 15 newly emerged female flies were treated with ds-Bddsxf, while the
same number of female flies given non-specific egfp dsRNAs was used as control. After mating, averaged egg-laying values were calculated from each treatment as an index of reproductive ability. In the ds-Bddsxf-treated group, each female could only produce approximately four eggs in average. This is a dramatic drop compared to the control group, with approximately 43 eggs to each female (Fig. 6). Since the dsx gene is at the bottom of the sex-determination process, sex ratios of F1 from both groups remained at 1:1 as expected.

The morphological changes were observed in progeny of ds-Bddsxf-treated flies. In 3 of 11 (~27%) F1 female flies, the ovipositors were deformed (Fig. 7). The abnormal ovipositors were covered in an oval sac and could not execute their normal function. F1 male flies had normal genital organs.

4. Discussion

4.1. Structural conservation and sex-specific expression of Bddsx

BdDSX contains a DBD/OD1 domain, which has a zinc-finger structure and is involved in DNA binding and oligomerization, as well as an OD2 domain known to enhance-specific dimer formation (An et al., 1996; Cho and Wensink, 1997, 1998). The amino acid sequences of these two domains of BdDSXF exhibited over 98% identity with those of A. obliqua, B. tryoni, and B. oleae (Fig. 1B), suggesting a conservation of configuration and function. The deduced amino acid sequence of BdDSX in this study provides another example showing that all known dipteran dsx genes have a common evolutionary origin and a similar function as the transcriptional regulator to the downstream genes. Similar DNA binding domain structures have also been identified in MAB-3 protein and DMRT1 which are DSX homologues in C. elegans and vertebrates, respectively (Raymond et al., 1998, 1999a, b). The conservation of domain structures crossing metazoan indicates the essential roles of DSX proteins as double-switch regulators in the sexual-determination pathway.

The four 13 nt repeat elements and a putative purine-rich element (PRE) in the 3′-UTR of Bddsx y transcript have also been observed in corresponding transcripts from D. melanogaster, B. tryoni, B. oleae, and M. domestica. Inoue et al. (1992) reported that the binding of TRA and TRA-2 proteins with these 13 nt repeat elements mediated
the dsx pre-mRNA alternative splicing in D. melanogaster. With the identification of transformer (tra) genes in C. capitata (Pane et al., 2002) and B. oleracea (Lagos et al., 2007), we believe the corresponding tra gene is present in B. dorsalis as well.

The presence of putative TRA/TRA2 binding site in the female-specific Bddsx transcript suggests the RNA splicing mechanism essential to sex differentiation is also conserved in B. dorsalis. Northern blotting (Fig. 2) further supports that the expression of Bddsx is sex-specific. The two different major transcripts 2.6 and 2.8 kb observed in female and male flies, respectively, are slightly larger than expected. This might have come from pre-transcripts not fully processed or sequence of unidentified regions, such as 5’-UTR, 3’-UTR, and poly(A) tail. Similar phenomena have been reported in Northern analyses of Btdsx (Shearman and Frommer, 1998) and Bodsx (Lagos et al., 2005). The 1.3 kb transcript in both sexes is likely the breakdown product of the Bddsx gene, but we do not exclusive that it might represent an alternative splice variant at the moment.

4.2. Specific repression of Bddsxf and down regulation of Bdyp1 by Bddsxf RNAi

In a comparison study of the directed abdominal injection and egg injection for RNAi efficiency, Amdam et al. (2003) stated that the adult abdominal injection is more efficient and can be used to analyze the genes which are specifically expressed in the fat body. Since the simplicity of this method, we used it to perform the RNAi examination. The early appearance of Bddsxf transcript implies that it is involved and critical at the developmental stages (Fig. 4A). Because the non-specific ds-egfp-treated group gave comparable data to the untreated flies (data not shown), we believe that the Bddsxf RNAi caused a significant decrease of its own transcripts during the first 6 days after eclosion. This is consistent with the results of RNAi that it is a sequence-dependent process. However, the inhibitory effect of ds-Bddsxf exhibited an enlarged variation on day 10. From days 0 to 10 after eclosion, the amount of Bddsxf transcript in fat body increased with time (Fig. 4A). Since the variation on Bddsxf signal in control flies is also high, we believe the observed changes might reflect its own dynamic status and instead of being related to the RNAi effect.

YP deposits are important for egg maturation, and as the specific gene silencing effect of Bddsxf leads to less Bdyp1 expression, this should be related to the decrease in ovary size. Since yp expression is localized mainly in the fat body and ovary (Isaac and Bownes, 1982), dsRNA injected into the abdomen of the female fly is more easily accessible to these tissues. To confirm that RNAi can interrupt the Bdyp1 gene expression in the fat body, the specific tissue was dissected and RNA extracted. The Bdyp1 expression pattern changes caused by the silencing effect of Bddsxf dsRNA treatment clearly indicate the close relationship between Bddsxf and Bdyp1 in the fat body. The time course of Bdyp1 expression agreed with that of the pre-existence of Bddsxf transcript (Fig. 4A and B). In addition, YP expression profiles of Bddsxf dsRNA-treated flies clearly indicate the efficacy of RNAi (Fig. 5), and the time course of YP expression agreed with that of Bdyp1 transcript synthesis (Fig. 4B). In abdominal Bddsxf RNAi experiments, we have provided another line of evidence to support that the Bdyp1 gene is a downstream target of the DSX protein in oriental fruit flies as reported in other dipteran insects.

4.3. Efficacy on reproduction by Bddsxf RNAi and duration of gene silencing

As shown in Fig. 3B, the injection of dsRNA of Bddsxf resulted in various degrees of inhibition on ovary size. In one extreme case, only two developing ovarioles were observed. This observation might reflect the diversity of individual treatment, such as the unpredictable diffusion effect to different tissues after injection. Although there are differential effects on target gene expression and ovary development, the interruption by dsRNA is a general trend in oriental fruit flies as reported in other organisms.

It is worthy to note that the effect of ds-Bddsxf injected into the abdomen of B. dorsalis resulted in an egg-laying rate 10 times less than that of the control flies (Fig. 6). Furthermore, RNAi was observed to be continued in the F1 progeny (Fig. 7). In past studies, physiological functions of DSXs have been broadened to the development of genital imaginal disc, morphogenesis and differentiation of fly (Keisman et al., 2001; Sánchez and Guerrero, 2001; Sánchez et al., 2001; Christiansen et al., 2002). The sustainability of single-dose RNAi in this study should be related to the stability of dsRNA molecules integrated into the eggs at early developmental stages. The small 20–22 bp dsRNA molecules from degradation of introduced dsRNA by a Dicer enzyme system serve as guide sequences to destroy-specific messenger RNAs (Bernstein et al., 2001; Myers et al., 2003). The incorporation of these guide RNAs into a distinct nuclease complex (RISC) might protect them from further breakdown and prolong their half-life in the cell. We believe the transient RNAi effect on ovary development, reproductive ability, and the deformation of ovipositors correlates to the presence of small interfering RNA of Bddsxf in flies. Our data also suggest that the BdDSXF protein is critical for egg maturation and female reproductive organ formation. The detailed mechanisms involved in these developmental processes require much more research to be understood fully.

This phenomenon resulting from single-dose dsRNA injection is intriguing, and we hope to generate a transgenic line which can prolong the effect of Bddsxf RNAi. If the transgenic flies show the desired inhibitory effects on females only by endogenously generated Bddsxf dsRNA, this approach will have great potential in becoming an important control method for oriental fruit fly.
In summary, we have cloned and characterized Bddsxx cDNA, and confirmed, using RNAi data, the biological role of Bddsxl in down-regulating the expression of Bdyp1 gene in B. dorsalis. This work not only shows the usefulness of RNAi on female reproductive ability through target gene silencing, but also its potential in being a control strategy for this important insect pest.

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