Insect Biochemistry and Molecular Biology 75 (2016) 32–44

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Role of *Bmznf-2*, a *Bombyx mori* CCCH zinc finger gene, in masculinisation and differential splicing of *Bmtra-2*

Gajula Gopinath [a, **], Kallare P. Arunkumar [a, *], Kazuei Mita [b], Javaregowda Nagaraju [a, †]

[a] Centre of Excellence for Genetics and Genomics of Silkmoths, Laboratory of Molecular Genetics, Centre for DNA Fingerprinting and Diagnostics, Hyderabad 500001, India
[b] State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing 400716, China

ARTICLE INFO

Article history:
Received 29 October 2015
Received in revised form
26 May 2016
Accepted 31 May 2016
Available online 1 June 2016

Keywords:
Bombyx mori
Zinc finger gene
Femaleness
Splicing
Masculinisation
Doublesex
Transformer

ABSTRACT

Deciphering the regulatory factors involved in *Bombyx mori* sex determination has been a puzzle, challenging researchers for nearly a century now. The pre-mRNA of *B. mori doublesex* (*Bmdsx*), a master regulator gene of sexual differentiation, is differentially spliced, producing *Bmdsxm* and *Bmdsxf* transcripts in males and females respectively. The putative proteins encoded by these differential transcripts orchestrate antagonistic functions, which lead to sexual differentiation. A recent study in *B. mori* illustrated the role of a W-derived *fem* piRNA in conferring femaleness. In females, the *fem* piRNA was shown to suppress the activity of a Z-linked CCCH type zinc finger (*znf*) gene, *Masculiniser* (*masc*), which indirectly promotes the *Bmdsxm* type of splicing. In this study, we report a novel autosomal (Chr 25) CCCH type znf motif encoding gene *Bmznf-2* as one of the potential factors in the *Bmdsx* sex specific differential splicing, and we also provide insights into its role in the alternative splicing of *Bmtra2* by using ovary derived BmN cells. Over-expression of *Bmznf-2* induced *Bmdsxm* type of splicing (masculinisation) with a correspondingly reduced expression of *Bmdsxf* type isoform in BmN cells. Further, the site-directed mutational studies targeting the tandem CCCH znf motifs revealed their indispensability in the observed phenotype of masculinisation. Additionally, the dual luciferase assays in BmN cells using 5′ UTR region of the *Bmznf-2* strongly implied the existence of a translational repression over this gene. From these findings, we propose *Bmznf-2* to be one of the potential factors of masculinisation similar to *Masc*. From the growing number of *Bmdsx* splicing regulators, we assume that the sex determination cascade of *B. mori* is quite intricate in nature; hence, it has to be further investigated for its comprehensive understanding.

1. Introduction

Sex determination is a fundamental biological process that determines two distinct sexes. A variety of sex determination mechanisms is observed in animal species, most of which follow the chromosomal/genetic sex determination, except in a few cases where the sex is determined by environmental factors like temperature (e.g. crocodiles, alligators and few lizards). Among insects, the mechanism of sex determination is well understood in *Drosophila* and serves as a reference for all insects. In *Drosophila*, (XX is female and XY is male) the sex is determined by the dose of X-linked signalling elements (XSE) (XSE are four transcription factors *Scute*, *SisA*, *Runt* and *Unpaired*) (Erickson and Quintero, 2007), which in turn is determined by the number of X chromosomes. XSE, whose expression threshold can be reached only in female embryos, confines the production of the Sex-lethal (SXL) protein to females. SXL produced in this way directs the female specific splicing of pre-mRNA of *transformer* (*tra*) gene resulting in functional TRA protein. The TRA interacts with non sex specific transformer2 (TRA2) protein and this complex binds to the *doublesex* repeat element (*dsx*RE) in the middle of fourth exon and forces the female specific splicing of *doublesex* (*dsx*) mRNA, producing the female DSX protein. These two proteins have been shown to exhibit antagonistic functions in the process of sexual differentiation (Christiansen et al., 2002). In a few insect species like *Megaselia scalaris* (Traut, 1994), *Ceratitis capitata* (Willhoeft and

* Corresponding author.
** Corresponding author.
E-mail addresses: gnath@cdfd.org.in (G. Gopinath), arun@cdfd.org.in (K.P. Arunkumar).
† Deceased.

http://dx.doi.org/10.1016/j.ibmb.2016.05.008
0965-1748/

Franz, 1996), *Bactotocera tryoni* (Shearman and Frommer, 1998), *Lucilia cuprina* (Bedo and Foster, 1985) and *Chironomus thummi* (Hagele, 1985), an epigenetic male factor from the Y chromosome decides the male development. *Culex tritaeniorhynchus* lacks the sex chromosomes and the maleness is conferred by an autosomal gene (Baker and Sakai, 1976). The sex in *Aedes aegypti* is determined by *Nix* gene from M locus on Y chromosome like region (Hall et al., 2015). In hymenopteran species, the sex is maintained through haplodiploidy, where haploids develop as males and diploids develop as females. In *Nasonia vitripennis*, *transformer* (*Nvtra*) gene plays a crucial role in development of females, where it maintains its concentration by an autoregulatory loop through a maternally supplied TRA protein (van de Zande and Verhulst, 2014; Verhulst et al., 2010).

In lepidopterans (butterflies and moths) ZZ/ZW or ZZ/ZO chromosomal system of sex determination is observed. The heterogametic sex (ZW and ZO) is female and the homogametic sex (ZZ) is male. Sex in the domesticated silkworm, *Bombyx mori* is determined by a feminizing piRNA, *fem* on the W-chromosome (Hashimoto, 1933; Kiuchi et al., 2014). It has been reported that SXL is not regulated in a sex specific fashion in *B. mori* (Niimi et al., 2006). *Tra* orthologue has not been identified so far in *B. mori*, probably owing to its rapid sequence divergence in the course of evolution (Concha and Scott, 2009; Hediger et al., 2010; Kulathinal et al., 2003; O'Neil and Belote, 1992; Ruiz et al., 2007). *Dsx* pre-mRNA has been shown to be lacking TRA/TRA-2 binding sites (Suzuki et al., 2001). Though the orthologues of *tra2*, *intersex* (*ix*) and *fruitless (fru)* genes have been identified in *B. mori*, their functions remained elusive. Previous studies have resulted in the identification of two RNA binding splicing inhibitors: 1) *B. mori* homolog of IGF-II mRNA binding protein (BmIMP) (Suzuki et al., 2010) and 2) *B. mori* homolog of P-element somatic inhibitor (BmPSI) (Suzuki et al., 2008), which are involved in differential splicing of *Bmdsx* pre-mRNA. The involvement of *Bmpsi* and *Bmimp* renders this mechanism to be unique from any other group of insects.

Recently, the mechanism of *B. mori* sex determination was reported to be governed by a piRNA (*fem*) from the W-chromosome. The W-derived *fem* piRNA negatively regulates a Z-linked CCCH type zinc finger gene, *Masculinizer* (*masc*). This has been shown to regulate the *Bmdsx* sex specific splicing by promoting the expression of male specific *Bmdsxm* type of splicing isoform and also dosage compensation by an unknown mechanism. Thus, this gene, *masc* is presumably non-functional in females, leading to female specific *Bmdsxf* type of splicing isoform (Kiuchi et al., 2014). Further studies have shown that the over expression of *masc* gene in BmN cells has enhanced the transcription of *Bmimp* gene and most probably through this the *masc* induces the expression of male specific *Bmdsxm* type of splicing isoform (Sakai et al., 2015). Thus, the reported studies have shown that the sex in *B. mori* is regulated by a W encoded *fem* piRNA that in turn negatively regulates the *masc* gene in females, which is a masculinisation factor in males.

In *B. mori*, studies attempting to discover the genes involved in sex determination pathway have resulted in the identification of a female specific CCCH type znf motif encoding gene, termed as *z1* on W-chromosome and its homologous copies namely z2 and *z3* on 25th chromosome (Ajimura et al., 2006; Satish et al., 2006). Further, the studies of translocation of W-chromosomal fragments to autosomes have supported the existence of a strong putative epistatic female determining region called “feminizer” on the W-chromosome (Hashimoto, 1933; Tazima, 1954). A preliminary analysis using FISH has indicated that probably these *znf* genes are linked to the “feminizer” region of W-chromosome (Ajimura et al., 2006; Satish et al., 2006).

In the current study, we provide functional insights into the role of an autosomal CCCH type *znf* gene, *z2* in the *B. mori* sex determination. For the sake of simplicity and ease of understanding, we refer the gene *z2* as *Bmznf-2* (GenBank accession: XP_004924549) in the entire manuscript. Through transient over-expression of *Bmznf-2* in BmN cells, we have demonstrated that *Bmznf-2* affects the differential splicing of *Bmdsx* and *Bmtra-2* pre-mRNA. Further, we provide a critical evidence (Luciferase assays) for an endogenously existing 5′UTR mediated translational repression over *Bmznf-2* in these cells. With this study, we provide clues regarding the functional aspects of *Bmznf-2* gene in *B. mori* sex determination.

2. Materials and methods

2.1. Plasmid construction

(i) The coding sequence (CDS) of *Bmznf-2* (1083 bp) was PCR amplified with the primers Zn2-F-SacI and Zn2-R-NotI (Please refer Table S1 for primer sequences) using cDNAs synthesized from the fat body of 5th instar male larvae to make sure the amplicons are derived from autosomal *Bmznf-2* gene. The resultant amplicon was cloned into the pIZT-V5 insect glow vector (Invitrogen) using SacI and NotI restriction enzymes, for pIZT-*Bmznf-2* plasmid. The clones were selected on low salt Zeocin LB plates and the sequences were confirmed by OpIE2 F and OpIE2 R sequencing primers. (ii) For the luciferase assay, a modified pmirGLO vector (Promega), which had OpIEI and OpIEII promoters from pIZT vector in places of human PGK and SV40 respectively was used. The 5′UTR regions (with two intact and scrambled putative target sites for the ovarian small RNAs) of *Bmznf-2* were cloned downstream of firefly luciferase CDS using restriction sites XbaI and SacI. The CDS of GFP (600 bp) was used as a negative control in this experiment. (iii) For the localisation study of BmZNF-2 protein, the CDS of m-cherry was cloned inframe downstream of *Bmznf-2* in pIZT-*Bmznf-2* using the restriction sites NotI and XbaI, to get fused protein of BmZNF-2 with m-cherry at C-terminal end. The positive clones were confirmed through sequencing.

2.2. Transfection of cells

BmN cells were cultured and passaged using TC-100 insect medium (SIGMA-ALDRICH) with 10% FBS (Thermo Scientific-Gibco). Transfections were carried out in 12 well plates for ectopic expression and RNAi experiments, and 24 well plates for luciferase reporter assays. Cells were seeded at a concentration of 1×10^5 per well (for 12 well) and 5×10^4 (for 24 well) followed by transfection after 24 h. Transfection mixtures were prepared by mixing plasmid DNA or dsRNA with (200 ng of plasmid for ectopic expression, luciferase assays and one μg of dsRNA for RNAi) 3 μl of transfection reagent TransIT2020 (Mirus Bio) in a final volume of 100 μl using serum free medium per well. This mixture was incubated at room temperature for 20 min for complex formation and distributed dropwise to each well containing cells in a volume of 1 ml (for 12 well plate) and 0.5 ml (for 24 well plate) medium, following manufacturer's instructions.

2.3. RNA isolation and RT-PCR

Total RNA from tissues (~25 mg) was isolated following TriZol (Invitrogen) method. DirectZol kit (Zymo Research) was used for isolation of total RNA from BmN cells. To remove DNA contamination, the total RNA was DNase-I (Zymo Research) treated as per manufacturer's instructions. The RNA concentration of samples was measured using Nanodrop 2000 (Thermo Scientific). cDNAs were synthesized using 1 μg of total RNA, using SuperScript III (Invitrogen). PCR was set using Emerald Amp GT PCR 2X master mix