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In vivo masculinizing function of the *Ostrinia furnacalis Masculinizer* gene

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ABSTRACT

The *Masculinizer* gene (*Masc*) encodes a CCCH tandem zinc finger protein essential for masculinization and dosage compensation in the silkworm *Bombyx mori*. Previously we identified a *Masc* orthologue from the crambid *Ostrinia furnacalis* (*OfMasc*) and observed its masculinizing activity in the *B. mori* cultured cell line BmN-4. However, the role of *OfMasc* in masculinization of *O. furnacalis* has not been assessed. In this study, we unexpectedly discovered that all of the male larvae that escaped from *Wolbachia*-induced embryonic male-killing by *OfMasc* cRNA injection expressed the female-type splicing variants of *O. furnacalis doublesex* (*Ofdsx*). To clarify the role of *OfMasc* in the masculinization process *in vivo*, we established a system to monitor both sex chromosome- and *dsx* splicing-based sexes from a single *O. furnacalis* embryo. Using this system, we investigated the effects of *OfMasc* knockdown in early embryos on *Ofdsx* splicing and found that depletion of *OfMasc* mRNA in male embryos induced the production of the female-type splicing variants of *Ofdsx*. This result indicates that *OfMasc* is required for masculinization in *O. furnacalis*, and that the Masc protein possesses masculinizing activity in an insect species that is phylogenetically distant from Bombycidae.

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1. Introduction

Wolbachia, an endosymbiotic bacterium, infects a wide range of insect species and manipulates their sexes or reproduction [1]. *Wolbachia*-induced phenotypes include parthenogenesis, feminization, cytoplasmic incompatibility, and male-killing [2]. *Wolbachia* infection is known to cause male-killing in the lepidopteran insects *Ostrinia furnacalis* and *O. scapulalis* (Lepidoptera: Crambidae) moths [3–5]. However, the molecular mechanism through which it accomplishes such male-killing in *Ostrinia* moths remains elusive, with the major reason being that the sex determination cascade of lepidopteran insects (including *Ostrinia*) has not been comprehensively understood until recently.

The silkworm *Bombyx mori* (Lepidoptera: Bombycidae) is a model insect used in studies on the sex determination cascade in lepidopteran insects. In *B. mori*, females have WZ sex chromosomes, whereas males have two Z chromosomes. Femaleness of this insect is determined by the presence of the W chromosome irrespective of the number of Z chromosomes, demonstrating that there is a dominant feminizing gene (*Feminizer, Fem*) on the W chromosome [6,7]. In 2014, our team showed that *Fem* is a precursor of a single W chromosome-derived PIWI-interacting RNA (piRNA) [8]. A bioinformatic approach also identified the target of *Fem* piRNA, located on the Z chromosome. RNA interference (RNAi)-mediated knockdown of this Z-linked gene, *Masculinizer* (*Masc*), in male embryos resulted in the production of the female-type splicing variants of *B. mori doublesex* (*Bmdsx*) [8], known to play essential roles in the sex differentiation cascade of *B. mori* [9,10]. Overall, these results indicate that the Masc protein acts as a masculinizing factor in *B. mori*.

During the knockdown experiments of *Masc* mRNA, we observed that depletion of *Masc* mRNA in *B. mori* embryos resulted in male-specific lethality [8]. RNA-seq of *Masc* RNAi embryos showed enhanced expression of Z-linked genes in males, indicating that the Masc protein is required for transcriptional repression of the Z-linked genes in males and that Masc protein-mediated control of dosage compensation at an early embryonic stage is crucial for male development in *B. mori* [8]. We hypothesized that this

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male-specific embryonic lethality is a phenocopy of male-killing by *Wolbachia* observed in *Ostrinia* moths. Using a transcriptomic approach, we revealed that the level of *O. furnacalis Masc* (*OfMasc*) mRNA markedly decreased and dosage compensation of Z-linked genes did not occur in *Wolbachia*-infected *O. furnacalis* embryos [11]. In addition, injection of *OfMasc* complementary RNA (cRNA) synthesized *in vitro* prevented male progeny killing at embryonic stages. These results suggest that *Wolbachia*-induced male-killing is caused by a failure of dosage compensation via repression of the host *Masc* gene [11].

We recently established a cell-based assay system to assess the masculinizing activity of *Masc* cDNAs [12]. We transfected *Masc* cDNAs into the *B. mori* ovary-derived cell line BmN-4 and examined the splicing patterns of *Bmdsx* and expression levels of *B. mori IGF-II mRNA-binding protein* (*BmIMP*), a splicing regulator of *Bmdsx* [13]. Using this system, we investigated the masculinizing activity of the *Masc* derivatives, including *Masc* cDNAs without nuclear localization signals, zinc fingers, or other functional residues [12,14], and other lepidopteran *Masc* cDNAs and observed the masculinizing activity of *OfMasc* [11] and *Trilocha varians Masc* (*TvMasc*) [15] in BmN-4 cells.

Trilocha varians (Lepidoptera: Bombycidae) is an insect closely related to *B. mori* [16]. Using this species, we performed embryonic RNAi of *TvMasc* and showed the *in vivo* masculinizing activity of *TvMasc* [15]. However, we have not yet examined the masculinizing activity of *OfMasc* in *O. furnacalis*. The aim of this study was to establish a system for monitoring sex chromosome- and *dsx* splicing-based sexes from a single *O. furnacalis* embryo and to investigate the *in vivo* masculinizing activity of *OfMasc*.

2. Materials and methods

2.1. Insects

O. furnacalis moths used in this study were collected at Nishi-Tokyo, Japan (35.4° N, 139.3° E), in early summer of 2016 and 2017, whereas *Wolbachia*-infected *O. furnacalis* moth was collected at Matsudo, Japan (35.8° N, 139.9° E), in early summer of 2014. *O. furnacalis* larvae were reared on an artificial diet (Insecta LFS, Nosan Corp.) at 23 °C under a photoperiod of 16L and 8D [11].

2.2. Molecular sexing

Molecular sexing of a single *O. furnacalis* embryo was performed by quantitative PCR (qPCR) using genomic DNA of the Z-linked gene *kettin* and autosomal gene *EF-1α* as previously reported [11,17].

2.3. Reverse transcription-PCR (RT-PCR)

Total RNA and genomic DNA were prepared from a single *O. furnacalis* embryo using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was subjected to RT using avian myeloblastosis virus (AMV) reverse transcriptase with an oligo-dT primer (TaKaRa). PCR was performed with KOD FX-neo DNA polymerase (TOYOBO), and sex-specific splicing of *Ofdsx* was examined by RT-PCR as previously reported [11].

2.4. cRNA and small interfering RNA (siRNA) injection

Injections of cRNA [11] and siRNA [8] were performed as previously described with some modifications. In brief, we injected 1–2 nl of *OfMasc* or *GFP* siRNA solution (100 μM in 100 mM potassium acetate, 2 mM magnesium acetate, 30 mM HEPES-KOH; and pH 7.4) into *O. furnacalis* embryos within 4 h after oviposition. The sequences of the siRNAs were as follows:

siGFP_gui: 5′-AUAGACGUUGUGGCUGUUGUA-3′
siGFP_pas: 5′-CAACAGCCACAACGUCUAUUU-3′
siMasc-1_gui: 5′-ACUUUUGGGAGCUUCCCUGUU-3′
siMasc-1_pas: 5′-CAGGGAAGCUCCCAAAAGUUU-3′
siMasc-2_gui: 5′-AAUGAAUCUAGCGAAUUGCUU-3′
siMasc-2_pas: 5′-GCAAUUCGCUAGAUUCAUUUU-3′

3. Results

3.1. Failure of larval masculinization in Wolbachia-infected O. furnacalis male progenies rescued by injection of OfMasc cRNA

We previously performed rescue experiments by injecting *OfMasc* cRNA synthesized *in vitro* into male-killing *Wolbachia*-infected *O. furnacalis* embryos, and the results showed hatching of male larvae from *OfMasc* cRNA-injected embryos but not from *GFP* cRNA-injected control embryos [11], indicating that a decrease in *OfMasc* mRNA is responsible for the male-killing phenotype of *Wolbachia* and that *OfMasc* is required for dosage compensation in *O. furnacalis* embryos. On the other hand, in this rescue experiment, we did not investigate the transcriptional sex, e.g., *Ofdsx* splicing, in *OfMasc* cRNA-injected, rescued male progenies. In this study, we first performed RT-PCR of *Ofdsx* using cDNA prepared from the hatched larvae injected with *GFP* cRNA or *OfMasc* cRNA. As shown in Fig. 1A, the hatched larvae from *GFP* cRNA-injected embryos, all of which were female, expressed the female-type splicing variants of *Ofdsx*. Although the hatched larvae from *OfMasc* cRNA-injected embryos included female and male, all of them expressed the female-type splicing variants of *Ofdsx* (Fig. 1B), indicating that the larvae with different genetic and transcriptional sexes can survive. Overall, these results do not support a conclusion that *OfMasc* is required for masculinization in *O. furnacalis*, although *OfMasc* overexpression was shown to induce masculinization at the transcriptional level in *B. mori* cultured cells [11].

3.2. Establishment of a system to monitor both genetic and transcriptional sexes using a single embryo of O. furnacalis

To assess the role of *OfMasc* on masculinization *in vivo*, it is

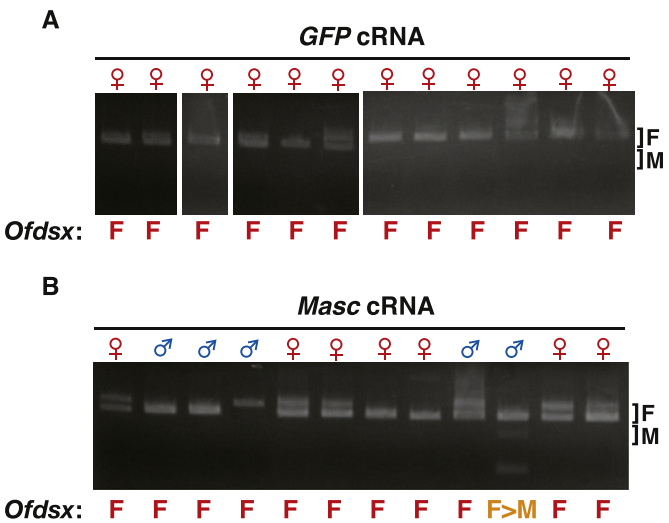

Fig. 1. Splicing pattern of *Ofdsx* in *OfMasc* cRNA-injected *Wolbachia*-infected *Ostrinia furnacalis* larvae. *OfMasc* cRNA was synthesized *in vitro* and injected into *Wolbachia*-infected embryos immediately after oviposition. The hatched larvae were collected and molecularly sexed by qPCR of the *kettin* and *EF-1α* genes. Splicing variants of *Ofdsx* in *GFP* cRNA-treated (A) and *OfMasc* cRNA-treated (B) progenies were amplified by RT-PCR. F and M indicate female- and male-type splicing of *Ofdsx*, respectively.

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