Review

Unique sex determination system in the silkworm, *Bombyx mori*: current status and beyond

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Abstract: The silkworm *Bombyx mori* has been used for silk production for over 5,000 years. In addition to its contribution to sericulture, *B. mori* has played an important role in the field of genetics. Classical genetic studies revealed that a gene(s) with a strong feminizing activity is located on the W chromosome, but this W-linked feminizing gene, called *Feminizer* (*Fem*), had not been cloned despite more than 80 years of study. In 2014, we discovered that *Fem* is a precursor of a single W chromosome-derived PIWI-interacting RNA (piRNA). *Fem*-derived piRNA binds to PIWI protein, and this complex then cleaves the mRNA of the Z-linked *Masculinizer* (*Masc*) gene, which encodes a protein required for both masculinization and dosage compensation. These findings showed that the piRNA-mediated interaction between the two sex chromosomes is the primary signal for the sex determination cascade in *B. mori*. In this review, we summarize the history, current status, and perspective of studies on sex determination and related topics in *B. mori*.

Keywords: sex determination, piRNA, silkworm, dosage compensation, *Wolbachia*

Diversity and conservation in insect sex determination

The mechanisms of sex determination in insects are surprisingly diverse. In dipterans, the signal for sex determination in *Drosophila melanogaster* is the number of X chromosomes, which reflects the dose of X-linked signaling elements.¹⁾ On the other hand, in hymenopteran insects, haploids and diploids become male and female, respectively.²⁾ In lepidopteran insects, the W chromosome is known to possess a strong feminizing factor in the silkworm *Bombyx mori*.^{3),4)} Recent progress in molecular biological techniques, such as deep sequencing by next-generation sequencers and genome editing methods, has enabled the identification of the primary genes

involved in sex determination cascades in various insects, including a small RNA,⁵⁾ a small protein (56 amino acid-long peptide),⁶⁾ and an RNA-binding protein.⁷⁾ These discoveries revealed that the primary factors for insect sex determination cascades are also diverse. However, in contrast to primary factors, the gene acting at the downstream end for sex differentiation is common between insects. This gene was first identified in *D. melanogaster* and is called as *doublesex* (*dsx*).⁸⁾ *dsx* is sex-specifically spliced and translated into sex-specific DSX proteins. DSX is a transcription factor that potentially binds to thousands of genome loci, leading to sex-specific gene expression and subsequent sexual differentiation.⁹⁾

The silkworm *Bombyx mori*: not only an industrial insect but also a model animal in genetic studies

Bombyx mori (“KAIKO” in Japanese; Fig. 1) is the only completely domesticated insect, which has been used for silk production for approximately 5,000 years.¹⁰⁾ In addition to its industrial use, *B. mori* had been a model insect in genetic studies, until the fruit fly, *D. melanogaster* was established as a model organism. For example, *B. mori* was the first animal

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Fig. 1. Photographs of *B. mori*. Photographs of (A) 5th instar larvae; (B) cocoons; (C) pupae; and (D) adult moths of *B. mori*. In all panels, the female is on the left, and the male is on the right. Note that this is a sex-limited strain possessing larval marks only in females (A).

through studies of which Mendelian laws were rediscovered. In 1906, Kametaro Toyama reported the Mendelian inheritance of the cocoon color of *B. mori*.¹¹⁾ In addition, Yoshimaro Tanaka discovered in 1916 that *B. mori* females have a W chromosome,³⁾ and Haruo Hasimoto reported in 1933 that one copy of the W chromosome is sufficient for determining femaleness, regardless of the copy number of Z chromosomes.⁴⁾ Thus, in the early 1900s, Japanese silkworm researchers made many epoch-making discoveries in the field of genetics.

History of studies on *B. mori* sex determination

The WZ sex determination system is found in a diverse range of animals, such as birds, reptiles, and lepidopteran insects. As mentioned above, Yoshimaro Tanaka discovered that *B. mori* females are heterogametic (WZ), whereas *B. mori* males are homogametic (ZZ).³⁾ In addition, Haruo Hasimoto reported that the W chromosome determines the femaleness of *B. mori* irrespective of the number of Z chromosomes.⁴⁾ After these major discoveries, however, no considerable developments were made in research on silkworm sex determination for about 70 years. In 2001, Toru Shimada *et al.* identified the *B. mori* homolog of *Drosophila dsx* from the database of the expressed sequence tags of *B. mori* and found that *B. mori dsx* (*Bmdsx*) is sex-specifically spliced as observed in *Drosophila dsx*.¹²⁾ This was the first discovery of a *dsx* homolog in an insect outside the diptera. Transgenic approaches revealed that BmDSX acts at the downstream end of the sex differentiation cascade in *B. mori*.^{13),14)} In addition, Hiroaki Abe *et al.* developed W chromosome-specific PCR markers and sequenced fragments of the W chromosome. They found that the W chromosome of *B. mori* is almost fully occupied by complete or incomplete units of transposable and repeat elements.¹⁵⁾ Furthermore, Ken Sahara *et al.* developed a method for detection of the W chromosome of *B. mori* by fluorescence *in situ* hybridization (FISH) with bacterial artificial chromosome (BAC) probes.¹⁶⁾ Furthermore, Masataka Suzuki *et al.* generated male and female cultured cell lines from embryos of a sex-limited black egg strain. Using these cell lines, they identified two factors, *Bombyx* homolog of IGF-II mRNA binding protein (*BmIMP*) and *Bombyx* homolog of P-element somatic inhibitor (*BmPSI*), both of which are involved in *Bmdsx* splicing.^{17),18)} Having made these discoveries, Japanese researchers have played central roles in the progress of research in the field of sex determination in *B. mori*. However, despite the huge amount of trials conducted by entomologists, geneticists, and molecular biologists all over the world, a putative *B. mori* feminizer *Fem* located on the W chromosome was not identified until 2014.

Major obstacles to the discovery of the *B. mori* feminizing factor

We experienced three big obstacles to the discovery of the feminizing factor of *B. mori*. First, in this species, crossing-over events do not occur in

females. Therefore, it was impossible to narrow down the *Fem*-linked genomic region by conventional DNA marker-based positional cloning strategies. Second, the W chromosome of *B. mori* is almost fully occupied by nested transposable and repeat elements,¹⁵⁾ which prevented the construction of long accurate sequence scaffolds for this chromosome. Furthermore, the lack of accurate nucleotide sequences of the W chromosome was fatal to the identification of *Fem* by nucleotide sequence-based approaches. Third, no established method existed to distinguish the sexes of *B. mori* embryos either visually or molecularly. Because *B. mori* sex determination was believed to be established at an early stage of embryogenesis, we had to prepare the sexed RNAs to search *Fem* candidates by transcriptome-based strategies.

Our strategy to identify the *B. mori* feminizing factor

In contrast to the Z chromosome, the W chromosome of *B. mori* is almost completely occupied by selfish repetitive elements,^{15),19)} most of which are considered to be precursors of PIWI-interacting RNAs (piRNAs). piRNAs are small RNAs whose length ranges from 23 to 30 nucleotides. They potentially act as sequence-specific guides for PIWI proteins that cleave target transposon-derived RNAs, resulting in repression of transposon activity.^{20),21)} Considering the transposon-dense nature of the W chromosome, we hypothesized that W chromosome-derived, female-specific or female-enriched piRNAs globally regulate the expression of the genes required for sexual differentiation in *B. mori*. To examine this possibility, we generated and characterized piRNA libraries prepared from pupal ovary and testis of wild-type and three *B. mori* strains with a unique truncated W chromosomes. We found that female-enriched piRNAs were produced from transposons or repetitive sequences that were located within the sex-determining region of the W chromosome.²²⁾ However, at that time, we did not know which piRNAs are actually involved in sex determination and how they function in the sex determination cascade.

Transcriptome profiling with molecularly sexed RNAs from *B. mori* early embryos

To identify the genes or non-coding RNAs (*i.e.*, piRNA precursors) involved in *B. mori* sex determination, it was necessary to establish a method to obtain molecularly sexed RNA from early embryos whose sexes were visually indistinguishable. First,

Fig. 2. Strategy for the identification of *B. mori* Feminizer. (A) Method for preparation of sexed RNA from a single egg. Total RNA and genomic DNA were prepared using a commercially available phenol/guanidine isothiocyanate reagent. Genomic DNA was utilized for molecular sexing, and total RNA of sexed individuals was subjected to RT-PCR and RNA-seq. (B) Determination of embryonic stages for transcriptome analysis. The stages at which a male-type *Bmdsx* appeared were examined by RT-PCR. The male-specific splice variants were clearly detected in male embryos at 21 hpo. (C) MA-plot of transcriptome data at 24 hpo. The *Fem* contig is indicated by the red circle. The axes indicate the following information: A (x -axis) = $(\log_2(\text{transcripts per million in male}) + \log_2(\text{transcripts per million in female}))/2$. M (y -axis) = $\log_2(\text{transcripts per million in male}) - \log_2(\text{transcripts per million in female})$.

we managed to simultaneously prepare total RNA and genomic DNA from a single embryo using a commercially available phenol/guanidine isothiocyanate reagent (Fig. 2A). Using the genomic DNA, we established a method to molecularly sex the individual embryo using three W chromosome-specific PCR markers. Next, we examined the splicing pattern of *Bmdsx* using total RNA of molecularly sexed embryos and found that female-

type splice variants of *Bmdsx* are the default transcripts during the early stages of development, and male-type splice variants appeared in male embryos at 21 h post-oviposition (hpo) (Fig. 2B). These results indicated that the feminizing factor is transcribed from the W chromosome before 21 hpo. Therefore, we performed deep sequencing of RNAs (RNA-seq) using molecularly sexed RNAs from embryos before and after 21 hpo (Fig. 2B), and identified a 767 base-long transcript that was expressed only in female embryos at any of the time points examined (Fig. 2C).

Identification of *Feminizer*, which produces a W chromosome-derived female-specific piRNA

Genomic PCR and RT-PCR experiments revealed that this female-specific transcript is a repetitive sequence on the W chromosome.⁵⁾ We measured the copy number of this sequence using genomic DNA from three *B. mori* strains (N4, C108T, and p50T) and *B. mandarina*, a putative ancestor insect of *B. mori*. Quantitative PCR experiments revealed that the copy number of this repeat was more than 150 in all strains examined (Fig. 3A). Next, to determine the location of this repeat on the W chromosome, we performed FISH using a plasmid containing the 767 base pair-long fragment (approx. 3.6 kilobase pairs in total) as a probe. We routinely use BAC or fosmid clones as a probe to obtain a clear signal in FISH, but in this case, we obtained an exceedingly clear signals on the W chromosome using a short DNA probe, strongly suggesting that this fragment is highly accumulated at a specific locus on the W chromosome (Fig. 3B).

The nucleotide sequence of this transcript showed no homology to any nucleotide sequences deposited in public databases. However, we noticed that the transcript was a precursor that produces a huge amount of a single 29-base-long female-specific piRNAs, when we applied this sequence to our in-house piRNA database (SilkBase, <http://silkbases.ab.a.u-tokyo.ac.jp>) (Fig. 4A). This piRNA was detected in piRNA libraries from *B. mori* ovaries, embryos, and the ovary-derived cell line BmN-4, but not in libraries from testes. To elucidate the function of this piRNA, we designed an inhibitor RNA and examined its efficacy using BmN-4 cells, which possess a complete piRNA biogenesis pathway²³⁾ and do not express male-type *Bmdsx* variants (control in Fig. 4B).^{5),24)} The transfection of the inhibitor RNA, however, resulted in the production of the male-type splice variant of *Bmdsx* (inhibitor in Fig. 4B),

Fig. 3. *Fem* is a repeat sequence located on the sex-determining region of the W chromosome. (A) The copy number of *Fem*, determined by qPCR using genomic DNA of *B. mori* N4, C108T, p50T, and *B. mandarina*. (B) Left: FISH identification of W chromosome (a green-signal painted chromatid) and *Fem* (yellow signals on the W: yellow arrow). Bar, 10 μ m. Right: A drawing of *Fem* cluster on the W chromosome.

indicating that this inhibitor functions properly, and the inhibition of this function of the piRNA leads to masculinization in ovarian cells. To determine the *in vivo* function of this piRNA, we injected the inhibitor RNA into early embryos and examined *Bmdsx* splicing. We found that the inhibition of this piRNA-mediated cascade resulted in the production of male-type *Bmdsx* variants in female embryos (Fig. 4C), indicating that this piRNA is the long-sought feminizing factor of *B. mori*. We renamed the 767 base-long piRNA precursor *Feminizer* (*Fem*).

piRNAs potentially act as sequence-specific guides for PIWI proteins that cleave target RNAs, such as transposable and repetitive RNAs. We next searched for targets of the *Fem* piRNA using a BLAST search with the genome information of male *B. mori* and identified one genomic locus that had significant homology with the *Fem* piRNA (see Fig. 5A). We experimentally confirmed the cleavage of this target mRNA at the predicted site in early embryos.⁵⁾ Surprisingly, this mRNA was not transcribed from the repeat sequence but from the protein-coding sequence of the Z chromosome. We named this novel gene as *Masculinizer* (*Masc*).⁵⁾

Fig. 4. *Fem* and *Masc* are ping-pong partners functioning at the starting point of the *B. mori* sex determination cascade. (A) Identification of *Fem* piRNA. piRNA mapping was performed using embryonic piRNAs at 24 hpo. (B) Effect of the inhibitor RNA in *Bmdsx* splicing in BmN-4 cells derived from ovary. BmN-4 cells were transfected with the inhibitor RNA or control RNA, and the splicing patterns of *Bmdsx* were examined by RT-PCR. The F and M indicate female- and male-type splicing of *Bmdsx*, respectively. (C) Effect of the inhibitor RNA in *Bmdsx* splicing in early embryos. Early embryos were injected with the inhibitor RNA or control RNA, and the *Bmdsx* splicing patterns were examined at 72 h post-injection. (D) Identification of *Masc* piRNA. piRNA mapping was performed using embryonic piRNAs at 24 hpo. (E) The ping-pong model between *Fem*- and *Masc*-derived piRNAs.

piRNAs are known to be produced by a unique biogenesis pathway called the “ping-pong” cycle. This pathway requires the involvement of two different PIWI proteins, Siwi and BmAgo3 in *B. mori* (Fig. 4E).^{23,25} If *Fem* piRNA is generated by this system, *Masc*-derived piRNA should be produced from the *Masc* mRNA in female cells. Mapping of embryonic piRNAs to the *Masc* sequence revealed that the *Masc* mRNA produced a single piRNA species in embryos (Fig. 4D). Immunoprecipitation experiments also showed that Siwi and BmAgo3 preferably bind to *Fem* piRNA and *Masc* piRNA, respectively.⁵ Based on these results and observa-

tions, we concluded that *Fem*- and *Masc*-derived piRNAs are produced by the cleavage involving the *Masc* piRNA-BmAgo3 and *Fem* piRNA-Siwi complexes, respectively, which function at the starting point of the *B. mori* sex determination cascade (Fig. 4E).

Masc protein is required for both masculinization and dosage compensation

The target of *Fem* piRNA, *Masc*, encodes a CCCH-tandem zinc finger protein that is conserved in lepidopteran insects (Fig. 5A).⁵ Transfection of *Masc-GFP* fusion cDNA into BmN-4 cells revealed

Fig. 5. Masc is a masculinizing factor of *B. mori*. (A) Structure of the Masc protein. The locations of the two zinc fingers, NLS, and two conserved cysteines are indicated. Sequence of *Masc-R* mRNA and cleavage site by *Fem* piRNA-Siwi complex are also displayed. (B) Intracellular localization of the Masc protein. BmN-4 cells transfected with expression plasmids containing *GFP* or *Masc-R-GFP* were photographed at 72 h after the transfection. (C) Effect of the depletion of *Masc* mRNA on *Bmdsx* splicing in early embryos. The *Bmdsx* splicing pattern of embryos injected with siRNAs for *GFP* or *Masc* was determined at 72 h post-injection. (D) Effect of the overexpression of *Masc* on *Bmdsx* splicing in BmN-4 cells originating from the ovary. The *Bmdsx* splicing pattern of *Masc* cDNA-transfected BmN-4 cells was examined. MR-CS is a *Masc-R* derivative (*Masc-R-C301S*) expressing a Masc protein with a substitution of Cys301 to serine. M1 is a male cell line that originated from a male embryo of a sex-limited black egg strain and was used as a control. (E) Effect of the overexpression of *Masc* on *BmIMP^M* expression in BmN-4 cells. The expression of *BmIMP^M* in *Masc* cDNA-transfected BmN-4 cells was examined by RT-qPCR. (F) Effect of *Masc-R* overexpression on egg maturation. Pupal ovaries of the wild-type and a transgenic strain overexpressing *Masc-R* in fat body cells were compared. Bar, 2 mm.

that Masc is a nuclear protein (Fig. 5B).²⁶⁾ Motif prediction and mutagenesis experiments identified a bipartite nuclear localization signal (NLS) located between residues 274 and 290 of the Masc protein (Fig. 5A).²⁷⁾ To establish the function of *Masc* in sex determination, we performed RNA interference (RNAi) experiments using *B. mori* early embryos and successfully achieved the depletion of *Masc* mRNA. We found that all of the *Masc*-knocked down embryos exclusively expressed female-type splice variants of *Bmdsx*, even though they were male (Fig. 5C).⁵⁾ In addition, the transfection of *Fem* piRNA-resistant *Masc* (*Masc-R*) cDNA, which possesses five nucleotide substitutions at the *Fem* piRNA-Siwi cleavage site but translates the same amino acid sequence as the Masc protein (Fig. 5A), resulted in the production of the male-type *Bmdsx* in BmN-4 cells (Fig. 5D).^{5),26)} These results clearly showed that Masc is the masculinizing factor of *B. mori*.

To identify the amino acid residues of the Masc protein required for the masculinizing activity, we constructed a series of *Masc* cDNA derivatives and transfected them into BmN-4 cells in order to examine *Bmdsx* splicing patterns and male-specific *BmIMP* (*BmIMP^M*) expression. We discovered that the two zinc finger domains (Fig. 5A) are dispensable for the masculinizing activity.²⁶⁾ We also revealed that the degree of nuclear localization is not associated with the masculinizing activity of the Masc protein.²⁷⁾ Furthermore, we identified two cysteines, Cys301 and Cys304, as the critical residues for the masculinizing activity (Fig. 5D, E), both of which are completely conserved in lepidopteran Masc proteins (Fig. 5A).²⁶⁾

To examine the role of Masc protein at the organism level, we generated transgenic *B. mori* strains expressing *Masc-R* cDNA. We found that a strain weakly expressing *Masc-R* exhibited a partial female-to-male transition, *i.e.*, abnormal ovaries with testis-like tissues containing sperm bundles.²⁸⁾ Another strain, in which *Masc* was abundantly transcribed in fat body cells, showed markedly reduced egg production in females, presumably because of a decrease in vitellogenin (egg yolk precursor protein) expression by *Masc-R* expression in fat body tissues (Fig. 5F). These results indicated that Masc is essential to the masculinization process in *B. mori*.

Furthermore, we found an intriguing phenomenon that the depletion of *Masc* mRNA resulted in male-specific embryonic death (Fig. 6A). To reveal

the reason for this male-specific lethality, we performed RNA-seq of *Masc*-knocked down embryos and found that most of the Z-linked genes, but not autosomal genes, were abnormally enhanced in *Masc*-knocked down male embryos (Fig. 6B). This clearly showed that the Masc protein is required for the repression of Z-linked genes, *i.e.*, dosage compensation in *B. mori* embryos, and that the failure of this dosage compensation may cause male-specific embryonic death. The existence of dosage compensation in lepidopteran insects has been controversial because conflicting experimental results were reported in several lepidopteran insects.^{19),29)–35)} Therefore, our discovery was the first report to provide experimental evidence on the existence and importance of dosage compensation in lepidopteran insects, at least in the embryonic stage, and to identify the key factor essential for dosage compensation. In addition, we found that a transgenic *B. mori* strain ubiquitously expressing *Masc-R* exhibited a female-killing phenotype.²⁸⁾ A similar phenotype was also observed in ovary-derived BmN-4 cells transfected with *Masc-R* cDNA (Fig. 6C). These results suggested that the strong repression of Z-linked genes by *Masc-R* overexpression is toxic and results in cell death in females, but visible phenotypic defects are not induced in males. The expression of the *Masc-R* derivative protein with a single amino acid substitution at Cys301 did not affect the cell viability of BmN-4 cells (Fig. 6C), suggesting that Cys301 is an essential residue not only for masculinization but also for dosage compensation of the Masc protein.

How does *Wolbachia* establish the male-killing trait in moths?

Parasites have been shown to manipulate the behavior, reproduction, and development of their host insects for their own benefit. For example, a baculovirus is known to control the behavior of host caterpillars to spread progeny viruses in nature.^{36)–38)} On the other hand, *Wolbachia*, an endosymbiotic bacterium that infects a wide range of insect species, is an example of a parasite that manipulates the sex of its host's progeny.³⁹⁾ Male-killing is one of the phenotypes that *Wolbachia* induces in host insects. Studies on *Wolbachia*-induced male-killing have been recently conducted mainly using *Ostrinia* moths.^{40)–42)} As described above, we observed that RNAi-mediated artificial depletion of *Masc* mRNA results in male-specific embryonic lethality in *B. mori*. We speculated that this phenomenon is the phenocopy of *Wolbachia*-induced male-killing in *Ostrinia*.

Fig. 6. *Masc* is required for dosage compensation in *B. mori*. (A) Sexing of newly hatched larvae injected with *GFP* or *Masc* siRNA. Red and blue portions represent the respective frequencies of females and males. Molecular sexing was performed by PCR of *Fem*. (B) Change of Z-linked (Z, blue) and autosomal (A, red) gene expression levels in *Masc* mRNA-depleted embryos. The data are illustrated by box-and-whisker diagrams. The boxes represent the median and 25–75 percentile ranges of the expression ratios. (C) Phenotypes of ovary-derived BmN-4 cells transfected with *Masc* cDNA derivatives. BmN-4 cells were transfected with an empty plasmid (vector), *Masc-R*, or *Masc-R-C301S* cDNA, and treated with Zeocin 3 days after transfection. Photographs were taken at 13 days after transfection.

To test this hypothesis, we first attempted to collect *Ostrinia* moths infected with male-killing *Wolbachia* in the field around Tokyo area and obtained one *Ostrinia* female moth (Fig. 7A) that was *Wolbachia*-positive and produced only female progeny when crossed with *Wolbachia*-uninfected male moths (Fig. 7B). We then examined the expression level of *Masc* mRNA in early embryos and found a marked decrease in *Masc* mRNA in *Wolbachia*-infected *Ostrinia* embryos.⁴³⁾ We also observed a failure of the dosage compensation in *Wolbachia*-infected embryos. The male-specific embryonic death was rescued by injecting *in vitro*-transcribed *Masc* cRNA.⁴³⁾ These results indicated that *Masc* is the *Wolbachia*'s target for male-killing in lepidopteran insects, and thus a failure of dosage compensation by depleting *Masc* mRNA results in male-killing during embryogenesis (Fig. 7C).

Conclusion and perspective

In this review, we summarized the history and recent progress of research on *B. mori* sex determination. We discovered *Fem*, the precursor of a W chromosome-derived female-specific piRNA, as the feminizing factor of *B. mori*, and *Masc*, the target of *Fem* piRNA, as the factor that is essential for both masculinization and dosage compensation (Fig. 8). Further studies are required to elucidate whether a piRNA-mediated sex determination cascade also operates in other lepidopteran insects with a WZ/ZZ sex chromosome constitution. In addition, we are interested in the mechanism of sex determination in lepidopteran species that have a Z/ZZ (female/male) sex chromosome system. Such insects do not possess female-specific piRNAs, because of the absence of the W chromosome, suggesting that the amount of

Fig. 7. *Wolbachia* targets the *Masc* orthologue to establish male-killing in *Ostrinia* moths. (A) *Ostrinia* female moth infected with male-killing *Wolbachia*. (B) Brood sex ratios in a *Wolbachia*-infected matriline through five generations. (C) A proposed model for *Wolbachia*-induced male-killing in *Ostrinia*. In uninfected male *Ostrinia*, the expression of *Masc* protein is sufficient for dosage compensation and masculinization during male development (left panel). In *Wolbachia*-infected male *Ostrinia*, *Wolbachia* infection reduces the *Masc* mRNA level in early embryos, resulting in failure of dosage compensation and masculinization (right panel).

Fig. 8. The sex of *B. mori* is determined by the piRNA pathway. The piRNA-mediated pathway plays a critical role in the sex determination pathway of *B. mori*. *Fem* RNAs are transcribed from the sex-determination regions and are cleaved by the maternally transmitted *Masc* piRNA-BmAgo3 complex. The *Fem* piRNA-Siwi complex cleaves *Masc* mRNA, resulting in the accumulation of *Masc* piRNA and feminization.

Z-linked *Masc* mRNA should be determined by the number of Z chromosomes. We are now in the process of obtaining experimental evidence that supports the Z-counting hypothesis using *Samia* species.

At present, it remains unknown how the Masc protein transmits its signal to male-specific *Bmdsx* splicing. The Masc protein possesses two CCCH zinc fingers that presumably bind to nucleic acids, *i.e.*, DNA or RNA. However, surprisingly, we showed that both of the two CCCH zinc fingers are dispensable for the masculinizing activity of the Masc protein.²⁶⁾ Instead, we found that two conserved cysteine residues are required for the masculinizing activity.²⁶⁾ It is necessary to investigate why these two cysteines are essential for the masculinizing activity. In addition, we still have not revealed how Masc protein establishes dosage compensation, *i.e.*, the means to repress the expression of Z-linked genes, but not that of autosomal genes. It is of substantial interest whether a common pathway mediates both of these different functions of the Masc protein or not.

Our findings on the *B. mori* sex determination cascade provided an important hint regarding *Wolbachia*-induced male-killing in lepidopteran insects. Based on the results of embryonic RNAi of *B. mori* Masc mRNA, we identified the *Ostrinia* Masc ortholog as the target of *Wolbachia* for male-killing in *Ostrinia* moths (Fig. 7C).⁴³⁾ Our experiments revealed that *Wolbachia* infection reduces Masc mRNA at early embryonic stages, leading to male-specific embryonic death by a failure of dosage compensation.⁴³⁾ These results indicated that the genome of the male-killing *Wolbachia* encodes a factor(s) that negatively controls the level of host Masc mRNA. The identification of this factor will shed light on how *Wolbachia* has evolved to hijack the Masc-dependent, lepidopteran insect-specific sex determination cascade in the history of *Wolbachia*-host coevolution.

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Profile

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