Guardian small RNAs and sex determination

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The W chromosome of the silkworm *Bombyx mori* has been known to determine femaleness for more than 80 years. However, the feminizing gene has not been molecularly identified, because the *B. mori* W chromosome is almost fully occupied by a large number of transposable elements. The W chromosome-derived feminizing factor of *B. mori* was recently shown to be a female-specific PIWI-interacting RNA (piRNA). piRNAs are small RNAs that potentially repress invading "non-self" elements (e.g., transposons and virus-like elements) by associating with PIWI proteins. Our results revealed that female-specific piRNA precursors, which we named *Fem*, are transcribed from the sex-determining region of the W chromosome at the early embryonic stage and are processed into a single mature piRNA (*Fem* piRNA). *Fem* piRNA forms a complex with Siwi (silkworm Piwi), which cleaves a protein-coding mRNA transcribed from the Z chromosome. RNA interference of this Z-linked gene, which we named *Masc*, revealed that this gene encodes a protein required for masculinization and dosage compensation. *Fem* and *Masc* both participate in the ping-pong cycle of the piRNA amplification loop by associating with the 2 *B. mori* PIWI proteins Siwi and BmAgo3 (silkworm Ago3), respectively, indicating that the piRNA-mediated interaction between the 2 sex chromosomes is the primary signal for the *B. mori* sex determination cascade. *Fem* is a non-transposable repetitive sequence on the W chromosome, whereas *Masc* is a single-copy protein-coding gene. It is of great interest how the piRNA system recognizes "self "*Masc* mRNA as "non-self" RNA.

The ZW sex determination system is found in a diverse range of animals including moths. In the mulberry silkworm *Bombyx mori*, females are heterogametic (ZW), while males are homogametic (ZZ).[1,2] Classical genetic studies have revealed that *B. mori* sex is dominantly determined by the presence of the W chromosome.[1,2] One copy of the W chromosome is sufficient for determining femaleness, regardless of the copy number of the Z chromosome.[1,2] This observation originally led to the simple assumption that the W chromosome encodes a dominant feminizing gene. However, neither the female-determining gene nor its candidate was isolated until 2014. There are 2 major reasons for this insuperability. First, a conventional positional cloning strategy is not applicable to this species because of the lack of crossing-over events in females. Second, the *B. mori* W chromosome is almost fully occupied by nested transposable and repeat elements,[3,4] which preventing construction of long accurate sequence scaffolds of this chromosome.

Controlling transposable elements in germ line cells is essential for the host genome to ensure a precise transmission of parental genomic information to the next generation.[5,6] Animals have evolved an excellent defense system to combat invading non-self elements lying concealed in their own genomes. At the core of this immune system are PIWI proteins and associated PIWI-interacting RNAs (piRNAs). piRNAs are small RNAs that range 23–30 nucleotides (nts) in length.[5,6] piRNAs potentially act as sequence-specific guides for PIWI proteins that cleave target RNAs.[5,6] The biogenesis of piRNAs is Dicer-independent and initiates with fragmentation of long single-stranded piRNA precursors by the endonuclease MitoPLD/Zucchini.[7-9] Fragmented RNAs that are longer than mature piRNAs are incorporated into a subset of PIWI
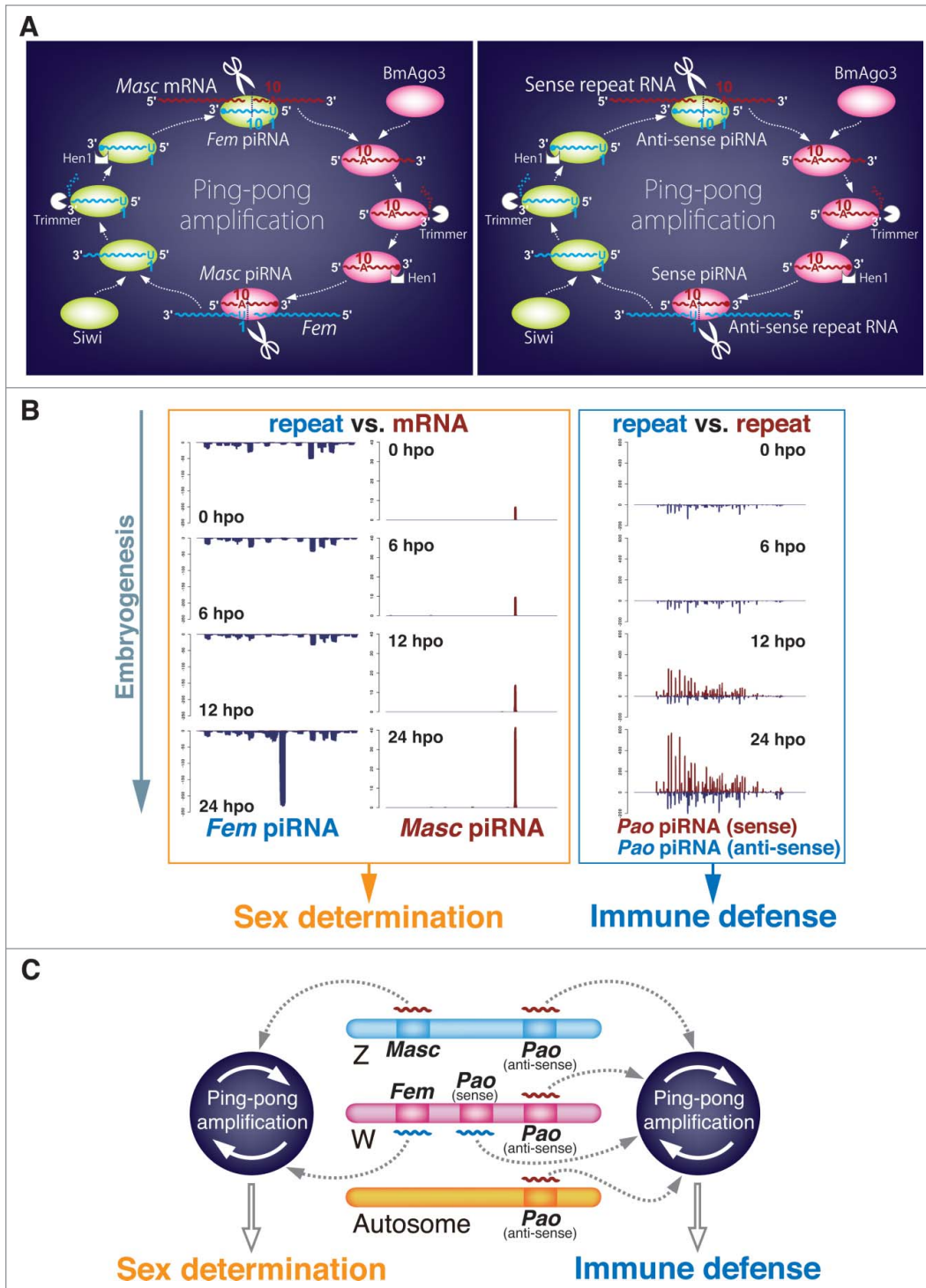
Figure 1 The *B. mori* sex is determined by the piRNA pathway. (**A**) Models of ping-pong amplification cycles of piRNAs in *B. mori* embryos. The left panel shows a ping-pong loop involving *Fem* and *Masc* piRNAs, which controls the sex determination pathway. The right panel shows a canonical ping-pong cycle, resulting in transposon silencing. (**B**) Zygotic amplification of repeat RNA- and protein-coding mRNA-derived piRNAs by maternally transmitted antisense piRNAs. Density plots of *Fem*-, *Masc*-, and *Pao*-derived piRNAs are shown at 0, 6, 12, and 24 hours post-oviposition (hpo), respectively. *Pao* was used as a representative transposable element. (**C**) Two different roles of the piRNA pathway in *B. mori* embryogenesis. In *B. mori* embryos, the piRNA system plays critical roles not only in immune defense against active transposable elements but also in the sex determination pathway. Full-length sense transposons, which are piRNA precursors, are transcribed frequently from the W chromosome.[20] They are silenced by Siwi, which is complexed with antisense piRNAs derived from truncated transposable elements located on each chromosome. In the sex determination pathway, *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.

proteins, Siwi in *B. mori* (**Fig. 1A**),[10,11] with a specific nucleotide preference for uracil (1U) at the 5′ end of loaded RNAs.[5,6,12-16] The 3′ ends of Piwi-associated RNAs are further trimmed to the mature length by an unidentified 3′ to 5′ exonuclease that we named Trimmer,[16] followed by 2′-*O*-methylation catalyzed by the methyltransferase Hen1.[17-19] This 1U piRNA is called primary piRNA. The Piwi-primary piRNA complexes cleave their targets from positions 10 and 11 of the guide piRNAs. The 3′ fragments of the cleavage products are incorporated into another subset of PIWI proteins, BmAgo3 in *B. mori* (**Fig. 1A**),[10,11] which do not show a first nucleotide bias. This Ago3-associated RNA is processed into mature secondary piRNAs with adenine at position 10 (10A). The secondary piRNAs precisely overlap with primary 1U piRNAs by 10 nts.[5,6,12-15] The secondary piRNA-Ago3 complexes, in turn, digest their complementary targets to generate secondary 1U piRNAs. This piRNA biogenesis pathway is called a ping-pong amplification cycle, and the 10-nt overlap is called a ping-pong signature[5,6] (see **Fig. 1A**). In animals, Piwi-bound 1U piRNAs are usually antisense to repetitive sequences, whereas secondary 10A piRNAs associated with Ago3 are identical to the sense strands of repetitive sequences. In other words, piRNA production occurs concomitantly with the repression of transposable elements in this ping-pong amplification loop.

The *B. mori* W chromosome but not the Z chromosome is almost completely occupied by selfish repetitive elements, most of which are considered piRNA precursors.[20] To determine if the difference in *B. mori* sex chromosome constitution affects piRNA profiles, we analyzed piRNA libraries prepared from pupal ovary and testis of wild-type and 3 *B. mori* strains with a unique truncated W chromosome.[20] We found that the sex-determining region of the W chromosome produces female-enriched piRNAs that originate from transposons or repetitive sequences;[20] however their biological functions remain to be elucidated. In 2014, we performed transcriptome analysis by deep sequencing (RNA-seq) using male and female RNAs from *B. mori* early embryos and discovered a single female-specific transcript. This transcript was a

non-transposable repetitive sequence expressed from the sex-determining region of the W chromosome and was a precursor of a single female-specific piRNA.[21] Injecting an RNA inhibitor for this piRNA into female embryos resulted in the production of the male-type splicing isoform of *B. mori doublesex* (*Bmdsx*), which acts at the downstream end of the sex differentiation cascade in *B. mori*.[22,23] These results demonstrated that this piRNA is required for the female-type splicing of *Bmdsx*, and we named the precursor of this piRNA as *Feminizer* (*Fem*).

To understand how *Fem* piRNA transmits the feminizing signal to downstream cascades in female embryos, we searched for the targets of *Fem* piRNA by bioinformatic analyses. We identified only one genomic locus where the *Fem* piRNA sequence was extensively complementary (26/29, 5′ 14 nts are perfectly matched). This locus was present in the coding region of a CCCH-tandem zinc finger gene located on the Z chromosome. We named this gene *Masculinizer* (*Masc*).[21] Injection of small interfering RNA (siRNA) of *Masc* into male embryos led to the production of the female-type *Bmdsx* transcripts, indicating that *Masc* is essential for silkworm masculinization. We also observed that the embryonic knockdown of *Masc* mRNA results in male-specific lethality. RNA-seq of *Masc* siRNA-injected embryos revealed that the Masc protein globally represses gene expression from the male Z chromosome at the embryonic stage, suggesting that a failure of dosage compensation on the Z chromosome possibly results in male-specific lethality in *Masc* mRNA-depleted male embryos. Taken together, the Masc protein controls both dosage compensation and masculinization.[21]

piRNA biogenesis occurs via a ping-pong mechanism, which is achieved by 2 different PIWI proteins, Siwi and BmAgo3, in *B. mori* (**Fig. 1A**).[10,11] Bioinformatic analysis revealed that the *Masc* mRNA-derived piRNA (*Masc* piRNA) is a ping-pong partner of *Fem* piRNA (**Fig. 1A**).[21] Deep sequencing of the immunoprecipitated piRNA libraries showed that *Fem* piRNA preferentially binds to Siwi, whereas *Masc* piRNA preferentially binds to BmAgo3 (**Fig. 1A**).[21]

Furthermore, a modified RNA ligase-mediated 5′ rapid amplification of cDNA ends (RACE) experiment indicated *Fem* piRNA-mediated cleavage of *Masc* mRNA.[21] These findings indicate a ping-pong amplification model for *Fem* and *Masc* piRNAs (**Fig. 1A**). *Fem* piRNA is *de novo* produced during *B. mori* embryogenesis, whereas *Masc* piRNA is maternally transmitted at moderate levels (**Fig. 1B**). Western blot analysis of egg lysates revealed that BmAgo3 is also maternally transmitted,[24] suggesting that the *Masc* piRNA-BmAgo3 complex is maternally inherited and exists in newly laid eggs, which cleaves zygotically synthesized *Fem* transcripts to generate *Fem* piRNA in female embryos. The resulting *Fem* piRNA then cleaves *Masc* mRNA by associating with Siwi (**Fig. 1A**), inhibiting masculinization of female embryos.[21]

The piRNA pathway is an evolutionarily conserved system that is found in a diverse range of organisms. Although this pathway was originally considered a limited system for protecting the host genome against non-self elements, it was recently revealed to be utilized in other biological roles. Rajasethupathy et al. reported that piRNAs are abundantly expressed in *Aplysia* brain, and some are markedly induced by serotonin, an important neuromodulator for learning and memory.[25] Further experiments revealed that the Piwi-piRNA complex mediates serotonin-dependent CpG methylation and transcriptional silencing of CREB2, a key protein that functions as a transcriptional repressor of memory. These results demonstrate that neuronal piRNAs are involved in long-term memory. Watanabe et al. also reported that a non-coding RNA transcribed from the differentially methylated region (DMR) of the imprinted mouse *Rasgrf1* locus is cleaved by repeat-derived piRNAs.[26] Although the relationship between cleavage by the PIWI-piRNA complex and DMR methylation is unclear, this result suggests a role of the piRNA pathway in genomic imprinting by *de novo* DNA methylation in mice. In addition, recent studies have suggested that somatic piRNAs are involved in tumorigenesis,[27] liver regeneration,[28] and genomic heterogeneity in the brain.[29] Together with our recent discovery that

the piRNA pathway is required for determining femaleness in *B. mori*, piRNA plays crucial roles in diverse biological processes of various animals.

Maternally inherited antisense piRNAs trigger acute amplification of secondary sense piRNA production in *B. mori* embryos, which is coupled with zygotic transcription of sense transposable RNAs (**Fig. 1B**).[24] This suggests that the Siwi-antisense piRNA complexes cleave sense transposable RNAs to reduce transposon activity, and sense piRNAs are generated as a consequence (**Figs. 1B, C**). Combined with the finding of the *Fem* piRNA-*Masc* pathway, the *B. mori* piRNA system plays 2 critical roles simultaneously during embryogenesis: immune defense and sex determination (**Figs. 1B, C**). How does *B. mori* sense *Masc* mRNA as a target of the Siwi-*Fem* piRNA complex? Previous studies in *B. mori*, *Drosophila*, and mice revealed that transcription from a piRNA cluster in the host genome possibly initiates *de novo* piRNA production against new insertion of "non-self" elements.[30-32] However, *Masc* is not likely to be localized within a distinct piRNA cluster of the *B. mori* genome. Furthermore, *Masc* is a single-copy protein-coding gene and is not a repeat-like element. Although *Drosophila* piRNAs are supposed to be involved in the silencing of the 2 protein-coding genes, *FasIII*[33] and *nos*,[34] the genomic regions targeted by these piRNAs are intronic and 3′-untranslated regions, respectively. The predicted cleavage site of *Masc* mRNA by the *Fem* piRNA-Siwi complex is located within the translated region (9th exon) of *Masc*, and this cleavage has been experimentally verified by a modified RACE procedure,[21] indicating that the *B. mori* embryonic piRNA pathway recognizes a protein-coding exonic region as a non-self element. We are now addressing the question of why the *B. mori* genome considers "self" *Masc* mRNA as "non-self" RNA.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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