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MiR-2 family targets *awd* and *fng* to regulate wing morphogenesis in *Bombyx mori*

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MicroRNAs (miRNAs) are post-transcriptional regulators that target specific mRNAs for repression and thus play key roles in many biological processes, including insect wing morphogenesis. *miR-2* is an invertebrate-specific miRNA family that has been predicted in the fruit fly, *Drosophila melanogaster*, to be involved in regulating the Notch signaling pathway. We show here that *miR-2* plays a critical role in wing morphogenesis in the silkworm, *Bombyx mori*, a lepidopteran model insect. Transgenic over-expression of a *miR-2* cluster using a Gal4/UAS system results in deformed adult wings, supporting the conclusion that *miR-2* regulates functions essential for normal wing morphogenesis. Two genes, *abnormal wing disc (awd)* and *fringe (fng)*, which are positive regulators in Notch signaling, are identified as *miR-2* targets and validated by a dual-luciferase reporter assay. The relative abundance of both *awd* and *fng* expression products was reduced significantly in transgenic animals, implicating them in the abnormal wing phenotype. Furthermore, somatic mutagenesis analysis of *awd* and *fng* using the CRISPR/Cas9 system and knock-out mutants also resulted in deformed wings similar to those observed in the *miR-2* overexpression transgenic animals. The critical role of *miR-2* in *Bombyx* wing morphogenesis may provide a potential target in future lepidopteran pest control.

Introduction

Insects are the only class of winged invertebrates and this is a key factor contributing to their abundance and distribution, and their impact on human welfare.¹ An understanding of the molecular basis of wing morphogenesis could provide a starting point for the development of novel pest management strategies. MicroRNAs (miRNAs) are a class of endogenous, non-coding, single-stranded RNAs, ~22 nucleotides (nt) in length, that regulate gene expression at the post-transcription level by binding to the 3'-end untranslated regions (UTR) of their mRNA targets, leading to their degradation, decreased stability or translational inhibition. We propose that a functional analysis of the roles of miRNAs in wing morphogenesis could provide the basis for developing tissue-specific developmental defects for controlling holometabolous insects of agricultural and medical significance.

Several miRNAs have been implicated in wing development of the fruit fly, *Drosophila melanogaster*.² For example *let-7* mutant flies have smaller wings,³ and the *bantam* miRNA can affect the stability of the dorsal-ventral affinity barrier in the wing disc.⁴ *miR-9a*-deficient mutants have wing margin defects, whereas their overexpression results in a severe loss of sensory organ precursors.⁵ miRNA *iab-4* regulates a homeotic transformation of halteres to wings.⁶ *miR-315* and *miR-8* can regulate

Wingless signaling,^{7,8} while *miR-1* can regulate Notch signaling.⁹ However, the role of miRNAs in wing development of in non-drosophilid insects is largely unknown.

Sequence analyses show that some mature miRNAs are conserved phylogenetically in different species and are often clustered in the genome.^{10,11} *miR-2* comprises a large invertebrate-specific family with most members clustered in the genome that is conserved in insects.¹² Members of *miR-2* in *D. melanogaster* have been predicted to regulate the Notch signaling pathway.^{13,14} The Notch pathway in the fruit fly is involved in the determination of wing vein cells and patterning of the wing margins.^{15,16} Furthermore, *miR-2/13* regulates embryonic development by repressing the proapoptotic factors *hid*, *grim*, *reaper* and *sickle*.^{17,18} This linkage of cell death and abnormal wing phenotypes is a key feature of many mutants in *D. melanogaster*.^{19,20} It remains to be verified in other holometabolous insects.

Genomic mapping of known miRNAs has enabled identification of orthologs in a number of species, including the silkworm, *Bombyx mori*.^{21–24} More than 487 miRNAs have been identified in *B. mori* (miRBase release 21, June 2014), but their functions are only beginning to be understood. We show here that *miR-2* and their target genes, *abnormal wing disc (Bmawd)* and *fringe (Bmfng)*, mediate wing morphogenesis in this lepidopteran model insect. Silkworms exhibiting severe wing abnormalities

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were produced by suppressing *Bmawd* or *Bmfng* expression products using CRISPR/Cas9-mediated genome engineering. The phenotypes of these mutant silkworms were indistinguishable from those produced by transgenic over-expression of *miR-2*.

Results

Ectopic overexpression of a *miR-2* cluster induces deformed wings

A search of the silkworm database (<http://sgp.dna.affrc.go.jp/KAIKObase/>) revealed a *miR-2* cluster located on chromosome 1 comprising 5 miRNAs, *miR-2a-1*, *miR-13a*, *miR-13b*, *miR-2a-2* and *miR-2b*, with similar seed sequences (Fig. 1A). A Gal4/UAS transgenic system was established to over-express *miR-2* to probe its functions *in vivo*. An *A3-Gal4* activator line²⁵⁻²⁷ was used to drive ubiquitously an effector UAS line that contained a pre-*miR-2* cluster 1004 base-pairs (bp) in length flanking genomic DNA sequences (Fig. 1A). The *mCherry* open reading frame (ORF) was placed at the 5'-end of the pre-*miR-2* sequence as a marker.²⁵ Transgenic animals (n = 32) were screened by fluorescence microscopy²⁵ (Fig. S1A–C), and genomic insertion sites identified using inverse-PCR (Fig. S2). The *UAS-miR-2* line crossed with *A3-Gal4* (Fig. 1A) animals, and their offspring (*A3 > miR-2*) show expression of both *EGFP* and *mCherry* (Fig. S1D–F). Quantitative gene amplification of transcripts (QPCR) of *miR-2* in samples derived from the epidermis, fat body, mid gut and wing discs of wandering-stage (3 days prior to pupation) hybrid larvae revealed that *miRs-2/13* transcript levels

were significantly higher ($P < 0.001$) throughout when compared to control animals (*UAS-miR-2*) (Fig. 1B).

The silkworm has a pair of meso- and meta-thoracic discs located laterally in larvae.²⁸ These discs evert during the prepupal stage of metamorphosis and the nascent wings form outside the pupal body.^{29,30} The wing buds expand and inflate during adult eclosion and give rise to the corresponding fore- and hind-wings of the adult.²⁸ All transgenic animals in the hybrid *A3 > miR-2* line developed to the adult stage supporting the conclusion that the ubiquitous overexpression of *miR-2* did not cause lethality in the subadult stages. However, 80% (n = 80) of *A3 > miR-2* heterozygotes moths had abnormal wings characterized by folding, curling and small size (Fig. 2). In addition, wing discs of late 5th instar *A3 > miR-2* larvae were small and appeared poorly developed with veins that failed to extend from the base of the discs and lacked tracheal invasion (Fig. 2). Adult hybrid animals had disordered and atrophied veins. Furthermore, both larval and adult wing margins were irregular in morphology.

miR-2 targets *Bmawd* and *Bmfng* to regulate wing morphogenesis

Three miRNA target-prediction tools, miRanda, PITA and microTar,^{25,31,32} were used in combination to identify 113 putative *miR-2* family targets in *B. mori* (Table S1). Since the overexpression of the *miR-2* cluster produced the abnormalities in wing disc development, we focused on 2 putative target genes, *Bmawd* and *Bmfng* (Table S2), which are well-known regulators in Notch signaling for insect wing development.

miR-2 reduced reporter activity up to ~42% and ~39% compared to controls using the wild-type *Bmawd* and *Bmfng* 3'-end

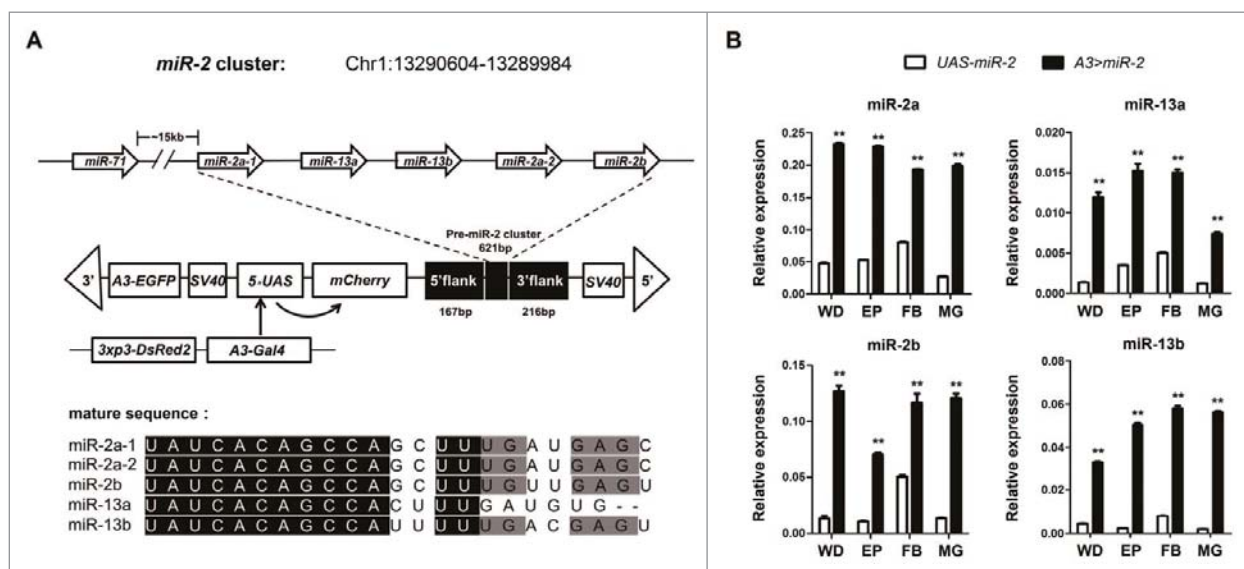


Figure 1. The *miR-2* cluster in *Bombyx mori*, transgene structures and abundance measurements of *miRs-2/13*. (A) Schematic representations of the genomic organization of the *miR-2* cluster, the *miR-2* over-expression vector *UAS-miR-2* and Gal4 vector *pBacA3-Gal4*. The members of *miR-2* family (*miR-2a-1*, *miR-13a*, *miR-13b*, *miR-2a-2* and *miR-2b*) cluster in *B. mori* with *miR-71*, an evolutionarily unrelated miRNA.¹² (B) *miRs-2/13* expression levels determined by QPCR in the tissues of wandering-stage transgenic and control larvae. mRNA abundance of *A3 > miR-2* was significantly higher than control *UAS-miR-2*, *miRs-2/13*. WD- wing disc, EP- Epidermis, FB- Fat body, MG- Mid gut. The asterisks (* or **) indicate the significant differences ($P < 0.05$ or $P < 0.01$, respectively) compared with the relevant control with a 2-tailed t-test. Error bars depict \pm SEM.

UTRs, respectively, in a Dual Luciferase Reporter Assay in HEK293T cells (Fig. 3A). These findings were supported further by observing no effect on reporter activity when using forms of *Bmawd* and *Bmfng* mutated in the seed-binding regions of the respective 3'-end UTRs (Fig. 3A; Fig. S3).

QPCR and immuno-blot analyses confirmed that overexpression of *miR-2* reduced significantly *Bmawd* and *Bmfng* transcript and protein levels in *A3 > miR-2* wandering-stage larval wing discs when compared to controls (Fig. 3B). Additionally, the transcript abundance of *BmWnt-1*, which is a *B. mori* homolog of *wingless* whose function is required later in the wing development morphogenetic pathway^{30,33} was reduced 91% (Fig. 3C, D).

Loss-of-function of *Bmawd* or *Bmfng* causes wing defect phenotypes

The CRISPR/Cas9 system was used to produce separate loss-of-function mutations in the *Bmawd* (ΔA) and *Bmfng* (ΔF) loci.³⁴ Two 23-bp sgRNA-targeting sites (S1 and S2) were identified by screening the *Bmawd* or *Bmfng* ORF followed the 5'-GG-N₁₈-NGG-3' rule³⁴⁻³⁶ (Fig. 4A). *Bmawd* lacks an intron and the interspace fragment between S1 and S2 was 172 bp in length (Fig. 4A). The *Bmfng* gene has 9 exons, and its S1 site is localized to the antisense strand of the 5'-end UTR and S2 is localized in exon-1 with a 101 bp interspace fragment between them (Fig. 4A). SgRNA-1 and SgRNA-2 for each gene were co-injected separately with Cas9 mRNA into 480 preblastoderm embryos as described previously.³⁴ Of these, 262 (55%, ΔA) and 235 (49%, ΔF) eggs hatched, and 201 ΔA and 197 ΔF larvae survived to the adult stage. Knockout of the same genes in *Drosophila* caused lethality,^{37,38} but less lethal mutations in *Bombyx*. A total of 61% ΔA (n = 122) and 40% ΔF (n = 78) moths showed abnormal wings identical to those observed in the *A3 > miR-2* line (Fig. 4B). PCR-based mutagenesis analysis revealed that targeting sites and their interspace fragments were deleted (Fig. 4C). *BmWnt-1* transcripts also were reduced in mRNA extracted from these wings (Fig. 4D).

Discussion

We provide molecular and genetic support for the conclusion that *mir-2* function is essential for wing development in

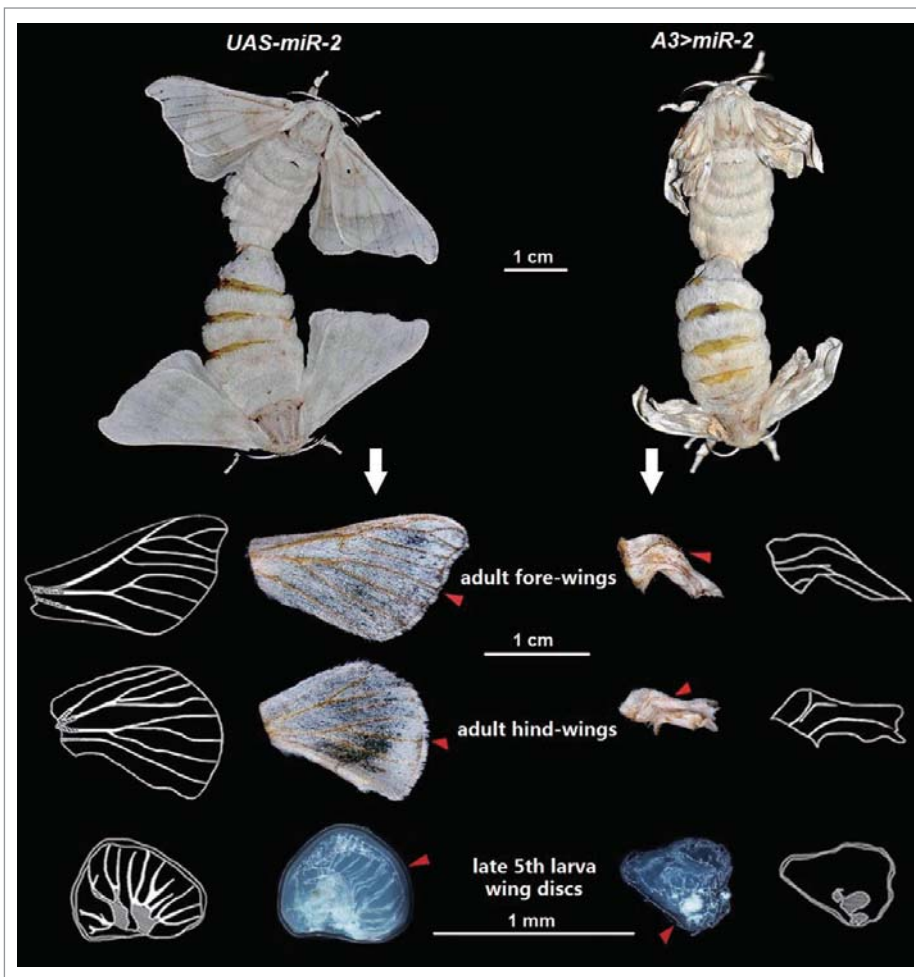


Figure 2. Effect of *miR-2* overexpression in *Bombyx mori*. The *A3 > miR-2* silkworms (right column) show abnormal wing phenotypes after *miR-2* overexpression, while the control group (left column) *UAS-miR-2* are normal. The wing veins of *A3 > miR-2* fail to extend at the late 5th larval instar stage (red arrowhead) and the wing margin is irregular. The fore- and hind-wings of *A3 > miR-2* moths are curled with disordered and atrophied veins (red arrowhead). Schematic diagrams show the wing margin and wing vein phenotypes.

B. mori. *miR-2* over-expression in transgenic *A3 > miR-2* results in mutant moths with abnormally small wing discs observable as early as the 5th instar larvae, and these result in aberrant adult wings.

The combined results also support the conclusion that *Bmawd* and *Bmfng* are major *miR-2* targets and are involved in normal wing development. The *awd* gene encodes a protein with nucleoside diphosphate kinase (NDP kinase) activity and is a homolog of human metastasis suppressor gene, *Nm23*.^{39,40} *awd* in *D. melanogaster* is required for proper Notch signaling⁴¹ and mutations in the gene results in adults with wing margin and vein-thickening defects similar to the *Notch* phenotype.⁴¹ Loss of *awd* function also represses *wingless* (*wg*) expression.⁴¹ dsRNA-mediated ablation of *awd* function in the lepidoptera, *Spodoptera litura*⁴² and *Diaphorina citri*,⁴³ also results in defects in wing formation. The *fng* gene in *D. melanogaster* encodes a glycosyltransferase that can modify the Notch receptor and thereby modulate

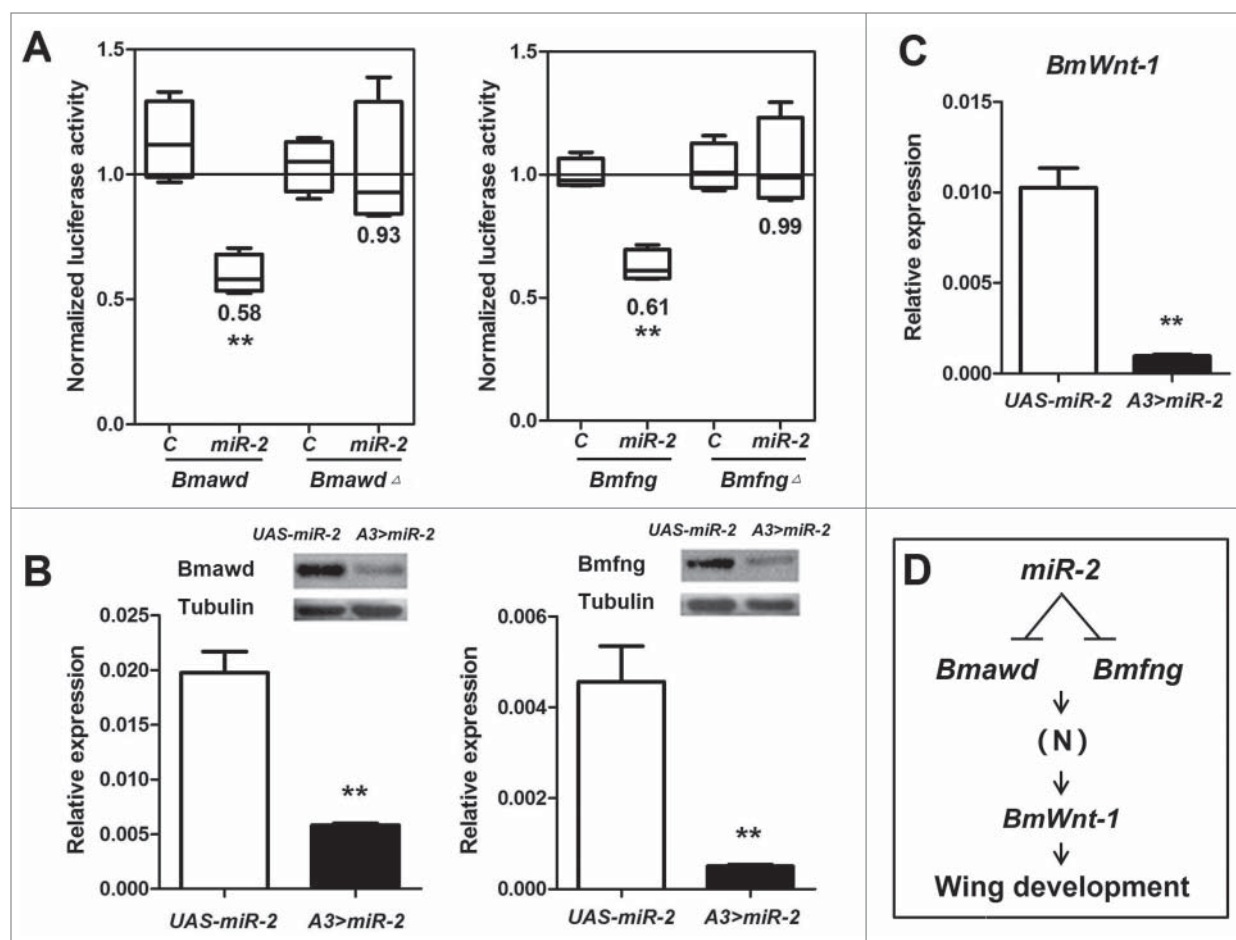


Figure 3. *Bmawd* and *Bmfng* are the direct targets of *miR-2*. (A) *miR-2* targets the *Bmawd* and *Bmfng* 3'-end UTR *in vitro*. The firefly luciferase activity was normalized to renilla luciferase activity and then normalized to the median of the control group (the line in the box and the number below the box show the median). *Bmawd* Δ and *Bmfng* Δ defined as forms of *Bmawd* and *Bmfng* mutated in the seed-binding regions of the respective 3'-end UTRs. (B) Over-expression of the *miR-2* cluster *in vivo* results in a decrease of the *Bmawd* (71%) and *Bmfng* (89%) at transcript levels and this is consistent with protein levels. (C) The transcript level of the downstream effector gene, *BmWnt-1*, is reduced by 91%. The asterisks (* or **) indicate the significant differences ($P < 0.05$ or $P < 0.01$, respectively) compared with the relevant control with a 2-tailed t-test. Error bars depict \pm SEM. (D) Model showing regulation of *miR-2* in *B. mori*. *miR-2* represses *Bmawd* and *Bmfng*, which then affects the expression of *BmWnt-1* by Notch signaling (N) leading to the wing development.^{30,33}

the signaling pathway during wing development.^{44,45} Loss of *Bmfng* function in *B. mori* also inhibits the activation of the Notch signaling pathway and results in an abnormal wing phenotype and reduced *BmWnt-1* transcript level observed in the *flügellos* mutants.³⁰ Although the seeds of *miR-2* family were conserved in insects,¹² their predicted targets changed in different species because of the diverse 3'-end UTRs. For example, the *Drosophila dme-miR-2* had target binding sites in the 3'-end UTRs of *Dmefng* but not in *Dmeawd* (Table S3).

dsRNA-mediated RNAi by using directly injection of dsRNA was seldom effective in lepidoptera, especially during larval stage. An alternative approach is to apply Cas9 system in *Bombyx*. The Cas9 sgRNA-mediated mutagenesis of the *Bmawd* and *Bmfng* loci in this study produced abnormal wing phenotypes similar to those in the *A3 > miR-2* overexpression line and a reduction of the transcript level of *BmWnt-1*.

These data are consistent with the conclusion that *miR-2* has a major role in wing morphogenesis in *B. mori* by regulating the expression of *Bmawd* and *Bmfng* to a proper level required for normal wing formation. Manipulating *miR-2* may be useful for developing novel methods to control lepidopteron pests by establishing a sex-specific flightless strain to permit genetic sexing.⁴⁶

Materials and methods

Silkworm strains and plasmids

A multivoltine, nondiapausing silkworm strain, Nistari, was used for germ-line transformation and subsequent experiments. Larvae were reared on fresh mulberry leaves under standard conditions.

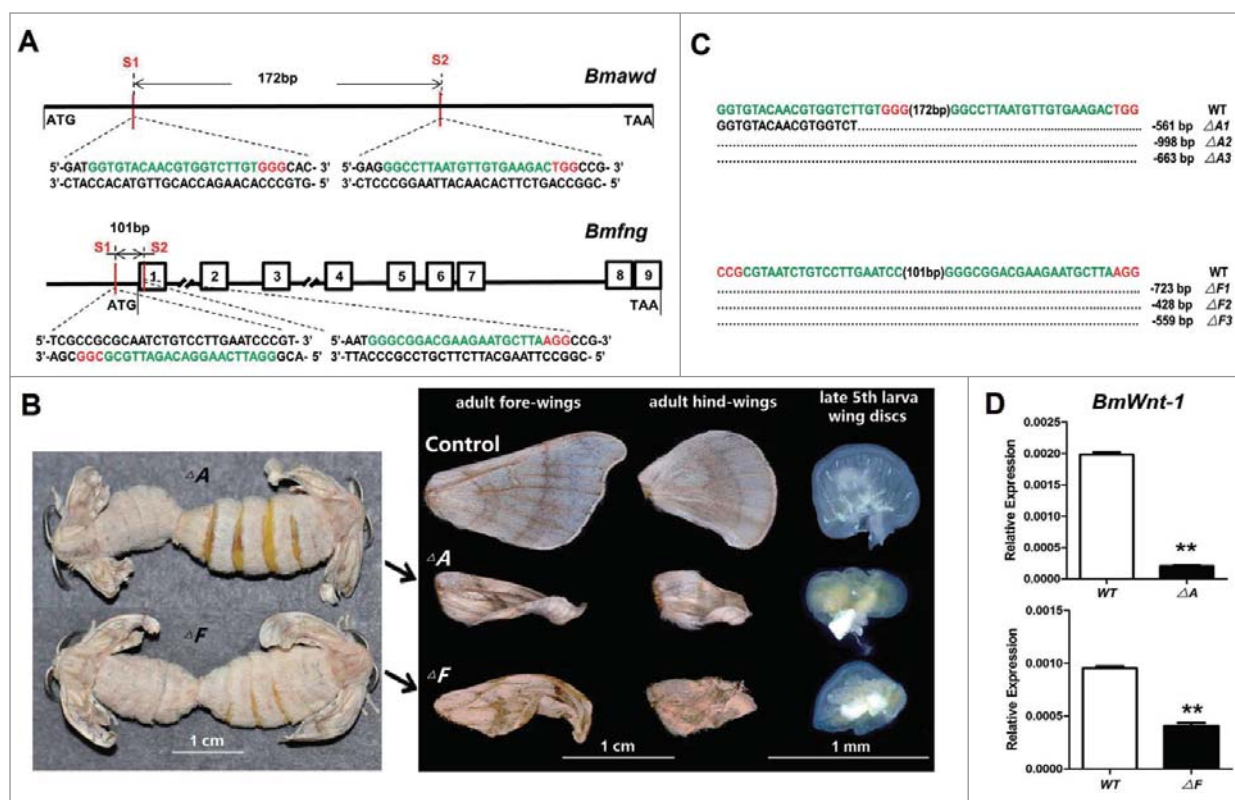


Figure 4. Abnormal wing phenotypes caused by *Bmawd* or *Bmfng* mutations in *Bombyx mori*. **(A)** schematic diagram of the sgRNA-targeting sites (red vertical line). The target site sequence is in green, and the protospacer adjacent motif sequence is in red. The *Bmawd* gene lacks introns and *Bmfng* gene has 9 exons (box). **(B)** ΔA or ΔF mutant moths have abnormal wings. **(C)** Primary structures of deletion mutations in the *Bmawd* or *Bmfng* loci. Different types of deletion events are shown with the number of deleted nucleotide. The target site sequence is in green, and the protospacer adjacent motif sequence is in red. **(D)** The transcript level of *BmWnt-1* is down-regulated significantly in ΔA (89%) and ΔF (57%). The asterisks (* or **) indicate the significant differences ($P < 0.05$ or $P < 0.01$, respectively) compared with the relevant control with a 2-tailed t-test. Error bars depict \pm SEM.

The pre-*miR-2* cluster and genomic 5'- and 3'-end flanking sequences were amplified by PCR. Complete products 1004 bp in length were sub-cloned into a *piggyBac* plasmid *pmR-IE1-mCherry* (Clontech). DNA sequences of *mCherry-5'F-premiR2-3'F* were PCR-amplified²⁵ using primers HRF and HRR (Table S4), and at the 3'-end of 5 \times UAS in the *XhoI* site of *pBacA3EGFP-UAS-SV40* to generate the final plasmid *UAS-miR-2*.

miRNA target predictions

Three miRNA prediction software packages, PITA, miRanda and microTar,^{25,31,32} were used jointly to predict the *miR-2* targets. Thresholds were set to a score of ≥ 140 for miRanda (default), $ddG \leq 0$ for PITA, and energy ≥ 0.5 for microTar.^{25,31,32} The 3'-end UTRs sequences of *B. mori* genes were downloaded from NCBI (<http://www.ncbi.nlm.nih.gov/>).

Dual luciferase reporter (DLR) assay

For high transfection efficiency and low background expression of targets, the mammalian HEK293T cell line was used for the DLR assay. A 1004 bp genomic fragment containing the pre-*miR-2* cluster was cloned into the pmR-mCherry (Clontech) multiple cloning site (MCS) for the expression of *miR-2* cluster

(*miR-2-F* & R, Table S4). Mutated sequences (Fig. S3) were introduced into the 3'-end UTRs of *Bmawd* and *Bmfng* by PCR. The wild-type or mutant 3'-end UTRs were cloned and inserted into the pGL3-promoter plasmid (Promega, Madison, WI) between the firefly luciferase ORF and SV40 poly (A) DNA fragments. HEK293T cells were transfected with 5 ng of the pGL3 reporter plasmid, 5 ng of the pRL-TK control plasmid and 100 ng of the pmR-mCherry-*miR-2* expression plasmid mixed with 0.5 μ l Lipofectamine[®] 2000 Transfection Reagent (Invitrogen) in 5 μ l Opti-MEM[®] I Reduced Serum Medium (Gibco) in each well of a 96-well plate. The Dual-Luciferase[®] Reporter Assay (Promega) was performed 24 h after the transfection according to the manufacturer's protocol. Experiments were performed triplicate, each with 4 technical repeats. The mean of the relative luciferase expression ratio (firefly luciferase/renilla luciferase) of the control was set to 1.

Molecular biology

Inverse PCR was carried out as described previously^{25,47} to investigate genomic insertion loci of transgenes. Both lines crossed with *pBacA3-Gal4* animals respectively, and their transformed G2 offspring showed the identical phenotype thus we

used the G2 offspring of B1 line in subsequent experiments. Real-time quantification of miRNAs⁴⁸ was performed to analyze the expression level of *miR-2* in transgenic and control individuals. Total RNA was extracted from the larval wing discs (n = 80) and other tissues (n = 3) from wandering-stage larvae using TRIzol Reagent (Invitrogen). cDNA was prepared using the ReverTaid First-Strand cDNA synthesis kit (Thermo). The small nuclear RNA *U6* of *B. mori* (*BmU6*) was used as an internal control. The stem-loop primers for cDNA synthesis from miRNA and the primers used for QPCR are listed (Table S4). QPCR conditions were performed as described previously.²⁵ *Bmrp49F* and *Bmrp49R* (Table S4) amplifies a 136 bp fragment from the *B. mori* ribosomal protein 49 (*Bmrp49*) as an internal control.

Wandering stage wing discs were collected, homogenized in lysis buffer (Beyotime), and samples resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel. The gel was blotted onto a PVDF membrane and this was incubated with rb-anti-Bmawd or rb-anti-Bmfng at 1:500 and HRP-anti-rabbit at 1:5000. The signal was detected using a Pierce ECL immuno-blotting substrate kit (Thermo). The detection of tubulin as a control was performed as described above using mouse-anti-tubulin as the primary antibody.

Cas9/sgRNA preparation and microinjection

The Cas9-sgRNA system was used to target *Bmawd* or *Bmfng* locus. S1 and S2 were identified by screening the *Bmawd/Bmfng* ORF and following the 5'-GG-N₁₈-NGG-3' rule.³⁴ The sgRNAs were prepared using the MAXIscript[®] T7 kit (Ambion) according to the manufacturer's instruction. The Cas9 mRNA was synthesized with mMACHINE[®] T7 kit (Ambion) from the linearized vector *pTD1-T7-Cas9* as previously described.³⁴

Fertilized eggs were collected within 1 hour after oviposition and microinjected within 3 hours. sgRNA-1 (150 ng/μl) and sgRNA-2 (150 ng/μl) of either *Bmawd* or *Bmfng* combined with Cas9 mRNA (300 ng/μl) were mixed and co-injected into 480 embryos each. Injected eggs were incubated at 25°C in a humidified chamber for 10–12 days until larval hatching. Larvae were reared with fresh mulberry leaves under standard conditions.

Statistical Analysis

Data were analyzed with GraphPad using 2-tailed t-test. Probability values of less than 0.05 are considered significant. Data are presented as mean with SEM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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