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Research

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miR-92a-1-p5 Modulated Expression of the *flightin* Gene Regulates Flight Muscle Formation and Wing Extension in the Pea Aphid, *Acyrthosiphon pisum* (Hemiptera: Aphidoidea)

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Abstract

Aphids exhibit wing polyphenism. Winged and wingless aphid morphs are produced by parthenogenesis depending on population density and host plant quality. Recent studies showed that microRNAs in alate and apterous individuals have differential expression and are involved in wing dimorphism of *Acyrthosiphon pisum*. From which miR-92a-1-p5 can target the mRNA of flight muscle gene *flightin* in vitro, but what effect they have on wing development of aphid is unclear. Here with the nanocarrier-delivered RNA interference (RNAi) method, *flightin* gene was knocked down in winged nymphs of *A. pisum*. Results showed that the majority (63.33%) of adults had malformed wings, the shape of dorsal longitudinal muscle (DLM) was deformed severely, the dorsoventral flight muscle (DVM) became wider and looser in aphids with *flightin* reduction compared with the negative control. Overexpression of miR-92a-1-p5 caused decreased expression of *flightin* and malformed wings of aphids, with a mutant ratio of 62.50%. Morphological analysis of flight musculature showed the consistent result as that with *flightin* knockdown. These results suggest that *flightin* is essential for flight musculature formation and wing extension in *A. pisum*, which can be modulated by miR-92a-1-p5.

Key words: aphid, wing, *flightin*, miR-92a-1-p5

Aphids are common pests in agriculture and forestry. They are distributed worldwide, with more than 5,000 species (Remaudiere and Remaudiere 1997, Blackman and Eastop 2016, Wang et al. 2016). Aphids exhibit wing dimorphism during parthenogenetic generations, the determination of winged and wingless viviparous morphs occurs during embryogenesis in the maternal ovary in response to environmental cues perceived by the mother, such as population density, microorganisms, temperature, photoperiod, and host quality (Braendle et al. 2006, Zhang et al. 2019a). This is a successful strategy used by aphids to adapt to environmental changes. Morph differentiation occurs during postembryonic development (Ishikawa et al. 2013). All parthenogenetic aphids are born with wing buds, these degenerate in the wingless morphs during the second instar, but continue to develop in the winged morphs (Brisson et al. 2010). Winged aphids develop flight apparatus, wings, and *flightin* muscles to disperse to new habitats (Kennedy 1950). Wings allow aerodynamic lift, and indirect flight muscles (IFM), which consist of dorsoventral muscles (DVM), dorsal longitudinal muscles (DLM), and oblique dorsal muscles (ODM), allow the wings to move up and down (Ogawa and Miura 2013). IFM develop from the fourth-instar period to the early adult stage in *Acyrthosiphon pisum*, after the winged morphs migrate to new host plants, or begin reproduction, the IFM degenerate (Johnson 1980, Ishikawa and Miura 2009).

Genes that participate in the regulation of wing development were identified in the bird cherry-oat aphid *Rhopalosiphum padi* (Hemiptera: Aphidoidea) (Zhang et al. 2019b). Meanwhile, microRNAs (miRNAs) are also involved in wing dimorphism of aphid. In total, 345 miRNAs were identified in *Sitobion avenae* (Hemiptera: Aphidoidea) by RNA-Seq (Li et al. 2016). The expression levels of 16 miRNAs were significantly up-regulated in winged

parthenogenetic individuals, and another 12 miRNAs were abundant in wingless individuals; The target genes of miRNAs were classified in 124 metabolic pathways, including genes in the Wnt, Notch, Hedgehog, and TGF-beta signaling pathways (Li et al. 2016). Moreover, miR-147b can modulate expression of *vestigial* to regulate wing development in *R. padi* (Fan et al. 2020). miR-9b mediates wing dimorphism by targeting the ABC transporter AcABCG4, a decrease of aci-miR-9b followed by an increase of ABCG4, this activates the insulin and insulin-like signaling pathway and produces a high proportion of winged offspring in the brown citrus aphid, *Aphis citricidus* (Hemiptera: Aphidoidea) (Shang et al. 2020).

In a previous study, miR-92a-1-p5 was found to have more expression in wingless nymphs of the fourth instar, at which the flight muscle gene *flightin* was highly accumulated in the winged ones of *A. pisum* (Fig. 1A) (Yang et al. 2019). The seed sequence of miR-92a-1-p5 matches well with the coding sequence (CDS) of *flightin*, and they can interact with each other in vitro (Ma et al. 2021). But what roles of *flightin* and miR-92a-1-p5 played in wing development of aphid are unknown. Here with a nanocarrier-mediated delivery system, the *flightin* and the miR-92a-1-p5 were knocked down or overexpressed in winged nymphs to study their effect on flight muscle formation and wing extension of *A. pisum*, which provides new clues for aphid control.

Materials and Methods

Insect Rearing and Sample Preparation

A single apterous viviparous parthenogenetic female *A. pisum* was reared on broad bean (*Vicia faba* L., Rosales: Leguminosae) seedlings in an incubator at 20°C, 60% RH, and a photoperiod of 16:8 (L:D) h. Modified crowding treatment (Vellichirammal et al. 2016) was used to induce winged morphs. Ten adult wingless aphids were placed in a plastic petri dish (32.5 mm × 15 mm) for 24 h (a total of 100 aphids were treated). The aphids were then transferred to a plastic cup (8 cm diam. 14 cm tall) planted with broad beans to induce winged aphids.

Quantitative Real-time PCR to Detect the Expression of *flightin* and miR-92a-1-p5

Total RNAs were extracted from winged or wingless aphids with TRIzol (Thermo Fisher, Waltham, MA). The first strand of cDNA was synthesized with 2 μg total RNA by the PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) with oligo $d(T)_{15}$. Quantitative reverse-transcription PCR (qRT-PCR) was conducted using FastStart Universal SYBR Green master mix (Roche, Cornwall, England, UK) on the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The PCR procedure was as follows: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s with 40 cycles, *actin* was used as an internal control (housekeeping gene) for *flightin*. Reverse transcription and quantitative real-time PCR of miRNA was done by an All-in-One miRNA qRT-PCR Detection Kit (GeneCopoeia, Rockville, MD), U6 was used as an internal control. The primers used are listed in Table 1. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) was used to calculated the relative expression of miRNA and *flightin* from the Cts obtained in the PCR quantification, Ct is the cycle threshold, which represented the number of cycles when the fluorescence signal in each reaction reached the set threshold; ΔCt represents the average Ct value of the sample minus the internal control. ΔCtΔCt represents the average Ct value of sample minus control sample. Three independent experiments were conducted, and each sample was repeated three times.

Synthesis of miRNA Agomir and Antagomir

Based on the mature miRNA sequence of miR-92a-1-p5, the agomir and antagomir of miRNA, as well as the negative control (NC), were synthesized by GenePharma Technology Co., Ltd. (Shanghai, China. Primer sequences are shown in Table 1). The sequences of them were listed in Table 1.

Preparation of the Nanocarrier/miRNA or dsRNA Complex

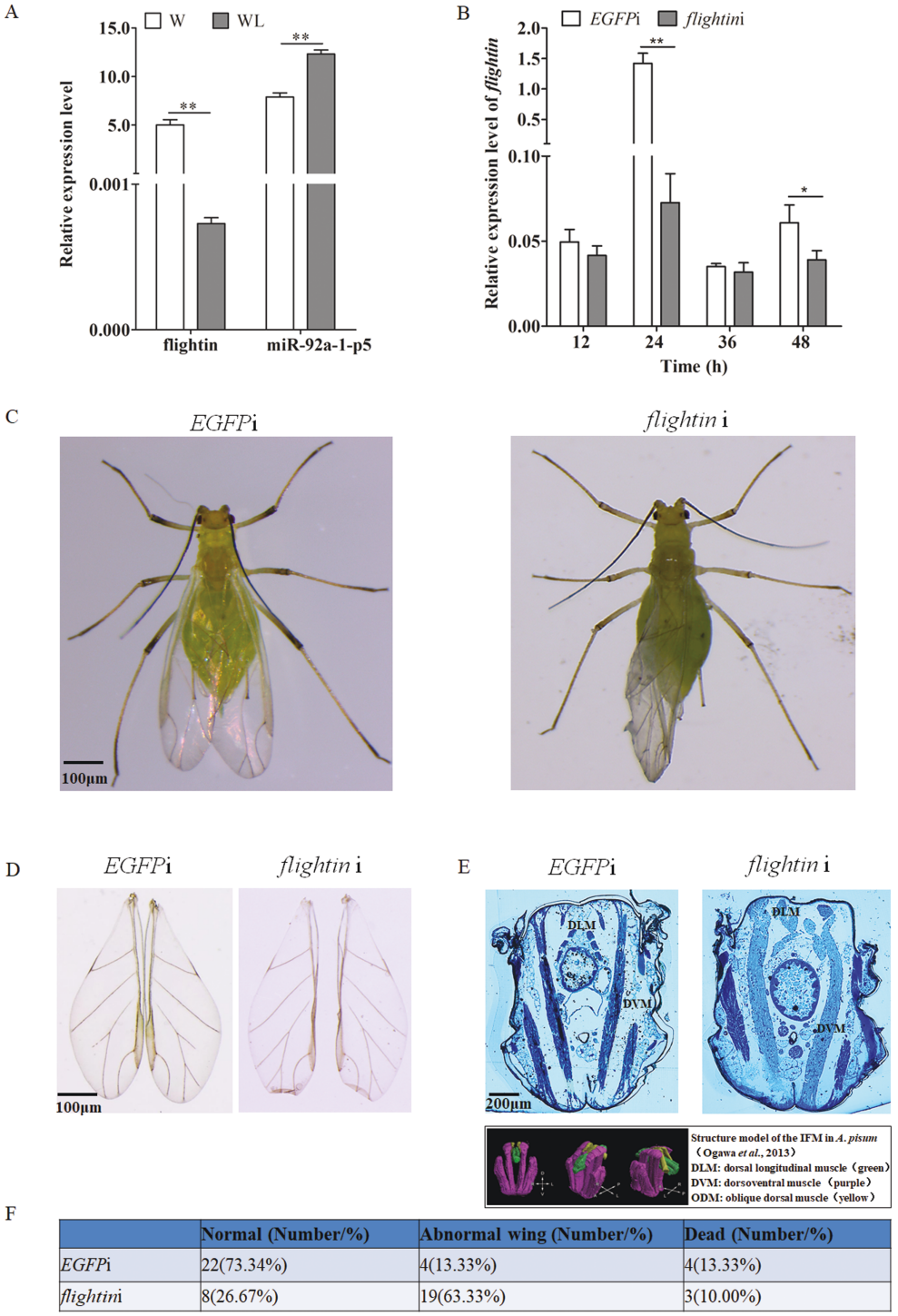
To determine the functional roles of *flightin* and miR-92a-1-p5 played in wing extension of aphids, a formulation of the nanocarrier/miRNA or double strand RNA (dsRNA)/detergent was developed with the method of Zheng et al. used (Zheng et al. 2019). The agomir or antagomir of miR-92a-1-p5 (mimic and inhibitor of miR-92a-1-p5, respectively, sequences were in Table 1) or dsRNA of *flightin* were mixed with the nanocarrier in a volume ratio of 1:1. The detergent was added at a volume of 0.1% and the mixture was maintained at room temperature for 15 min to create the nanocarrier/miRNA (or dsRNA)/detergent formulation. The same amount of NC or dsRNA of EGFP was used as negative controls for miR-92a-1-p5 and *flightin*, respectively. Three independent experiments were conducted, and each sample had 10 individuals.

Infiltration of Nanocarrier Delivery System

The dsRNAs of *flightin* or the agomir of miR-92a-1-p5 were injected into the fourth instar nymphs from the winged lines of *A. pisum* with nanocarrier-mediated delivery system. The antagomir of miR-92a-1-p5 was injected into the second instar nymphs of wingless *A. pisum*. The nanocarrier/miRNA (dsRNA)/detergent formulation was delivered by a microinjector. We applied 50 nL droplets to the notum of the *A. pisum* nymphs. The treated nymphs were isolated until the droplet had completely penetrated the cuticle. The nymphs were then transferred to seedlings of broad bean under long-day conditions using the method described above. The whole body of aphids was collected and frozen in liquid nitrogen at different time points. RNA was extracted with TRIzol (Thermo Fisher, Waltham, MA) method. Phenotype of aphid was detected in the microscope SZX7 (Olympus, Tokyo, Japan).

Image Analysis of Flight Muscle

The winged aphid with *flightin* knockdown or miR-92a-1-p5 overexpression were prefixed at 4°C for 3 d with paraformaldehyde (2%) and glutaraldehyde (2.5%) in 0.1 M phosphate buffered saline (PBS, pH 7.2), then post-fixed for 2 h with 1% OsO4 in 0.1 M PBS (pH 7.2), and followed by dehydration in ethanol series solutions (50, 70, 80, 90, 100%) for 10 min each. After being dehydrated with pure acetone two times for 30 min each, the samples were embedded in SPI-PON 812 (SPI Suppliers, West Chester, PA) through mixtures of 3: 1, 1: 1, 1: 3 of acetone and SPI-PON 812 and then kept in pure SPI-PON 812 overnight. Polymerization was accomplished with heating at 30°C 24 h, at 60°C for 48 h in tightly closed gelatin capsules filled completely with the resin monomer. Semithin sections were cut with a diamond knife (Leica, Wetzlar, Germany) on a Leica EM UC7 microtome (Wetzlar, Germany). The sections were stained with toluidine blue and observed on Zeiss Axio Imager A2 (Oberkochen, Baden-Württemberg, Germany). Photographs were only adjusted for brightness and contrast.



F

	Normal (Number/%)	Abnormal wing (Number/%)	Dead (Number/%)
EGFPi	22(73.34%)	4(13.33%)	4(13.33%)
flightini	8(26.67%)	19(63.33%)	3(10.00%)

Fig. 1. Knockdown of *flightin* in winged *A. pisum*. (A) Expression level of *flightin* and miR-92a-1-p5 in the fourth instar nymphs from winged and wingless lines of aphids (Yang et al. 2019). W and WL represent winged and wingless nymphs, respectively. (B) RNAi-mediated suppression of *flightin* at different time points. Three independent experiments were conducted, and each sample was repeated three times. Significance analysis was conducted with ANOVA and Student's *t*-test. The data were from three independent experiments (means ± SEM; $*P < 0.05$, $**P < 0.01$). (C) Phenotype of the fifth instar nymphs after injected with dsRNA of *flightin* for 48 h. The deformed wings were tilted to one side, curled at the distal end, and hard to peel after *flightin* knockdown. Scale bar represents 100 μm. (D) Phenotype of malformed wings after *flightin* knockdown for 48 h. Scale bar represents 100 μm. (E) Morphological change of flight musculature after *flightin* knockdown for 48 h. DLM means dorsal longitudinal muscle, DVM means dorsoventral flight muscle. The DVM deformed severely, and became wider and looser, the DLM was also loosed in aphids with *flightin* knockdown compared with the negative control. Scale bar represents 200 μm. The 3D reconstruction images of the flight muscle in *A. pisum* (Ogawa et al. 2013) were provided in order to get a better observation of the flight muscle structure. DLM was in green, DVM was in purple, ODM (oblique dorsal muscle) was in yellow. (F) Statistics of the aphids with *flightin* knockdown. Three independent experiments were conducted, and each sample had 10 individuals. EGFPi means aphids being injected with dsRNA of EGFP, flightini means aphids being injected with dsRNA of *flightin*.

Table 1. Primers used in the study

Genes (Genebank no.)	Primer sequences(5'–3')
U6 (KU050837)	F: CGCAAGGATGACACGCAA
miR-92a-1-p5 (NR_040129)	F: TGGTCGACGACTTGTGCAACTATT
flightin-dsRNA	F: GAAATTAATACGACTCACTATAGGAGCCGAATGGAGGAAAACTT
	R: GAAATTAATACGACTCACTATAGGAAGGTGTACCGATGAGCTGG
EGFP-dsRNA	F: TAATACGACTCACTATAGGGTACGGCGTGCAGTGCT
	R: TAATACGACTCACTATAGGGTGATCGCGCTTCTCG
actin (NM_001142636)	F: CAGAAGAGCACCCAATCC
	R: GAGACACCGTCACCAGAG
flightin (XM_001944085)	F: CATGAACAGGCGACAGAGGG
	R: GATGGCCGTGGTACATCCAA
miR-92a-1-p5 agomir	UGGUCGACGACUUGUGCAACUAUU
agomir NC	UUCUCCGAACGUGUCACGUTT
miR-92a-1-p5 antagomir	AAUAGUUGCACAAGUCGUCGACCA
antagomir NC	GUGCAGAGUAAACCGACCUAUCUA

Data Analysis

The data were processed by the software SPSS Statistics 22.0, and the mean standard error was visualized in the graphs. Significance analysis ($P < 0.05$) was conducted with a one-way analysis of variance (ANOVA) and a Student's *t*-test.

Result

Knockdown of *flightin* Lead to Malformed Wings and Flight Muscle in *A. pisum*

Flightin is the main protein expressed uniquely in IFM And it maintains the integrity of the IFM sarcomere in *Drosophila* (Vigoreaux et al. 1993). Ma et al. found that the seed sequence of miR-92a-1-p5 matched to the bases 66 to 91 of *flightin* CDS with high complementarity, dual luciferase reporter assay revealed that they can interact with each other in vitro (Ma et al. 2021). To further elucidate the functional roles of *flightin* and miR-92a-1-p5 during wing development of aphid, dsRNA of *flightin* were injected into the fourth instar nymphs of the winged *A. pisum*. After 24 h injection, the expression of *flightin* decreased by 95% ($P < 0.01$) (Fig. 1B). The mortality of aphids was 10% and 13.33% after 48 h injection with dsRNA of *flightin* and *EGFP*, respectively (n =30, Fig. 1F). Interestingly, the majority of adults (n = 19) had malformed wings after *flightin* knockdown, with a ratio of 63.33% (Fig. 1C and F), whose wings were tilted to one side, curled at the distal end, and hard to peel (Fig. 1C and D). The flight behavior of them also decreased (data not shown). To gain insights into changes of flight musculature, semithin section was cut from the thoracic segment of aphids with *flightin* knockdown. The morphology of dorsal longitudinal muscle (DLM) was deformed severely after *flightin* retardation (Fig. 1E), and the dorsoventral flight muscle (DVM) became wider and looser compared with the negative control (Fig. 1E), these suggested that knockdown of *flightin* changed the morphological structure of flight muscle, especially the DLM, which might result in the malformed wings of aphids.

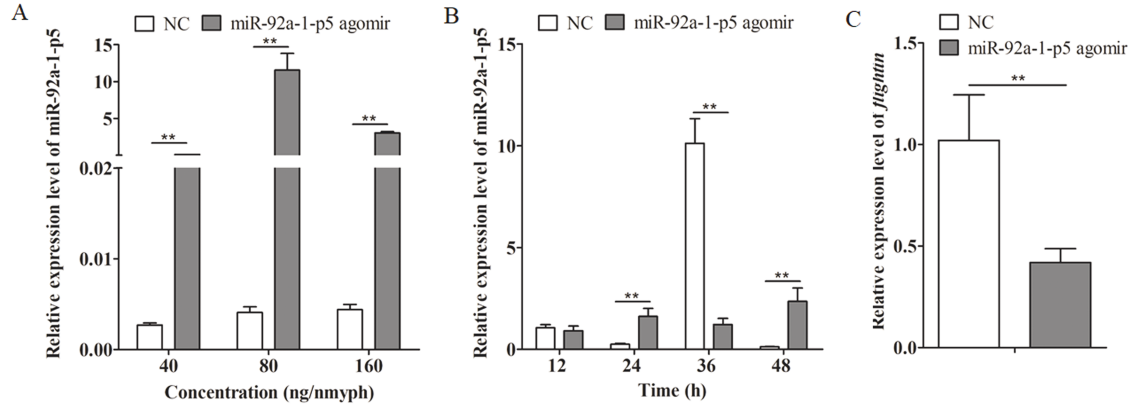
miR-92a-1-p5 Modulates Flight Muscle Formation and Wing Extension of Aphid Through *flightin*

To determine whether the wing abnormality of *flightin* knockdown was modulated by miR-92a-1-p5, the agomir (modified mimics) of miR-92a-1-p5 at different concentrations (40 ng, 80 ng, 160 ng per nymph) was also injected into the fourth instar nymphs from the winged lines of *A. pisum* by nanocarrier-mediated delivery system. The expression of miR-92a-1-p5 increased significantly at the concentration of 80 ng per nymph ($P < 0.01$), but higher concentration of agomir could not induce more accumulation of miR-92a-1-p5 (Fig. 2A). The appropriate sampling time was also determined with 80 ng/nymph treatment. The expression level of miR-92a-1-p5 increased significantly by 85% after 24 h injection ($P < 0.01$, Fig. 2B), at which the expression level of *flightin* decreased by 59% (Fig. 2C).

The phenotype of aphid was also recorded. The shape of wings changed greatly in aphids injected with the miR-92a-1-p5 agomir, with a mutant rate of 62.50% (Fig. 2G). The wings were also tilted to one side and became curled at the distal end, exhibiting consistent results as that of *flightin* knockdown (Fig. 2D and 2E). Morphological change of flight musculature was further observed, the DLM became wider, and looser after *miR-92a-1-p5* overexpression, the DVM also became looser compared with the negative control (Fig. 2F), exhibiting the consistent result to what observed in the aphids with *flightin* knockdown. These results suggested that miR-92a-1-p5 can play roles in flight muscle formation and wing extension of aphids through modulating *flightin*, overexpression of miR-92a-1-p5 decreased the mRNA level of *flightin*, which caused loose and malformed flight muscle, especially the DLM, then result in the abnormal wings of aphids.

Injection of miR-92a-1-p5 Antagomir in Parthenogenetic Wingless Aphids Did Not Promote Wing Development in Adult Females or Offspring of *A. pisum*

To verify whether knockdown of miR-92a-1-p5 can promote wing production of *A. pisum*, the antagomir of miR-92a-1-p5 was injected into the second instar nymphs using the nanocarrier-mediated delivery system. Different concentrations (40 ng, 80 ng, 160 ng per nymph) and sampling times were also studied. The expression of miR-92a-1-p5 decreased significantly at the 80 ng per nymph treatment ($P < 0.01$) (Fig. 3A) and 24 h to 48 h postinjection ($P < 0.01$), especially at 36 h postinjection, the expression of miR-92a-1-p5 decreased significantly by 94% (Fig. 3B). The expression level of *flightin* significantly increased by 40% after 36 h injection ($P < 0.01$) (Fig. 3C). But there was no obvious change of phenotype was detected aftermiR-92a-1-p5 repression in either adult females or offspring (Fig. 3D). This suggested that knockdown of miR-92a-1-p5 can increase the expression of *flightin*, but could not induce wings in the offsprings.



G

	Aphids with normal wings (Number/%)	Aphids with abnormal wings (Number/%)	Dead aphids after injection (Number/%)
NC	20 (66.67%)	6 (20.00%)	4 (13.33%)
miR-92a-1-p5 agomir	7 (21.87%)	20 (62.50%)	5 (15.63%)

Fig. 2. Overexpression of miR-92a-1-p5 in winged *A. pisum*. The expression levels of miR-92a-1-p5 in the fourth instar from winged aphids injected with agomir of miR-92a-1-p5 at different concentrations of agomir (A) and sampling time (B). (C) The expression level of *flightin* after injection of miR-92a-1-p5 agomir for 24 h. Three independent experiments were conducted, and each sample was repeated three times. Significance analysis was conducted with ANOVA and Student's *t*-test. The data were from three independent experiments (means ± SEM; $*P < 0.05$, $**P < 0.01$). (D) Phenotypes of winged aphids after injection with agomir and negative control (NC) of miR-92a-1-p5 for 48 h. Scale bar represents 100 μm. Deformed wings were tilted to one side, curled at the distal end, and hard to peel after miR-92a-1-p5 overexpression. (E) Phenotype of malformed wings after miR-92a-1-p5 overexpression for 48 h. Scale bar represents 100 μm. (F) Morphological changes of flight muscle after miR-92a-1-p5 overexpression for 48 h. DLM means dorsal longitudinal muscle, DVM means dorsoventral flight muscle. The DVM was deformed and became wider and looser compared with the negative control. Scale bar represents 200 μm. (G) Statistics of the aphids with miR-92a-1-p5 overexpression. Three independent experiments were conducted, and each sample had 10 individuals. NC means negative control, miR-92-1-p5 agomir means aphids being injected with agomir of miR-92-1-p5.

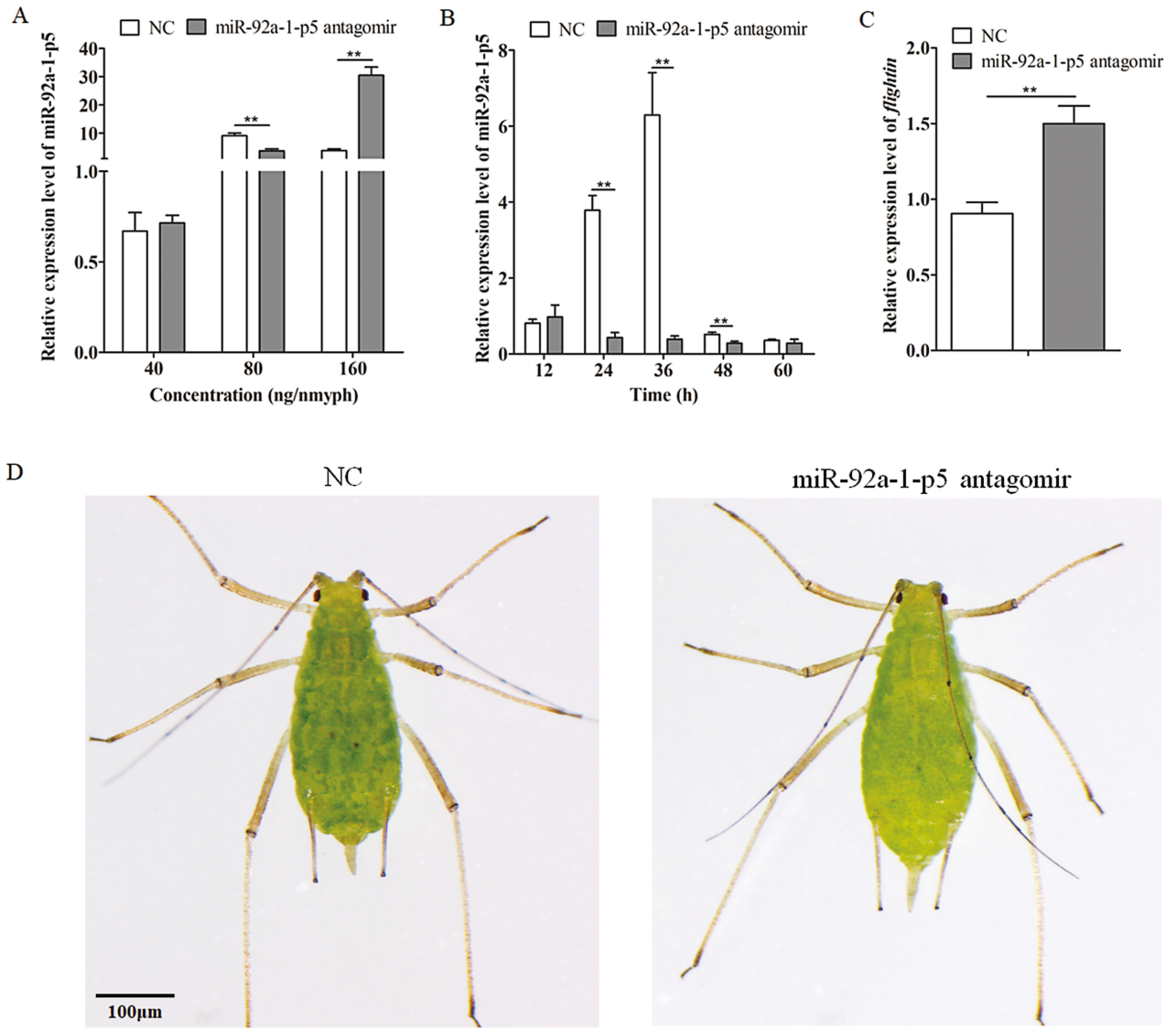


Fig. 3. Knockdown of miR-92a-1-p5 in wingless *A. pisum*. The expression levels of miR-92a-1-p5 in the second instar nymphs from the winged aphids injected with agomir of miR-92a-1-p5 at different concentrations of antagomir (A) and different sampling time (B). (C)The expression level of *flightin* at 36 h after injection of miR-92a-1-p5 antagomir. Three independent experiments were conducted, and each sample was repeated three times. Significance analysis was conducted with ANOVA and Student's *t*-test. The data were from three independent experiments (means ± SEM; $*P < 0.05$, $**P < 0.01$). (D) Phenotypes of adult aphids after injection with antagomir and negative control (NC) of miR-92a-1-p5. No obvious change was observed in wingless aphids after miR-92a-1-p5 knockdown. Scale bar represents 100 μm. Three independent experiments were conducted, and each sample had 10 individuals. NC means negative control, miR-92-1-p5 antagomir means aphids being injected with antagomir of miR-92-1-p5.

Discussion

Flightin was first identified in *Drosophila* (Vigoreaux et al. 1993). It is conserved across Pancrustacea species, including winged insects, nonwinged insects, noninsect hexapods, and several crustaceans (Xue et al. 2013). *Flightin* is expressed in juvenile instars and adults, and was localized in the antenna muscles of *Daphnia magna* (Diplostraca: Daphniidae), played key roles in maintaining IFM and male DLM structure in *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae) (Xue et al. 2013). The indirect flight muscles (IFM) are the power-producing muscles that move the wings indirectly by deformation of the thoracic exoskeleton (Crossley et al. 1978), which contains dorsoventral muscles (DVM), dorsal longitudinal muscles (DLM), and oblique dorsal muscle (ODM) in aphid (Ishikawa et al. 2008, Ogawa et al. 2012, Ogawa and Miura 2013). The DLM function as wing depressors (downstroke) and their contraction stretches the DVM, which in turn contract to elevate the wings (upstroke) and stretch the DLM (Tsuji and Kawada 1987, Ogawa and Miura 2013). Here we found that knockdown of *flightin* or overexpression of miR-92a-1-p5 both result in deformation of aphid wings, the shape of flight musculature, especially the DLM, changed severely after impairment of *flightin*, the DLM and DVM became widener and looser compared with the negative control, which caused the abnormal flight apparatus, and result in the tilt-to-one-side and curled wings of aphids. These results indicated that *flightin* is necessary to maintain normal morphology of flight muscle and wing in aphid, which can be regulated by miR-92a-1-p5.

RNA interference (RNAi) is a potential 'green' pest management strategy. The main method for dsRNA/small interfering RNA (siRNA) delivery is microinjection (Baum et al. 2007, Hammell and Hannon 2016) or ingestion (Katoch et al. 2013, Niu et al. 2018).

However, the high mortality caused by microinjection limits its practical application. A nanocarrier-mediated transdermal dsRNA delivery system was recently developed in soybean aphids for efficient RNAi (Zheng et al. 2019, Yan et al. 2020, Yan et al. 2021, Zhang et al. 2021). This technology may accelerate the development of sprayable RNA pesticides. With the nanocarrier-mediated delivery system, the protein-coding gene *flightin* and the non-coding RNA miR-92a-1-p5 were all knocked down efficiently within 48 h treatment in *A. pisum*, which may provide a new direction for pesticide development.

In summary, we found that miR-92a-1-p5 participates in the wing formation of *A. pisum* through regulating the expression of the flight muscle gene *flightin*. Repression of *flightin*, or overexpression of miR-92a-1-p5 resulted in abnormal wings and flight muscle of aphids, which might provide new clues for aphid control.

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Author Contributions

Meiling Chang: Visualization, Investigation, Data curation, Writing-Original draft preparation. Hao Cheng: Visualization, Investigation, Data curation. Zhiyan Cai, Yuxin Qian: Software, Validation. Kun Zhang, Linlin Yang: Validation. Dandan Li: Conceptualization, Methodology, Software, Writing, Supervision.

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