miR-147b-modulated expression of vestigial regulates wing-morph differentiation in the bird cherry-oat aphid Rhopalosiphum padi

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Abstract

Background The wing polyphenism occurs under crowding and nutrition-deficiency conditions in most aphid species. Although the influence of environmental factors on wing polyphenism of aphids have been extensively investigated, molecular mechanisms underlining wing-morph differentiation has not been fully understood. Results The expression levels of the twenty genes involved in wing patterning network were examined, and only vestigial (vg) showed significantly different expression levels in both whole-body and wall-body of third nymphal instars, with 5.4- and 16.14- fold higher in winged lines compared to wingless lines, respectively in Rhopalosiphum padi. Moreover, vg expressions were higher in winged aphids compared to that in wingless aphids of third, fourth instar nymphs and adults, and larger difference ratio were observed in third (21.38-fold) and fourth (20.91-fold) instar nymphs relative to adult (3.12-fold) between wing morphs. Suppression of vg using RNAi repressed the wing development of third winged morphs. Furthermore, modulation of miR-147b levels by microinjection of its mimics decreased vg expression levels and repressed wing development. Conclusions Our findings suggest that vg is essential for wing development and that miR-147b modulates its expression. To our knowledge, our results provide an empirical evidence that miRNA is involved in the regulation of wing morphs in aphids.

Background

All organisms show phenotypic plasticity depending on the environmental conditions they experienced [1]. Polyphenism is an extreme case of phenotypic plasticity in which discrete multiple phenotypes are produced based on a single genome [2]. Most aphid species exhibit wing polyphenism occurring during the asexual portion of the aphid life cycle, in which winged and wingless female are produced depending on environmental stimulus, e.g. population density and host nutrition [3]. The wingless morph specializes in reproduction, allowing rapid colony growth. In contrast, the winged form specializes in dispersal which enable aphids to seek out new habitats, mates, and food resources [4].

The bird cherry-oat aphid, *Rhopalosiphum padi* (L.), is one of the most globally abundant cereal aphid pests. In addition to directly feeding on plants, *R. padi* damages cereal crops by transmitting *Barley yellow dwarf virus*, which causes economically important crop losses [5, 6]. *R. padi*, like most aphids, can produce wing dimorphism when experiencing the crowding and poor nutrition conditions [7, 8]. It is easy to produce winged lines owing to its short life cycles and high reproductive rate [9]. Winged aphids are able to travel long distances and carry viruses in autumn which are considered as a major epidemiological factor for determining the disease incidence[10-12]. To date, the control of *R. padi* remains to rely on the application of chemical insecticides, which have leaded to insecticide resistance and environmental pollution [13].The fight abilities of winged forms and high fecundity of unwinged forms have made aphids control more difficult. Therefore, understanding the molecular mechanisms of wing morphs is important for controlling *R. padi* effectively.

Generally, wing morphs include determination and differentiation processes that occur at completely different time during aphid development. Mostly, morph determination occurs during embryogenesis in

the maternal ovarian cavity and morph differentiation (i.e. wing development/degeneration) occurs during postembryonic development [14]. Nowadays, the influence of external cues on wing dimorphism have been extensively investigated, especially environmentally regulated maternal hormone in aphids can mediate phenotype production of next generation in the wing-morph determination. Recently, molecular mechanisms of ecdysone signaling controlling wing morph determination was discovered [15]. However, definitive molecular mechanisms in wing-morph differentiation, especially the roles of wing patterning genes at early wing development stages have been less reported.

Gene networks underlying wing patterns of *Drosophila melanogaster* have been well investigated. Based on the genomic sequences, the principal wing development gene homologs are largely conserved across holometabolous and hemimetabolous insect [16]. Brisson et al. [16] examined the expression levels of 11 genes involved in wing pattering, in which 6 genes showed significantly stage-specific expression level effects and only *apterous1*(*ap1*) exhibited different expression levels (< 4- fold) during the first and second nymphal instars in *Acyrthosiphon pisum*. This is the first and only report about the wing development gene repertoire in aphids, and did not consider the expression levels of wing patterning genes in the body wall, where the wing buds extend in wing differentiation. We depicted a gene network involved in major wing patterning events for *R. padi*, including anterior-posterior (A-P) patterning genes such as *engrailed* (*en*), *hedgehog* (*hh*), *decapentaplegic* (*dpp*), *brinker* (*brk*), *optomoter-blind* (*omb*), *spalt-major* [17] [18], dorsal-ventral (D-V) patterning genes such as *apterous* (*ap1, ap2*), *Notch* (*N*), *serrate* (*ser*), *delta* (*dl*), *suppressor of hairless* (*su*(*h*)), *wingless* (*wg*), *distalless* (*dll)*, *scalloped* (*sd*), and *vestigial* (*vg*) [19], a wing hinge development gene *homothorax* (*hth*) [20, 21], a Hox gene *ultrabithorax* (*Ubx*) [22], a wing notch and blade differentiation gene *extradenticle* (*exd*) [21], and a wing intervein development gene *serum response factor* (*srf*) [23]. In this study, the expression levels of the 20 genes in the wing patterning network were examined, and only *vg* showed significantly different expressions in both whole body and the body wall in third instar nymphs between the two wing morphs of *R. padi*. The role of *vg* in wing differentiation in *R. padi* was further investigatedacross the nymphal instars and adult and by *vg* RNAi. Our results demonstrate that *vg* is essential for wing development during wing differentiation in *R. padi*. More importantly, the expression of *vg* is regulated by miR-147b. These findings provide strong empirical evidence that at least one miRNA is involved in the regulation of wing morphs in aphids.

Results

Expression profiles of wing patterning genes in wing morphs.

To determine which genes may be involved in wing differentiation during post-embryonic development in *R. padi*, we evaluated the expression levels of 20 known wing patterning genes (Fig. 1) between wingless and winged lines in the third instar nymphs using qRT-PCR. Most of genes that contribute to wing patterning and development had similar expression levels in individuals between wingless and winged lines except for *vg*, in which expression was 5.4-fold higher in the winged line than in the wingless line

(Fig. 2). Also, owing to wing bud extends from the body wall of thoracic part in winged line, these genes expression levels were also examined in body wall. Expression levels of *vg*, *sal*, *omb* and *srf* were significantly higher in body-walls. They were 16.14-, 3.16-, 4.07- and 2.77-fold higher in body-walls of winged lines, respectively, relative to body-wall of wingless lines. Altogether, our results suggest that *vg, sal, omb,* and *srf* might play roles in wing differentiation in *R. padi*.

Conserved domains of *vg* and expression patterns in wing morphs.

We obtained the full length 2471-bp *vg* cDNA that included a 456-bp 5'-untranslated region (5'UTR), a 956-bp 3'UTR and an open reading frame (ORF) of 1059-bp. The ORF encodes 670 amino acids with a predicted molecular weight of approximately 39 kDa. The cDNA sequence has been deposited in GenBank under the accession number MH168385. The VG protein contains the Vg_Tdu domain, which is highly conserved among holometabolous and hemimetabolous insects (Additional file 1).

The expression patterns of *vg* were determined in different tissues, developmental stages, and wing morphs using qRT-PCR. The results showed that the relative levels of *vg* transcripts were the highest in the body wall of the winged lines (Fig. 3A) and the lowest in the body wall of the wingless lines (Fig. 3B). This strongly indicates that *vg* plays an important role in early wing development in *R. padi*.

Wing development in the winged lines is associated with various developmental stages, and the expression levels of *vg* were stable from the first to the second nymph stage, then increased sharply from the third nymph to the adult stage in the wingless morphs (Fig. 3C). In contrast, *vg* expression increased from the first to the third instar nymphs and then decreased in the adult stage in the winged morphs (Fig. 3D). Altogether, the highest expression of *vg* was found in the third nymphal instar, and it was 9.58-fold higher relative to the first instar nymphs, during winged nymph development.

The relative expression of *vg* was higher in winged aphids than in wingless aphids in the third and fourth instar nymphs as well as in adults (Fig. 3E), and higher difference ratios were observed in third (21.38-fold) and fourth (20.91-fold) instar nymphs compared with the adult (3.12-fold) between wing morphs. However, we observed no difference in the first and second instar nymphs between wing morphs. In addition, we also investigated the VG protein expression levels, and found that there were higher levels of the protein in the body wall of third nymphal winged lines compared to that in third nymphal wingless morphs (Fig. 3F). These results suggest that the third nymphal stage is the key period of aphid wing dimorphism and *vg* might be involved in wing differentiation.

RNAi knockdown of *vg* suppresses wing development.

RNAi experiments were performed to understand the relationship between wing development and *vg* gene expression. Third nymphal instar aphids of the winged lines were injected with dsRNA. At 24 h after injection with *vg* dsRNA, the mRNA levels of *vg* decreased significantly by 44% compared to control insects injected with dsEGFP (Fig. 4A). After 48 h, all aphids molted into adults, and injection of *vg* dsRNA resulted in 68% under-developed wing aphids compared to the dsEGFP control aphids, which were 100% normal (Fig. 4B). The RNAi aphids showed under-developed wings (Fig. 4B). These results further demonstrate that *vg* plays an important role in wing development in *R. padi*.

miR-147b putatively regulates the expression of *vg*.

There was no significant difference in *vg* DNA expression levels between wing morphs from body walls of third instar nymphs (Additional file 2). Bioinformatic analysis predicted that miR-147b targets *vg*. In the *vg* ORF, there is one potential binding site that shows high sequence complementarity with miR-147b (Fig. 5A). The transcriptional levels of miR-147b in winged lines were significantly lower than in wingless lines, while its predicted target gene *vg* showed higher expression in winged lines relative to expression in wingless lines (Fig. 5A).

Because aphid wing polyphenism is associated with colony density, we examined the effect of density on the expression levels of *vg* and miR-147b in third instar nymphs of the wingless morph. No significant differences in *vg* and miR-147b expressions were observed between the body walls of third nymphal instar wingless lines from LD and HD conditions (Additional file 3).

To determine whether miR-147b can bind to *vg*, the predicted target sequences of *vg* were inserted into the pmirGLO vector to construct the recombinant vector pmirGLO-miR-147b. Firefly luciferase activity normalized against *Renilla* luciferase was significantly reduced when pmirGLO-miR-147b was co-transfected with the miR-147b agomir (mimic). However, the luciferase activity levels of the pmirGLOmiR-147b-mut construct were not dramatically affected by the miR-147b agomir compared with the unmutated constructs (Fig. 5C). These results suggest that miR-147b can regulate the expression of *vg* by binding to the target sequence in the mRNA.

miR-147b can modulate wing development.

To verify that the expression of *vg* is regulated by miR-147b, miR-147b agomir was injected into the third nymphal instars winged nymph larvae of *R. padi*, and we then examined the expressions of miR-147b and *vg* after 24 h, respectively. Compared with control group, expression levels of *vg* was decreased by 47% after injection for 24 h. Wing development was dramatically repressed in the group injected with the miR-147b agomir, which exhibited two types of phenotypes at rates of 75% and 25% (Fig. 6D); however, wing development in the control group injected with the dsRNA negative control was normal at rates of 100% after 48 h (Fig. 6C). These results demonstrated that miR-147b can affect *vg* expression and modulate wing development.

Discussion

The growth of the *Drosophila* wing has been well studied, and it is regulated by signaling molecules that are produced along the anterior-posterior (A-P) and dorsal-ventral (D-V) compartment boundaries, most notably DPP, WG, and the Notch ligands SER and DL [24, 25]. The *vg* gene is regulated by these compartment boundary signals and plays a central role in the development and patterning of the wing [26]. In the absence of *vg* gene expression, cells within the larval wing and haltere imaginal discs fail to proliferate normally in *D. melanogaster* [27]. Although there are few studies describing the role of *vg* in wing development in other insects, principal wing development gene homologs are very conserved among insects [16, 28]. Our results show that only *vg*, out of 20 genes examined in this study, shows higher expression levels in both the whole body and the body wall of winged lines, compared to wingless morphs, in *R. padi*. Importantly, the expression of *vg* shows a larger difference ratio between the two wing morphs, 16.14-fold for the body wall and 5.4-fold for the whole body between the winged and wingless morphs, respectively. The difference of *vg* expression in the body wall may play a key role in wing development. The wing buds at birth are present as slight protrusions and develop slowly in the nymphs of winged aphids [16]. This point is further supported in aphids by our results showing that *vg* transcript levels were the highest in the body wall of the winged lines, while they were the lowest in the body wall of the wingless lines. These results demonstrate that *vg* plays a role in wing differentiation in *R. padi*, and they were subsequently verified by showing that *vg* dsRNA can suppress wing development. The higher expression levels of *sal* and *srf* found in the body wall of winged lines compared to wingless lines may be caused by the significant morph effect of *vg*. This is because *sal* and *srf* act downstream of *vg*, and *vg* can regulate their expression [28]. *Optomotor-blind* (*omb*), the downstream target of *dpp*, is required for distal wing development in *Drosophila* [18]. *Omb* is expressed at higher levels in third nymphal instars of winged aphids compared to wingless aphids, while the genes from *dpp* signaling (i.e. *dpp*) show no significant differences between the two morphs. Whether the increased expression of *omb* results from higher levels of *vg* in winged aphids needs further verification. The other 16 genes had no significant morph effect in third nymphal instars. Similar results were also observed by Brisson et al. (2010), who reported that the expression of *en*, *hh*, *dpp*, *ubx*, *ap*,*wg*, *hth*, and *dll* showed no significant differences between wing morphs of third instar nymphs in *Acyrthosiphon pisum,* but they did not examine expressions of *vg*, *sal*, *omb*, or *srf*. Although the expression levels of *en*, *hh*, *sal*, *wg*, *exd*, and *Ubx* were found to be significantly different between macropterous (migratory) and brachypterous forms of *Nilaparvata lugens* [29], our study showed that there are no significant differences in the expressions of these genes between aphid morphs. There is the possibility that brachypterous adults still have short wings, while wingless aphids have no wings because they degenerate by the second larval instar during development [29, 30]. This suggests that different developmental divergence times likely require different molecular mechanisms.

By the late third nymphal instars, the VG protein is expressed in cells on both sides of the D/V compartment border in wing and haltere imaginal discs in *D. melanogaster* [31]. Expression of the *vg* gene was highest in third instar nymphs of the winged morph, while expression of *vg* was highest in wingless adults during development. Third instar nymphs showed the greatest difference ratios of *vg*

expression during development compared to other stages between the wing morphs (Fig. 3). These results provide evidence that the third nymphal stage is the key point for wing differentiation and that *vg* plays an important role in this process. It is possible that the lower expression levels of *vg* in the wing primordia of early stage (i.e. third instarnymph) leads to wing degeneration in wingless lines of *R. padi*.

Gene expression can be regulated by both transcriptional and post-transcriptional mechanisms. Transcriptional regulation is often determined by *cis*-elements located within a gene's promoter as well as by the epigenetic status of the gene and the adjacent DNA sequences [32]. The expression of *vg* during wing development is regulated by two enhancers, a boundary enhancer (vgBE) and a quadrant enhancer (vgQE) [33, 34]. The *Suppressor of Hairless* (*Su*(*h*)) gene initiates the expression of *vg* through binding with vgBE and *wg* to collaborate with Notch/Su(H) signaling in the wing margin primordium [26]. Both *wg* and *dpp* mediate vgQE to elevate and maintain the levels of *vg* expression during development of the wing blade [26, 34]. The DNA duplication of *vg* in the two morphs shows no significant difference (Additional file 2), and we found no differences in expression levels for *wg*, *dpp*, and *su*(*h*) between the wing morphs in this study (Fig. 2). Therefore, we hypothesized that *vg* expression may be post-transcriptionally regulated by MicroRNAs (miRNAs). miRNAs are endogenous non-coding RNAs that post-transcriptionally regulate transcript levels and translational status of mRNA by degrading mRNA or terminating translation [35]. In addition, several reports have shown that mRNA-miRNA interactions may lead to the stabilization of mRNA [36]. miRNAs have been shown to regulate a variety of physiological and pathological processes throughout insect development including molting, metamorphosis, oogenesis, embryogenesis, behavior, and host-pathogen interactions [37]. However, few studies have investigated the potential role of miRNAs in wing polyphenism. Yang et al. (2014) found that miR-133 controls dopamine synthesis to control the production of solitarious versus gregarious forms in *Locusta migratoria*, and direct evidence showing that miRNAs regulate wing development in aphids has yet to be reported. At present, there are few studies describing how miRNAs regulate expression of the *vg* gene. We used bioinformatics to predict that miR-147b could potentially regulate the expression of *vg*. In humans, miR-147b regulates some cellular effects including proliferation, migration, and apoptosis [38]. Importantly, miR-147b is involved in endothelial barrier function and is a potent inducer of intestinal epithelial cell differentiation [39, 40]. We found that *vg* expression is reduced and wing development is repressed after injecting the miR-147b mimic into *R. padi*. This is consistent with the target experiments in which the co-transfection of miR-147b mimics with the corresponding target plasmids significantly decreased the relative luciferase activity. Our results are the first to provide direct evidence that miR-147b-meditated regulation of *vg* expression controls wing development in aphids.

Although we determined here that *vg* plays an important role in wing differentiation in *R. padi*, wing polyphenism is involved in both initial determination and subsequent differentiation [41]. Physical contacts (tactile stimulation) caused by crowding (high density) or poor nutrition can increase aphid dispersal [4]. In *Nilaparvata lugens*, two insulin receptors regulate wing bud development by responding to an insulin-like peptide secreted by the brain, and produce long-winged or short-winged forms [42]. Recently, the molecular mechanisms of ecdysone signaling in the control of wing morph determination were also determined in *A. pisum* [15]. High density has no effect on the expression of either *vg* or miR-

147b in third instar nymphs (Additional file 3). Therefore, we propose a hypothesis to explain wing polyphenism in aphids that includes four processes; 1) environmental factors cause endocrine changes, 2) the increase in the hormone signal results in increased expression of miR-147b in wing primordia, 3) miR-147b negatively regulates expression of *vg* by binding to the mRNA, and 4) wing discs degenerate in the wingless lines owing to the lack of *vg* expression (Fig. 7). The opposite occurs in the winged lines, where *vg* is expressed at high levels in the wing primordia.

Conclusions

In this study, the expression levels of the 20 genes in the wing patterning network were examined, and only vg showed significantly different expressions in both whole body and the body wall in third instar nymphs between the two wing morphs of *R. padi*. The role of *vg* in wing differentiation in *R. padi* was further investigated across the nymphal instars and adult and by vg RNAi. Our results demonstrate that vg is essential for wing development during wing differentiation in *R. padi.* The results indicate that *vg* is involved in wing differentiation and is overexpressed in the winged morphs due to increased transcription rather than *vg* gene duplication in *R. padi.* We have shown that *vg* transcription is post-transcriptionally regulated by miR-147b, which binds to its target sequence present in the *vg* mRNA. These results provide an empirical evidence that miRNA is involved in the regulation of wing morphs in aphids.

Methods

Insects and cell line culture.

Colony of *Rhopalosiphum padi* was collected from a wheat field at the Agricultural Experiment Station of China Agricultural University (N40°03', E116°28') in May 2005 [9]. The stock parthenogenetic colony was derived from a single apterous female from the colony and maintained > 10 generations in plastic petri dishes containing wheat seedlings in a climate controlled chamber under the following conditions: a temperature of 22±1°C, relative humidity [1] of 50±10 %, and a photoperiod of 16 h:8 h (day:night). Both wing morphs were induced by manipulating the colony density. The stock parthenogenetic colony was divided into two groups. For the high-density [43] condition to induce the winged morph, >30 adult wingless aphids were reared on wheat seedlings in each plastic peri dish (9 cm diameter, 20 cm tall), and the induction ratio of winged aphids under HD conditions was 43.0% ± 17.4 % (n=300 ± 38.4). Under the low-density (LD) condition, only one wingless adult was reared on wheat seedlings, and 100% (n=63 ± 4.8) wingless aphids were induced. All of the wingless morphs used in our study were obtained from the LD condition, and the winged morphs were induced under HD conditions except for the effect of density on gene expression in which the wingless morphs from HD conditions are also used.

The mammalian HEK293T cell line was maintained at 37°C under a 5% CO_2 atmosphere in DMEM high-glucose medium (Gibco) containing 10% fetal bovine serum (Gibco).

RNA extraction and cDNA synthesis.

To determine the expression profiles of genes at different developmental stages, and in wing morphs and body parts, the following samples were collected for RNA isolation: 20 individuals from each developmental stage (each instar nymph and adult) of apterous and alate aphids for whole body and body wall. Body cavity, and body wall were dissected from the third instar nymphal morphs under a binocular microscope and washed in cold phosphate-buffered saline (PBS: 130 mM NaCl, 7 mM $Na_2HPO_4 \cdot 2H_20$, 3 mM $NaH_2PO_4 \cdot 2H_2O$; pH 7.0). Total RNA was isolated using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. An additional DNaseI digestion was performed using RNase-Free DNaseI (Takara, Dalian, China). First-strand cDNA synthesis was carried out with a Reverse Transcription System (Takara) according to the manufacturer's instructions.

Small RNAs were isolated from aphids using the miRNeasy Mini Kit (Qiagen, Germany) following the manufacturer's protocol. First-strand cDNA was synthesized from 2 μg of total RNA using the miScript II RT kit (Qiagen) as directed by the manufacturer. Genomic DNA was extracted from body wall of third nymphal wing morphs, and performed as previously described methods [44].

Quantitative real-time PCR (qRT-PCR).

qRT-PCR was performed on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems) using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) kit (Takara, Japan). The cycling program for qRT-PCR assays for miRNA or mRNA was as follows: initial incubation at 50°C for 2 min and then at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s according to the manufacturer's protocol. Analysis of the qRT-PCR data was carried out using the $2^{-\Delta\Delta Ct}$ method of relative quantification. As an endogenous control, the EF-1α and U6 snRNA transcripts were used to normalize the expression level of mRNA (or DNA) and miRNA, respectively [44, 45]. Three biological replicates over at least two days and two dish aphid samples were carried out for each test. All primers were designed based on information from the transcriptome library of *R. padi* and were listed in additional file 4.

Cloning and sequence analysis of *vg*.

Total RNA from a mixed sample consisting of 60 aphids from various developmental stages and morphs was isolated as described above. For amplification of a partial *vestigial* cDNA sequence, PCR primers were designed based on information from the transcriptome library of *R. padi*. The 5'- and 3'-ends of the cDNA molecules were amplified using the rapid amplification of cDNA ends [46] method with the Gene-RACE Kit (Takara Biotechnology, Dalian, China) following the manufacturer's instructions. BLAST searches for homologous sequences and the prediction of conserved regions were performed on the National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

RNAi.

The TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific, Wilmington, DE, USA) was used for double-stranded RNA (dsRNA) synthesis following the manufacturer's instructions. Samples of approximately 13.8 nL (1000 ng/μL) were injected into thorax segments of each aphid using a micro-injector (Nanoliter 2000 Injector, WPI Inc.). Controls were injected with double-stranded Enhanced Green Fluorescent Protein (dsEGFP). More than 100 injected aphids were placed on wheat seedlings to recover and were then reared under laboratory conditions. A total of 20 injected aphids were randomly collected at 24 h post-injection for the subsequent qRT-PCR analyses as described above. Photos were taken with a Leica M165C microscope (Leica Microsystems, Wetzlar, Germany) 48 h after injection. All experiments were independently repeated at least three times.

Quantification of miR-147b and *vg* expression.

miR-147b was predicted to target *vg* by the two miRNA target prediction programs miRanda (http://www.microrna.org/microrna/getDownloads.do) and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/welcome.html) using miRNA library of *R. padi*. The expression of both miR-147b and *vg* were validated in the body walls of third nymphal wing morphs using qRT-PCR. A total of 20 individuals were used as a biological replicate for total RNA extraction and qRT-PCR, and three replicates were performed.

Dual luciferase reporter (DLR) assay.

The agomir (mimic) of miR-147b was designed and synthesized by GenePharm Co. Ltd (Shanghai, China). The miRNA agomir is a dsRNA formed from the miRNA and its complimentary sequence a chemical modification. The negative control [47] was designed based on a *Caenorhabditis elegans* miRNA with no similarity to insect miRNAs. Two 226-bp fragments containing the miR-147b predicted target sites and the mutated miR-147b target DNA sequence were amplified by PCR and inserted downstream of the luciferase gene in the pmirGLO vector (Promega, USA) between the PmeI and XhoI restriction sites to give the pmirGLO-miR-147b and pmirGLO-miR-147b-mut target constructs. The dual luciferase reporter (DLR) assay was performed as previously described [45]. HEK293T cells were cultured in a 24-well plate and transfected with the target plasmids and either the miRNA agomir or NC using the Calcium Phosphate Cell Transfection Kit (Beyotime, Nanjing, China) according to the manufacturer's instructions. Each well contained 0.2 μg plasmid DNA with 100 nM final concentration of the miRNA agomir. Luciferase assays were performed using the Dual-Glo® Luciferase Assay System (Promega) 24 h post-transfection. Normalized firefly luciferase activity (firefly luciferase activity/ *Renilla* luciferase activity) was compared to that of the control pmirGLO Vector. The mean of the relative luciferase expression ratio (firefly luciferase/ *Renilla* luciferase) of the control was set to 1. For each transfection, the luciferase activity was averaged from five replicates.

Modulation of miRNAs and the subsequent impacts on wing development.

The third instar winged aphids were selected for injection. Each aphid was injected with 13.8 nL of a 40 μM agomir solution, and the control was injected with agomir-NC. At 24 h post-injection, the 20 nymphs in each sample were collected and total RNA was extracted using TRIzol. The relative expression levels of *vg* and miR-147b were determined using qRT-PCR. The wing morphs were recorded 48 h after injection. All experiments were performed in triplicate.

Abbreviations

A-P: Anterior-posterior; D-V: Dorsal-ventral; qRT-PCR: Quantitative real-time reverse transcription polymerase chain reaction; UTR: Untranslated region; ORF: Open reading frame; RNAi: RNA interference; miRNAs: microRNA.

Declarations

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Availability of data and materials

The cDNA sequences from the study has been deposited in GenBank under the accession number MH168385.

Author Contributions

XWG and YJF conceived and designed the experiments. YJF performed the experiments. AAHM and XXL analyzed the data. YL conducted miRNA predication. XWG contributed reagents/materials. YJF, XWG and XXL wrote the paper. All authors have read and approved the final manuscript.

Competing Interests

The authors declare they have no competing interests.

Consent for publication

Not applicable

Ethics approval and consent to participate

Not applicable

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Figures

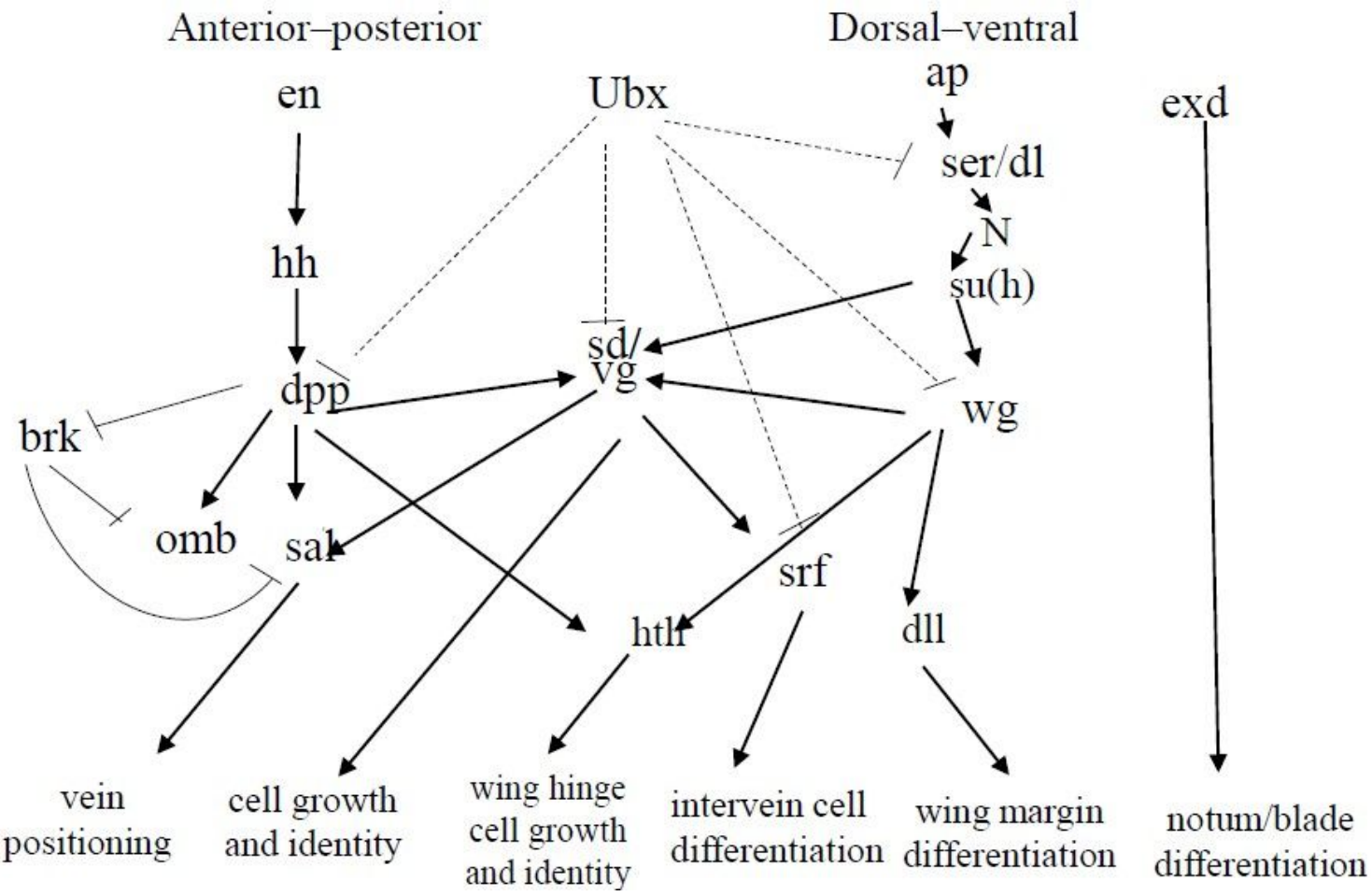

Figure 1

The presumptive wing-patterning network in Drosophila [16, 48]. The aphid orthologs of these Drosophila genes were examined in this study. Abbreviations: en, engrailed; hh, hedgehog; dpp, decapentaplegic; brk, brinker; omb, optomoter-blind; sal, spalt-major; Ubx, ultrabithorax; sd, scalloped; vg, vestigial; ap, apterous; ser, serrate; dl, delta; N, notch; su(h), suppressor of hairless; wg, wingless; dll, distalless,; hth, homothorax; srf, serum response factor; exd, extradenticle. Dashed lines indicate regulatory interactions specific to the hindwing disc, arrowheads and bars indicate activation and repression, respectively.

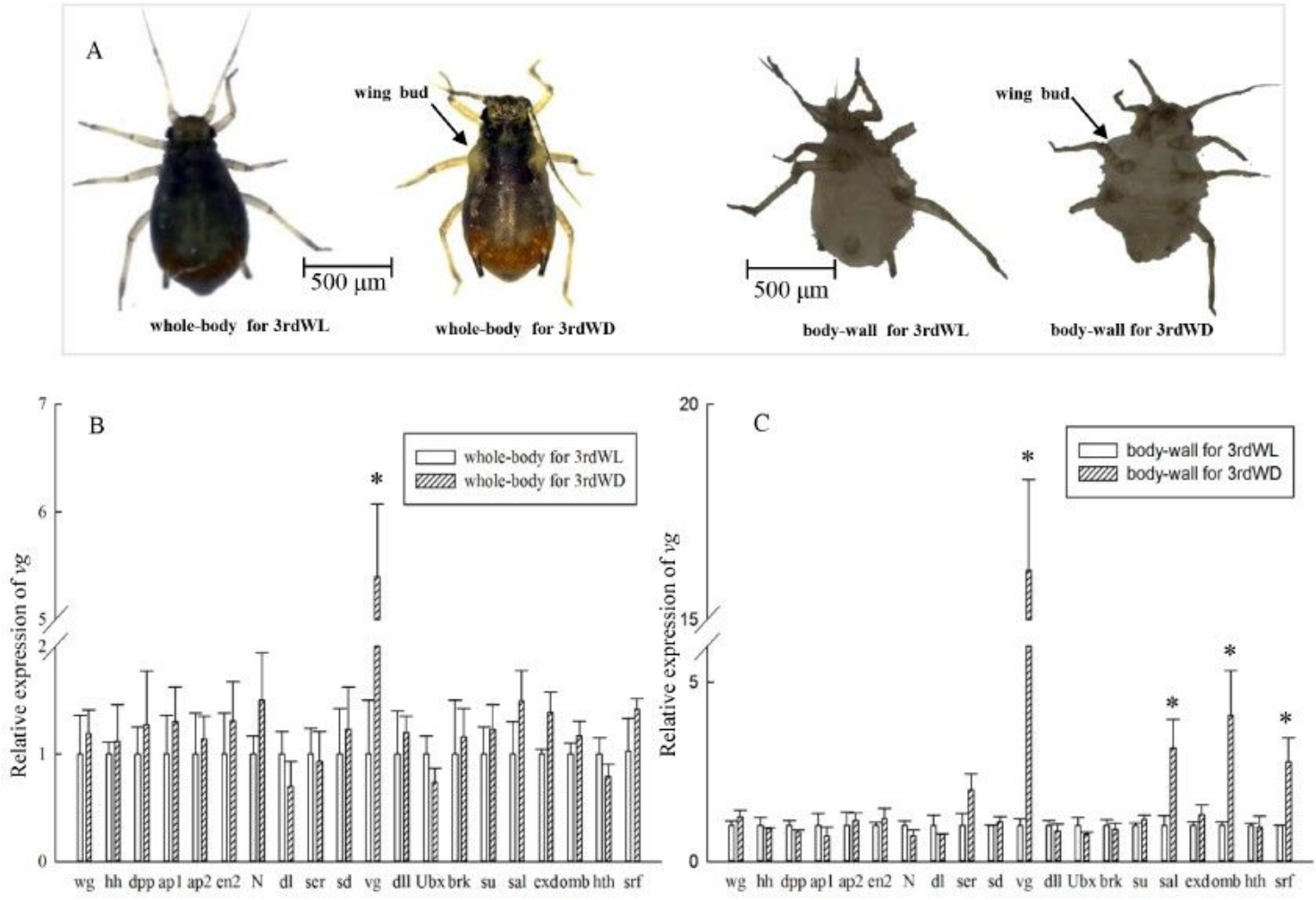

Figure 2

Expression profiles of wing patterning genes in third instar nymphs of wingless (3rdWL) and winged (3rdWD) morphs of R. padi. (A) Phenotypes of whole body and body walls of third nymphal instar wing morphs in R. padi. Expression profiles of wing patterning genes in whole bodies (B) and body walls (C). Date are means ± SD. An asterisk (*) indicates significance according to Student's t-test ($P < 0.05$).

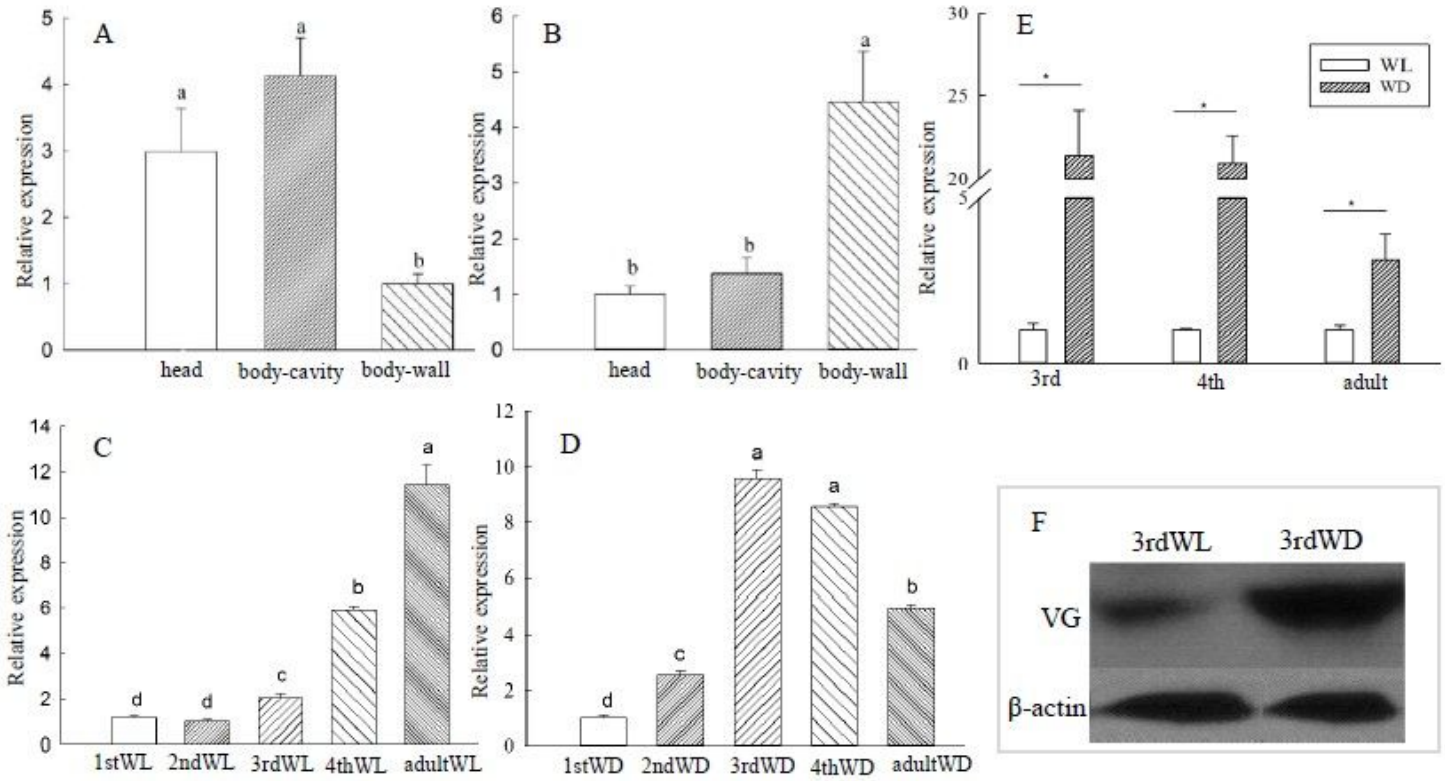

Figure 3

Expression profiles of vg in different body parts, developmental stages, and wing morphs of R. padi. Vg expression levels in different tissues of third instar wingless (A) and winged morphs (B). Expression levels of vg in wingless (C) and winged lines (D) during development. (E) Comparison of vg expression levels between wing morphs during development. (F) Expression levels of VG in body walls of third nymphal instar wingless (3rdWL) and winged (3rdWD) morphs. Abbreviations: first (1st), second (2nd), third nymphal instars (3rd), and fourth (4th) instar nymphs, wingless (WL), winged (WD). Different letters on the histogram bars indicate significant differences based on ANOVA followed by Tukey's HSD multiple comparison test (P <0.05). *Significant difference according to Student's t-test (P <0.05).

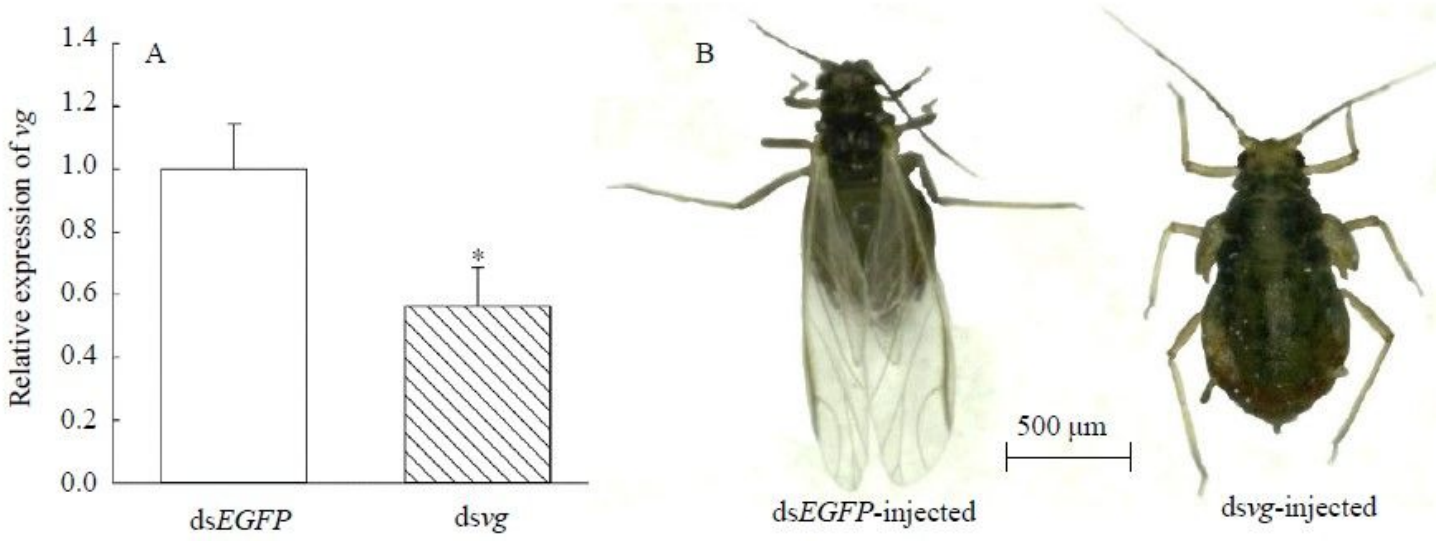

Figure 4

RNAi of vg and its effects on wing development of R. padi. (A) RNAi-mediated suppression of vg transcripts in third instar winged aphid injected with dsRNA-vg for 24 h. (B) Phenotypes of third nymphal instars winged aphid after injected with dsRNA of vg for 48 h.*indicated significant difference according to Student's t-test (P < 0.05).

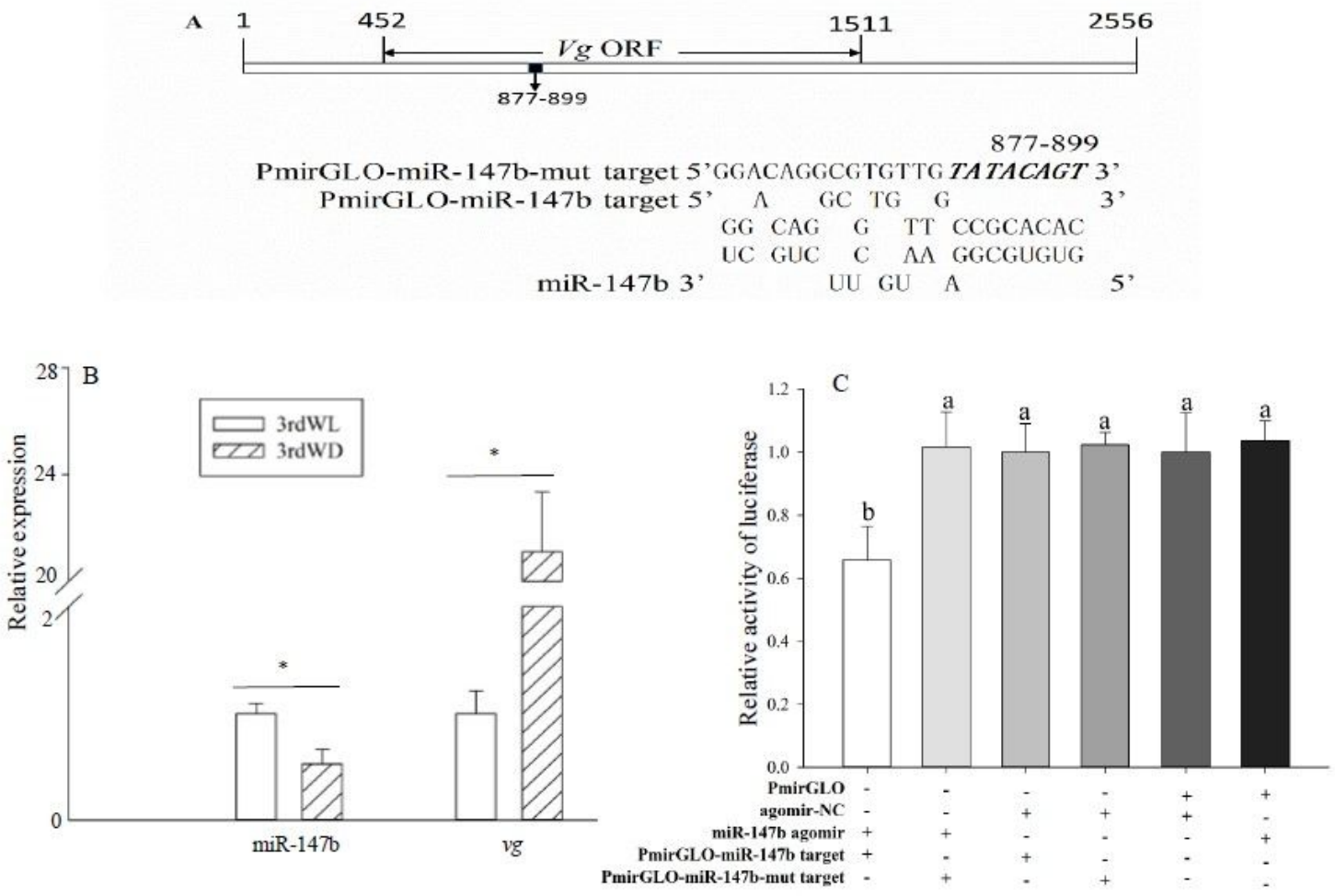

Figure 5

Functional validation of the miR-147b target sites in vg. (A) Predicted sites of miR-147b in the ORF of vg. (B) Relative expression levels of miR-147b and vg in the body walls of third wingless (3rdWL) and winged (3rdWD) lines. (C) Luciferase reporter assays were performed by co-transfecting the miR-147b agomir with a luciferase reporter gene linked to the vg targets. Mutated nucleotides are shown in bold italics. Different letters on the histogram bars indicate significant differences based on ANOVA followed by Tukey's HSD multiple comparison test (P <0.05). * indicates a significant difference based on Student's t-test (P <0.05).

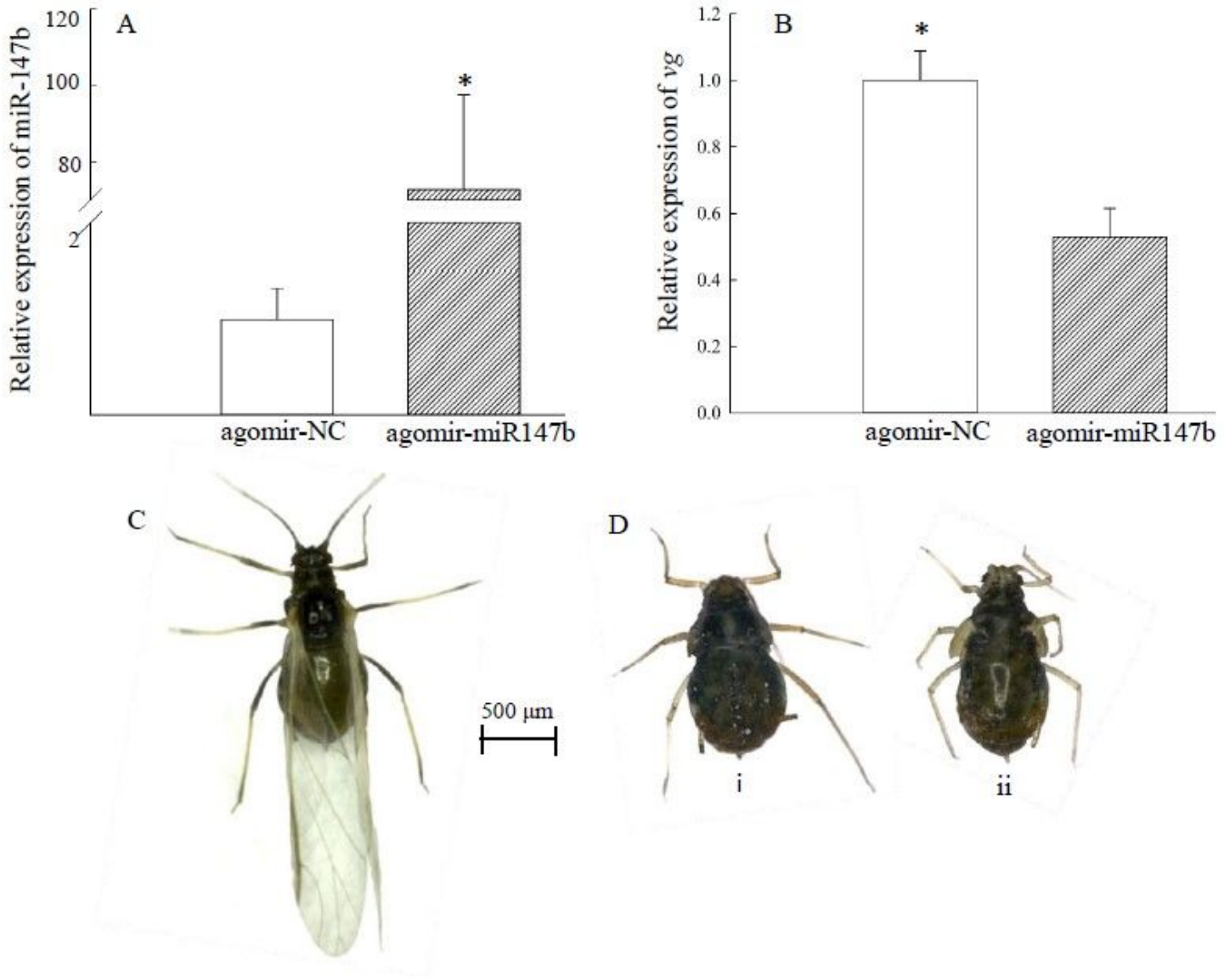

Figure 6

The effect of miR-147b on wing development. The expression levels of miR-147b (A) and vg (B) in third instar nymph winged lines after injection of miR-147b agomir for 24 h, resepectively. Phenotypes of third nymphal winged aphid after injecting with agomir-NC (C) and miR-147b agomir (D) for 48h, D (i) and [43] phenotypes are at rates of 75% and 25%, respectively. *Significant difference according to Student's t-test ($P < 0.05$).

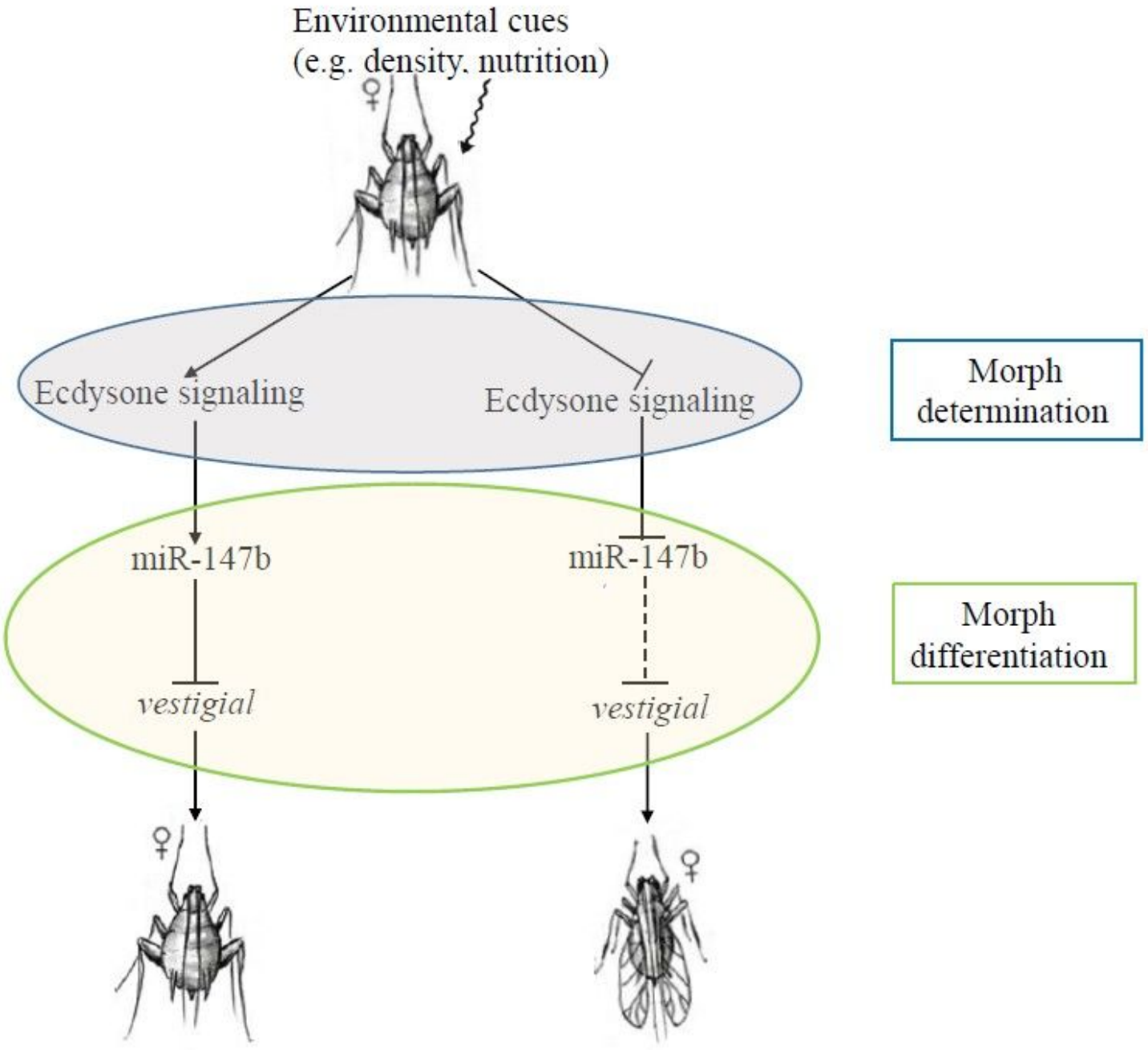

Figure 7

A proposed molecular mechanism for wing polyphenism in aphids. The process includes morph determination and morph differentiation shown in blue and pink, respectively. The dashed line indicates inactivation. Arrowheads and bars indicate activation and repression, respectively. Environmental factors (low density or good nutrition) activate ecdysone signaling in parthenogenetic aphids. The increase in ecdysone signaling leads to increased expression of miR-147b which then negatively regulates the expression of vg by binding to its mRNA in wing primordia, leading to degeneration of the primordia in the wingless morph. The opposite occurs in the winged morph when ecdysone signaling is repressed by external conditions such as high density or poor nutrition.

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