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Subtle roles of microRNAs let-7, miR-100 and miR-125 on wing morphogenesis in hemimetabolan metamorphosis

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ABSTRACT

In most insect species, the microRNA (miRNA) let-7 clusters with miR-100 and miR-125 in the same primary transcript. The three miRNAs are involved in developmental timing in the nematode *Caenorhabditis elegans* and in the fly *Drosophila melanogaster*. In the cockroach *Blattella germanica*, the expression of these miRNAs increases dramatically in the wing pads around the molting peak of 20-hydroxyecdysone (20E) of the last instar nymph. When let-7 and miR-100 were depleted with specific anti-miRNAs in this instar, the resulting adults showed wings reduced in size (when miR-100 was depleted) or with malformed vein patterning (when let-7 and miR-100 were depleted). Depletion of miR-125 induced no apparent effects. Interestingly, the wing phenotype obtained after depleting let-7 and miR-100 is similar to that resulting from silencing the expression of Broad-Complex (BR-C) transcription factors with RNA interference (hindwings with a short CuP vein, with the vein/inter-vein pattern disorganized in the anterior part and showing anomalous bifurcations of the A-veins in the posterior part).

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1. Introduction

MicroRNAs (miRNAs) are small non-coding RNAs of about 21– 22 nucleotides that modulate gene expression at the post-transcriptional level, frequently in the context of developmental and morphogenetic processes (Ambros, 2004; Bartel, 2009; Bushati and Cohen, 2007). MiRNAs are transcribed as part of a primary transcript (pri-miRNA), which contains one or more miRNA precursors (pre-miRNAs). In the nucleus, the pri-miRNAs are processed into hairpin-structured pre-miRNAs by the ribonuclease drosha, and exported to the cytoplasm, where they are cleaved by the ribonuclease dicer-1 into an imperfectly paired duplex. The 5'- and 3'strands of the paired duplex can give either one or two respective mature miRNAs (Bartel, 2004; Ghildiyal and Zamore, 2009).

Historically, lin-4 (an ortholog of miR-125) was the first miRNA discovered, using *Caenorhabditis elegans* as model (Lee et al., 1993). However, these kinds of factors were not recognized as a distinct class of biological regulators until the early 2000s, when let-7 was found to be a developmental-time regulator in *C. elegans* (Reinhart et al., 2000) and it was recognized that its sequence and possibly its functions are conserved across animal phylogeny (Pasquinelli et al., 2000). With the exception of the silkworm *Bombyx mori* and the pea aphid *Acyrthosiphon pisum*, in all studied insects including *Drosophila melanogaster* (Bashirullah et al., 2003;

Sempere et al., 2003), let-7, miR-100 and miR-125 cluster together in the same primary transcript. In that of *B. mori*, the precursor of miR-125 is completely absent and a different miRNA (miR-2795) clusters with let-7 and miR-100. In the primary transcript of *A. pisum*, only a part of the miR-125 precursor sequence is present together with the complete precursors of let-7 and miR-100 (Legeai et al., 2010).

In *D. melanogaster*, expression of let-7, miR-100 and miR-125 is enhanced by 20E (Chawla and Sokol, 2012; Garbuzov and Tatar, 2010), and the 20E response is mediated by Broad-Complex (BR-C) transcription factors (Sempere et al., 2002). In *D. melanogaster*, the BR-C gene is expressed in the last instar larvae and in the prepupae, and triggers pupal morphogenesis (Kiss et al., 1988). Moreover, expression of let-7 and miR-125 (miR-100 was not studied) follows the BR-C expression pattern (Sempere et al., 2002). In *B. mori*, expression of let-7 significantly increases in late larval instars, which shows maximal levels in the prepupal and pupal stages (Liu et al., 2007).

The mentioned expression data suggest that these miRNAs may be related to metamorphosis (Belles et al., 2011), and a number of studies have shown that different morphogenetic processes of *D. melanogaster* are affected by let-7 and/or miR-125 depletion, such as the terminal cell-cycle exit in wing formation and the maturation of neuromuscular junctions (Caygill and Johnston, 2008). Let-7 also plays an important role in abdominal neuromusculature remodeling (Sokol et al., 2008) as well as in innate immunity (Garbuzov and Tatar, 2010). By contrast, there is little functional data

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on miR-100 as no significant effects were observed after depleting it in *D. melanogaster* (Sokol et al., 2008). A dramatic demonstration that miRNAs, in general, are involved in insect metamorphosis was reported by Gomez-Orte and Belles (2009), who observed that knockdown of dicer-1 with RNA interference (RNAi) in the last nymphal instar of the cockroach Blattella germanica depleted miR-NAs and led to the formation of supernumerary nymphs, instead of adults, after the following molt (Gomez-Orte and Belles, 2009). To gain more information about which miRNAs may be important for B. germanica metamorphosis, we recently constructed two miRNA libraries, one with RNA extracted around the molting peak of 20hydroxyecdysone (20E) of the penultimate nymphal instar, and the other equivalently extracted from the last nymphal instar (Rubio et al., 2012). We identified a number of miRNAs that are differentially expressed in these two stages. Among them, let-7, miR-100 and miR-125 emerged as being clearly upregulated in the last. metamorphosing nymphal instar (Rubio et al., 2012). The present paper reports the particular role of these three miRNA in B. germanica metamorphosis.

2. Materials and methods

2.1. Insects, dissections and RNA extracts

Specimens of *B. germanica* were obtained from a colony reared in the dark at 30 ± 1 °C and 60-70% relative humidity (r.h.) Freshly ecdysed female nymphs were selected and used at the appropriate ages. All dissections and tissue sampling were carried out on carbon dioxide-anesthetized specimens. We performed total RNA extraction from the whole body (excluding the head and the digestive tube to avoid ocular pigments and intestine parasites) and from the wing pads, using the miRNeasy extraction kit (QIAGEN).

2.2. Cloning of miRNA precursors

In order to enhance the concentration of miRNA precursors, we used specimens with dicer-1 expression depleted by RNAi. Following this strategy, RNA was extracted from the last instar female nymphs that had been treated with dsDicer-1 as described by Gomez-Orte and Belles (2009), and retrotranscribed with the NCode™ miRNA first-strand synthesis and quantitative Real Time PCR (gRT-PCR) kit (Invitrogen) following the manufacturer's protocol. Then, we performed a 3'-rapid amplification of cDNA ends (3' RACE) using the mature sequences of let-7, miR-100 and miR-125 of B. germanica as forward primers. In the case of let-7 we obtained two fragments, one of 21 bp and the second one of 69 bp, for miR-100 we obtained two fragments of 21 and 67 bp, and for miR-125 we also obtained two fragments of 21 and 67 bp. All PCR products were subcloned into the pSTBlue-1 vector (Novagen) and sequenced. Folding of the putative precursors obtained was predicted using RNA fold (Gruber et al., 2008).

2.3. qRT-PCR studies

To establish the expression patterns of miRNAs, three biological replicates were used. Samples dissections and total RNA extraction was carried out with the procedure described above, and quantification of miRNA levels was performed by qRT-PCR. Amplification reactions were carried out using IQTM SYBR Green Supermix (BioRad) and the following protocol: 95 °C for 2 min, and 40 cycles at 95 °C for 15 s and 60 °C for 30 s, in a MyIQ Real-Time PCR Detection System (BioRad). A dissociation curve was carried out to ensure that there was only one product amplified after the amplification phase. All reactions were run in triplicate. The U6 from *B. germanica* (accession number FR823379) was used as a reference

gene and all procedures were made as previously reported (Cristino et al., 2011). Results are given as copies of RNA per copy of U6. Primer sequences are indicated in Table S1. Quantification of BR-C mRNA levels was carried out with equivalent procedures, as described by Huang et al. (2013), using the primers indicated in Table S1.

2.4. miRNA depletion

To deplete let-7, miR-100 and miR-125 levels in *B. germanica*, we used miRCURY LNA[™] microRNAs Power Inhibitors (Exigon). We performed two abdominal injections of **1 µl of LNA at 50 µM, the first injection carried out in the third day, and the second in the fifth day of the last instar nymph. Samples were collected 24 h after the second injection. Controls were injected equivalently with miRCURY LNA™ microRNA Inhibitor Negative Control A (Exigon). We used 20 specimens per experiment for phenotypic studies, and 3 biological replicates to measure miRNA depletion. RNA extraction and quantification of miRNA and mRNA levels were performed as described above. Statistical analyses between groups were tested by the REST 2008 program (Relative Expression Software Tool V 2.0.7; Corbett Research) (Pfaffl et al., 2002). This program makes no assumptions about the distributions, evaluating the significance of the derived results by Pair-Wise Fixed Reallocation Randomization Test tool in REST (Pfaffl et al., 2002).

2.5. Wing biometrics

Biometric measurements of the wings were performed in 1day-old control and treated female adults. The wings were dissected from carbon dioxide-anesthetized specimens and mounted in slides with Mowiol. Axiovision software was used to obtain photographs and measures. Maximal length (Lmax) and maximal width (Wmax) were measured on the forewings (tegmina) and hindwings.

2.6. RNAi of BR-C

The detailed procedures for RNAi of BR-C have been described by Huang et al. (2013). A dsRNA encompassing a 326-bp fragment of the BR-C core region (dsBR-C) was designed to deplete all isoforms simultaneously. The primers used to generate the fragment to prepare dsBR-C are indicated in Table S1. The fragment was amplified by PCR and cloned into the pSTBlue-1 vector. A 307 bp sequence from *Autographa californica* nucleopolyhedrosis virus (Accession number K01149, from nucleotide 370 to 676) was used as control dsRNA (dsMock). A volume of 1 μ l of the respective dsRNA solution (3 μ g/ μ l) was injected into the abdomen of freshly ecdysed fifth instar female nymphs (Huang et al., 2013). BR-C transcript decrease resulting from the treatment was determined on day six of the next (last) instar nymph. Dissections, RNA extraction, retrotranscription and RNA quantification was carried out as described above.

3. Results

3.1. The sequences of let-7, miR-100 and miR-125 precursors are conserved in B. germanica

Cloning of let-7, miR-100 and miR-125 precursors (pre-let-7, pre-miR-100 and pre-miR-125) was accomplished by a 3' RACE approach using the mature sequences of the miRNAs as primers, which had been obtained from a *B. germanica* small RNA library (Cristino et al., 2011). The amplifications rendered cDNAs of 69 bp for pre-let-7, 67 bp for pre-miR-100 and 67 bp for pre-

miR-125. Alignment of these sequences with the precursors of let-7, miR-100 and miR-125 of all insect species recorded in the miR-Base (Griffiths-Jones, 2004) (Release 18) showed that they have a high degree of conservation (Fig. S1). The most conserved region is that corresponding to the canonical mature miRNA sequence in each pre-miRNA. The region corresponding to the passenger strand is also considerably conserved in the case of pre-let-7 and pre-miR-125, although it is less conserved in the pre-miR-100. The inferred folded structure of these three sequences shows the typical hairpin structure that characterizes miRNA precursors (Fig. S2).

3.2. Expression of let-7, miR-100 and miR-125 peaks in the last nymphal instar

The expression of let-7, mir-125 and miR-100 was studied in whole body samples in penultimate (N5) and last (N6) nymphal instars, as well as in selected days of younger nymphal instars (from N1 to N4) and in the adult. Results showed that the three miRNAs are detectable in all life cycle stages of *B. germanica*, although expression levels increase in N5 and N6, and peak on day 6 of the latter instar, coinciding with the 20E peak (Fig. 1A). In addition, we studied the expression of the three miRNAs in the forewing and hindwing pads in the three days encompassing the 20E peak (days 5–7) of N6, and results showed that expression of all three miRNAs increase from day 5 to day 7 (Fig. 1A).

3.3. LNA treatment effectively depletes let-7, miR-100 and miR-125 levels

To study the function of let-7, miR-100 and miR-125, we depleted the corresponding miRNA levels with specific LNAs. We injected two doses of 1 μ l of LNA at 50 μ M in the N6, one 3 days after the molt (N6D3) and another 2 days later (N6D5). The LNAs were injected separately so that the specific effect on each miRNA (LNA for let-7, LNA for miR-100 and LNA for miR-125) could be observed. On day 6 of the last instar nymph (N6D6) the levels of the respective miRNAs had specifically decreased in the wing pads according to the specific LNA treatment (Fig. 1B). LNA treatment for let-7 and miR-100 specifically reduced these miRNAs by 96% and 91%, respectively. The treatment against miR-125 led to a decrease of the three miRNAs although it was less dramatic for miR-100 (80%) and let-7 (50%) than for miR-125 (96%).

3.4. Functions of let-7, miR-100 and miR-125 are associated with wing development

The treatment with LNA for miR-100 affected the size of the wings. All (n = 30) adult specimens that received a treatment with this LNA showed forewings and hindwings somewhat although significantly smaller than those of controls (Fig. 2A). Moreover, 18 out of 30 specimens had wrinkled wings that were not properly extended, a feature that we categorized as defect A. The same defect was found in 20 out of the 40 specimens treated with LNA for let-7 (Fig. 2B). A closer examination of the hindwings of individuals treated with LNA for let-7 or LNA for miR-100 with defect A, led to the observation of a number of vein patterning defects, including disorganization of the vein/inter-vein pattern in the anterior part of the hindwing (defect B), and atypical A-vein bifurcations in the posterior part (defect C; Fig. 2C). Only those individuals that were not able to properly extend the wings showed one or more of these defects (Table S2). It is worth noting that the wings of these specimens are significantly more fragile than controls as they easily break during the process of manual extension on a slide to view under a microscope. Depletion of miR-125 did not elicit any



Fig. 1. Expression patterns and depletion of let-7, miR-100 and miR-125 in *B. germanica*. (a) Expression patterns of let-7, miR-100 and miR-125 in female whole body (black points and continuous line, left side ordinates) and in female wing pads (black bars, right side ordinates) during the sixth nymphal instar. qRT-PCR data represent the mean \pm SEM and each point represents 3 biological replicates. The discontinuous line represents the 20-hydroxyecdysone titers according to Romaña et al. (1995). (b) Levels of let-7, miR-100 and miR-125 measured on day six of sixth instar female nymphs after the respective depletion with anti-miR treatment. Experimental females received two injections of 50 µM of LNA on day 3 and day 5 of last nymphal instar; treatments were with LNA for let-7, LNA for miR-100 or LNA for miR-125; control females received an equivalent treatment with miRCURY LNATM microRNA Inhibitor Negative Control qRT-PCR data represent 3 biological replicates statistically significant differences with respect to controls (p < 0.05) according to the REST software tool (Pfaffl et al., 2002).

defect as the adults emerging from the treated nymphs were practically identical to control specimens.

3.5. Depletion of BR-C results in lower levels of let-7, miR-100 and miR-125

Interestingly, the phenotype obtained with the LNA treatments for let-7 and miR-100 resembles that obtained when BR-C mRNA levels are depleted, as described by Huang et al. (2013). The phenotype of BR-C knockdowns concentrates in the hindwings, which, with respect to controls, are smaller, have a shorter CuP vein (leaving a notch in the wing edge at the CuP end), and show disorganized vein/inter-vein patterning in the anterior part and broken A-veins, especially in the posterior part (Huang et al., 2013). In light of these data, we tested whether expression of let-7, miR-100 and miR-125 might be influenced by BR-C in B. germanica. Our experiments showed that BR-C RNAi treatment on freshly emerged N5 nymphs led to an affective depletion of BR-C mRNA levels (Fig. 3A) and concomitant, albeit relatively modest, decreases of let-7, miR-100 and miR-125 levels in N6 (Fig. 3B). Specimens with depleted BR-C molted to the adult stage with malformed wings, showing the typical defects of BR-C knockdowns



Fig. 2. Effects of let-7, miR-100 and miR-125 depletion in *B. germanica*. (a) Forewing and hindwing biometrics in adult specimens resulting from the treatments; maximal length (Lmax) and maximal width (Wmax) were measured in the forewing (tegmina) and the hindwing (membranous wing). Data represent 8 biological replicates; the asterisk indicates statistically significant differences (*t*-test, *p* < 0.01). (b) General aspect of the adult female of a control specimen and specimens that had been treated with LNA for let-7 and LNA for miR-100, showing the wings wrinkled, not properly extended (defect A). (c) Typical adult hindwings in control females (dsMock-treated) and in specimens that were treated with LNA for let-7 and LNA for miR-100. Arrows indicate the main defects observed: vein-intervein patterning disorganized in the anterior part (defect B), and A-veins broken in the posterior part (defect C).



Fig. 3. Effects of BR-C depletion by RNAi in *B. germanica*. (a) BR-C mRNA levels on day 6 of sixth instar female nymphs in specimens that were treated with dsBR-C on freshly emerged fifth nymphal instar. qRT-PCR data represent 3 biological replicates and are normalized against the control females (reference value = 1); the asterisk indicates statistically significant difference with respect to controls (p < 0.05) according to the REST software tool (Pfaffl et al., 2002). (b) let-7, miR-100 and miR-125 levels on day 6 of sixth instar female nymphs in specimens that were treated with dsBR-C on freshly emerged fifth nymphal instar. qRT-PCR data, replicates and statistics as in panel B. (c) Typical adult hindwings in control females (dsMock-treated) and in specimens that were treated with dsBR-C on freshly emerged fifth nymphal instar. qRT-PCR data, replicates and statisticates the main defects observed: notch in the wing edge at the CuP end, vein-intervein patterning disorganized in the anterior part, and A-veins broken in the posterior part.

summarized above and described in detail by Huang et al. (2013) (Fig. 3C). Indeed, the wing defects are similar to those found when depleting miR-100 and let-7, except for the absence of the character of the shorter CuP vein and associated edge notch, which is typical of BR-C knockdowns.

4. Discussion

As first demonstrated in *D. melanogaster* by Bashirullah et al. (2003) and Sempere et al. (2003), let-7, miR-100 and miR-125 are clustered in the same primary transcript in many insects. There are some exceptions, however. For example, miR-125 has been lost

from the primary transcript in *B. mori* and *A. pisum* (Legeai et al., 2010). Although we conducted a number of attempts, we were not able to clone and sequence the let-7 primary transcript from *B. germanica*. Indeed, the generally very short half-life of primary transcripts, which are being cleaved by the enzyme drosha almost simultaneously with transcription (Kim and Kim, 2007; Morlando et al., 2008), makes cloning them extremely difficult. Therefore we cannot confirm whether let-7, miR-100 and miR-125 precursors cluster in the same primary transcript in *B. germanica*. However, given: (1) that the let-7 cluster is generally well conserved across metazoans (Chawla and Sokol, 2012; Pasquinelli et al., 2000), (2) that in *B. germanica*, the precursors and the sequences of mature let-7, miR-125 and miR-100, are also very well

conserved with respect to other metazoans, and (3) that in *B. germanica*, the three miRNAs have parallel expression patterns, we presume that these three miRNAs also cluster in the same primary transcript in our model species.

The increase of expression of let-7, miR-100 and miR-125 in B. germanica wing pads in days 5-7 of the last instar nymph already suggests that these miRNAs play a role in this particular tissue during metamorphosis. Depletion of miR-100 led to adult specimens showing the hindwings smaller that controls and with a subtle disorganization of the vein patterning compared to those of controls. Depletion of let-7 gave a similar phenotype concerning vein patterning. Depletion of miR-125 was not specific as it also triggered a significant decrease of let-7 and miR-100 levels, but the resulting adults did not show any apparent difference with respect to controls. This result might suggest that miR-125 have a stimulatory effect on let-7 and miR-100 and that medium-low levels of these two miRNAs resulting from LNA treatment for miR-125 are enough to promote normal wing development. In D. melanogaster and in mammals, there is evidence of an indirect stimulatory effect of miR-125 on let-7 mediated by the protein lin-28 (Liu et al., 2007; Moss and Tang, 2003; Nolde et al., 2007). The RNA-binding protein lin-28 blocks let-7 biogenesis with the uridylation of the let-7 precursor at its 3' end (Heo et al., 2008). In turn, miR-125 blocks the expression of lin-28 by binding to the 3' UTR region of its mRNA. Although we do not know whether B. germanica possesses a lin-28 homolog, an equivalent mechanism, including a stimulatory effect of miR-125 on miR-100, might operate in our model species and explain the observed results.

The targets of let-7, miR-100, and eventually miR-125, which are likely to be involved in vein-intervein patterning in the forewings, remain to be elucidated. In *D. melanogaster*, vein organization rely on a number of developmental signals and corresponding pathways like those mediated by EGF, Wnt, BMP, Notch and Hedgehog (Blair, 2007). As the genome sequence of *B. germanica* is not available, we cannot undertake bioinformatic predictions of miRNA binding sites in the members of these pathways and subsequent functional validation. This should be the direction of further efforts to elucidate the let-7/miR-100 targets involved in vein organization in our cockroach model.

In D. melanogaster, let-7 has not been reported to be involved in wing morphogenesis but it appears to be important to ensure the appropriate remodeling of abdominal neuromusculature during metamorphosis (Sokol et al., 2008). In 2008, Caygill and Johnston reported that the absence of let-7 and miR-125 results in temporal delay of maturation of neuromuscular junctions of imaginal muscles (Caygill and Johnston, 2008), and identified abrupt (ab) as the let-7 target in this context. In the same work, these authors found that let-7 is required for terminal exit from the cell cycle of the wing imaginal disc, and loss of let-7 led to adult specimens with small wings with more and smaller cells. This effect of let-7 in D. melanogaster is reminiscent of what we observed after depletion of miR-100 in B. germanica. Concerning effects on neuromuscular junctions, we did not carry out specific neuroanatomical studies, but the gradual metamorphosis that characterizes B. germanica does not involve extensive tissue remodeling, as occurs in the case of D. melanogaster.

Huang et al. (2013) have recently described in detail the phenotype obtained after depleting BR-C factors with RNAi in *B. germanica*. The specific features of this phenotype occur in the adult wings, especially in the hindwings, which are smaller, have a shorter CuP vein which leaves a notch in the wing edge, have the vein/intervein disorganized in the anterior part, and show anomalous bifurcations of the A-veins in the posterior part, when compared to controls. Interestingly, the phenotype of BR-C knockdowns is similar to that obtained with the LNA treatments for let-7 and for miR-100, except for the character of the shorter CuP vein and the associated edge notch, which was not induced by let-7 and miR-100 depletion. Moreover, our experiments of BR-C depletion resulted in lower values of let-7, miR-100 and miR-125, which suggest that the expression of the three miRNAs is regulated by BR-C and, hence, by 20E.

In D. melanogaster, 20E is necessary for the expression of let-7, miR-100 and miR-125, and the response appears to be mediated by BR-C transcription factors (Chawla and Sokol, 2012; Garbuzov and Tatar, 2010; Sempere et al., 2002). This reminds our results but the difference between D. melanogaster and B. germanica is that while BR-C is only expressed in the last instar larvae and in the prepupae of the fly (Kiss et al., 1988), in the cockroach BR-C is constantly expressed along the nymphal stages, and there is no apparent correlation between let-7, miR-100 and miR-125 levels (present results) and BR-C mRNA levels (Huang et al., 2013). Indeed, the three miRNAs are present in the whole life cycle of *B. germanica*, from the first instar nymph to the last instar nymph when juvenile hormone (JH) vanishes. Although the reduction of let-7, miR-100 and miR-125 levels observed after depleting BR-C is rather modest, we speculate that 20E enhances the expression of them (through BR-C factors) when JH vanishes, on the basis of our previous experiments showing that treatments with 20E alone tend to stimulate the expression of these three miRNAs, whereas treatments of 20E plus JH tend to inhibit it (Rubio et al., 2012).

Whether or not the upregulation of let-7, miR-100 and miR-125 in the last nymphal instar is due to the action of 20E in the absence of JH, what is clear is that the phenotype obtained after depleting these miRNAs is rather mild, which indicate that the contribution of them to the supernumerary nymph phenotype obtained after dicer-1 depletion (Gomez-Orte and Belles, 2009) is marginal. This means that we must keep searching other miRNAs that would explain such a dramatic phenotype. Work along this line is currently in progress in our laboratory.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2013. 09.003.

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Supplementary data to:

Subtle roles of microRNAs let-7, miR-100 and miR-125 on wing morphogenesis in hemimetabolan metamorphosis

by

Mercedes Rubio and Xavier Belles

let-7

	*	20	*	40	*	60	
ame-let-7 nvi-let-7 bge-let-7 tca-let-7 kmo-let-7 aga-let-7 aga-let-7 api-let-7 dme-let-7	ACGAUGC GCGACGC GGUACUGCCGUCGG GCCUACUCC	UGGGUGAGGUAGU UGAGGUAGU UGUUUGAGGUAGU UGUUGAGGUAGU AGGUUGAGGUAGU GUGUUGAGGUAGU GUGUUGAGGUAGU AAAUUGAGGUAGU UGAGGUAGU	AGGUUGUAU AGGUUGUAU AGGUUGUAU AGGUUGUAU AGGUUGUAU UGGUUGUAU UGGUUGUAU AGGUUGUAU GGUUGUAU	AGUA GGGAAUG IAGU GGGGAAUG IAGU UAUGAAC- IAGU AGUAUUA IAGUA CGGAAA- IAGUA CGGACU- IAGUA CCG-UG- IAGUA CCG-UG- IAGUA CUUUAU- IAGUA GUUUAU- IAGUA GUA	GAAAUUC AAAAUUC UAC	GCGAUA : GUCAU- : AUCAUU : CACC : ACAACA : GAU : GUU : UAU :	54 53 37 40 54 36 44 31 37
ame-let-7 nvi-let-7 bge-let-7 tca-let-7 kmo-let-7 aae-let-7 aga-let-7 api-let-7 dme-let-7	LACGAAGUCC CUGU UAUGGAAUCC CUGU UUUGGAAD-ACUGU UAUUGGGAGA-ACUGU CAUAGGAGGGCUGU CAUAGGUGGGACUGU CAAAUAUCUG-CUAU CCAAUACUCG CUAU UGACUUUACUAU UACACAUCAUACUAU U ACU U	80 ACACUUGCUAA ACACUUGCUAA ACACUUGCUAA ACACCUGCUAA AUAGCCUGCUAA AUAGCCUGCUAA ACAUCUGCUAA ACAUCUGCUAA ACAUCUGCUAA ACAUCUGCUAA ACAUCUGCUAA	* 1 2000 C C G C 2000 C C G C 2000 C C A C 2000 C C A C 2000 C C A C 2000 C C A C 2000 C C C C C C 2000 C C C C C C C 2000 C C C C C C C 2000 C C C C C C C C C C C C C C C C C C	.00 ICGUCGACGC IUGUCGGCGC ICC IUGACGGAAUGA IGGGUAGAGU IUUGA	* : 10 : 9 : 6 : 7 	0 9 9 8 4 1 0 3 8	
miR-1 aga-mir-100 : dme-mir-100 : tca-mir-100 : bge-mir-100 : ame-mir-100 : nvi-mir-100 : api-mir-100 :	00 		40 50 A G A D C C G A A C 50 A G A D C C G A A C 50 A G A D C C G A A C 50 A G A D C C G A A C 50 A G A D C C G A A C 50 A G A D C C G A A C 50 A G A D C C G A A C 50 A G A D C C G A A C 50 A G A D C C G A A C	* 60 00606 00606 00606 00606 00606 00606 00606 00606 00606 00606 00606 00606 00606 00606 00606	UUCACAUGU ACAACUGGA UAUAUCUGU CGUUGGGGA UUCGUACGA UUCUAUAUGCA AAAGUUUAG	Unc An Chanh Unc An CU coucc Unc coucc Unc coch Unc Coch	73 59 53 46 68 40 64 64 63
aae-mir-100 : aga-mir-100 : dme-mir-100 : tca-mir-100 : kmo-mir-100 : bge-mir-100 : ame-mir-100 : api-mir-100 :	80	00 * CCCGAL GUAUGCCUAJ GUCGACGUUGCC GUCGALGCAAACAACU ACUGG AGAA ACUAU CAAUGCAAC AUUCCA UUGAG CAGGCCAGCJ UGUAG CAGGCCAGCJ UGUAG CAGGCCGCC GCCAC CAGUCCAUU U	120 AAGCAUCGAUCG GGUUUUUGGCA CAAUAUCGAUUC	* 14 AUUUUGU	0 	* 124 : 92 : 100 : 77 CAAA : 142 : 67 : 100 : 100 : 99	5 1 5 2 7 5 5 9
miR-1 aae-mir-125 aga-mir-125 dme-mir-125 tca-mir-125 nvi-mir-125 bge-mir-125	25 UAUUGUUCGUUUU GACAUGUCGAAUGU GACAUGUCGAAUGU 	20 UUGUUUCUGUGUGAU UUGUAUG-GCUGAU UUGUAUG-GCUGAU C	* 4 UCCCUGAGAG UCCCUGAGAG UCCCUGAGAG CCCUGAGAG UCCCUGAGAG UCCCUGAGAG UCCCUGAGAG	0 * CCUAACUUGUGA CCUAACUUGUGA CCUAACUUGUGA CCUAACUUGUGA CCUAACUUGUGA CCUAACUUGUGA	6 CUAUAGU CUAUCGU CUUUUAAU UGUUAAA CGUCGCGA CGUCGCGU CGUAUUA CGUCGCGU CGUAUUA	0 GAAG : GCAAAG : AC-CAG : GGAC : CCGAUA : ACGAUA : GUAAAA :	54 65 63 44 59 62 36
aae-mir-125 aga-mir-125 dme-mir-125 tca-mir-125 ame-mir-125 nvi-mir-125 bge-mir-125	* 80 : UUUCACAACUUUUGA : UUUCACAACUUUUGA : UUUCACAACUUUGA : UCUCACAACUUUGA : UCUCACACCUAGAU : UCUCACACCUAGAU : UCUCACACCUAGAU U UCACA G U	* UCUC CGUAULACC UCUC CGUAULACC UCUC CGUAULCC UCUC CGUAULCC UCUC UGUAULCCC UCUC UGUAULCCC UCUC GGUAULCCC UCUC GGUAULCCC	100 GGUCGAGAUU GGUUGAGAUG GGAU GAUGAGUGCU GAUGAGUGCU	CA : CCAACGGUA- : CUGAUGUU- : GCCUUUUGC : GCCUUUUGA :	95 112 110 77 107 110 61		

Fig. S1. Alignment of the different precursors of let-7, miR-100 and miR-125 in insects. Stemloop sequences from the precursors of let-7, miR-100 and miR-125 were obtained from the miRBase (aae: *Aedes aegypti*, ame: *Apis mellifera*, aga: *Anopheles gambiae*, bmo: *Bombyx mori*, bge: *Blattella germanica*, cqu: *Culex quinquefasciatus*, dme: *Drosophila melanogaster*, nvi: *Nasonia vitiprennis*, tca: *Tribolium castaneum*). Alignment was carried out using ClustalW.

pre-let-7

5' - <u>UGAGGUAGUAGGUUGUAUAGU</u>UAUGAACUACAUCAUUUUUGGGAGAACUGUACAACUUGCUAACUUUAC - 3'



pre-miR-125

5' - <u>UCCCUGAGACCCUAACUUGUGA</u>CGUAUUAUGUAAAAUCUCACAGGCUAGAAUCUCCGGUAU - 3'



pre-miR-100

5' - AACCCGUAGAUCCGAACUUGUGUGACUUCGUACGAUUUGAUUUCACGGUUUCUAAAGUUUAUUCGCAAAAUCAUCGACCUGUAC - 3'



Fig. S2. Sequences and folding structures of let-7, miR-100 and miR-125 precursors of *Blattella germanica*. Sequences were obtained by 3' RACE using RNA extracts from specimens depleted for dicer-1. The respective primers correspond to sequences of the corresponding mature miRNAs. Folding structures were obtained using the RNAfold. Sequences of the mature miRNAs are indicated by a grey line.

	Transcript or miRNA	Primer name	Primer sequence
А		BRcoreF7	5'-CGCAACAAGCCGAAGACAGA-3'
	DK-C	BRcoreR5	5'-GCTATTTTCCACATTTGCCG-3'
		BRCoreF	5'-CGGGTCGAAGGGAAAGACA-3'
	BK-C	BRCoreR	5'-CTTGGCGCCGAATGCTGCGAT-3'
	let-7	let-7	5'-TGAGGTAGTAGGTTGTATAGT-3'
В	miR-100	miR-100	5'-AACCCGTAAATCCGAACTTGTG-3'
	miR-125	miR-125	5'-CCCTGAGACCCTAACTTGTGA-3'

Table S1. Primer sequences. (A) Primers used to generate dsBR-C. (B) Primers used for quantification by qRT-PCR.

Table S2. Phenotypes found with the LNA treatments. Defect A: Wings wrinkled, not properly extended; Defect B: Disorganization of the vein-intervein pattern in the anterior part of the hindwing; Defect C: A-vein bifurcations in the posterior part of the hindwing.

	Treatment	LNA for let-7	LNA for miR-100	LNA for miR-125	
	Individuals	40	30	30	
	No phenotype	20 (50%)	12 (40%)	30 (100%)	
Defect A	Plus defect B	4 (10%)	9 (30%)	0	
	Plus defect C	4 (10%)	6 (20%)	0	
	Defect B+C	12 (30%)	3 (10%)	0	