Journal of Insect Physiology 59 (2013) 1089–1094

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

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

Mercedes Rubio, Xavier Belles *

Institute of Evolutionary Biology (CSIC-UPF), Passeig Marítim de la Barceloneta 37, 08003, Barcelona, Spain

ARTICLE INFO

Article history:
Received 31 July 2013
Received in revised form 12 September 2013
Accepted 14 September 2013
Available online 24 September 2013

Keywords:
let-7
miR-100
miR-125
Wing morphogenesis
Insect metamorphosis
Blattella

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).

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

* Corresponding author. Tel.: +34 932309636.
E-mail address: xavier.belles@ibe.upf-csic.es (X. Belles).

0022-1910/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jinsphys.2013.09.003

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 miRNAs 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 20-hydroxyecdysone (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 (qRT-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 (Exiqon). 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 (Exiqon). 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 1-day-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 μl of the respective dsRNA solution (3 μg/μl) 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-