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The microRNA pathway core genes are differentially expressed during the development of *Helicoverpa armigera* and contribute in the insect's development

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ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs (18–25 nt) that are produced by all animals and plants as well as some viruses. Their roles have been revealed in many physiological processes including development, cancer, immunity, apoptosis and, host–microbe interactions through post-transcriptional regulation of gene expression. In this study, we predicted, characterized and transcriptionally analyzed the core miRNA pathway genes in *Helicoverpa armigera*. Our results showed that the canonical miRNA biogenesis pathway genes including *Pasha, Drosha*, *Loquacious, Exportin-5, Dicer-1 and Argonaute-1* are differentially expressed in different tissues and during the development of this insect. Considering the essential role of *Dicer-1* in this pathway, we used RNA interference to silence the expression of this gene in *H. armigera*. Silencing of *Dicer-1* decreased the levels of cellular miRNAs, let-7 and miR-184. Together, our results showed that the miRNA pathway functions during the development of *H. armigera*, and silencing of *Dicer-1* resulted in the miRNA pathway blockage and depletion of the miRNA contents leading to mortalities in the immature stage and abnormalities in the mature stage. Blockage of this pathway can therefore be considered in future attempts for interrupting/suppressing populations of this important crop pest.

1. Introduction

RNA interference (RNAi) is a regulatory mechanism that decreases the expression of target genes by inhibition of transcription or translation, or activation of the degradation process of specific sequences of target RNA (Bartel, 2009). As part of the RNAi machinery, microRNAs (miRNAs) are a class of naturally occurring, small non-coding RNA molecules, about 18–25 nucleotides in length that regulate gene expression post-transcriptionally (Bartel, 2009). miRNAs are partially complementary to one or more messenger RNA (mRNA) target molecules, and their main function is to downregulate gene expression in a variety of ways, including translational repression, mRNA cleavage, and deadenylation (Bartel, 2009; Cullen, 2006; Pasquinelli, 2012).

miRNA genes in the nucleus are transcribed by RNA polymerase II as large primary transcripts (pri-miRNA) that are processed by a protein complex containing the RNase III enzyme Drosha, in association with Pasha (equivalent to DGCR8 in mammals) to form an approximately 70 nucleotide precursor miRNA (pre-miRNA) (Cullen, 2006). This precursor is subsequently transported into the cytoplasm by Exportin-5 where it is processed by a second RNase III enzyme, Dicer-1, producing a miRNA:miRNA duplex intermediate of ~22 nucleotides with five phosphates and 2-nucleotide 3 overhangs (Bartel, 2009). Mature miRNA incorporated into RNA-induced silencing complex (RISC) containing Argonaute-1 is the main component to guide the complex to the target sites in mRNAs that are partially supplementary to the miRNA sequence, and induce repression of gene expression at the level of mRNA stability or translation (Ender et al., 2010).

The role of miRNAs in different biological processes of insects including immunity, apoptosis, development, and host–microorganism interactions have been reported (Asgari, 2015, 2012; Baradaran et al., 2019; Hussain and Asgari, 2014; Mehrabadi et al., 2013). Considering the conserved function of miRNAs in animal development, their roles in insect development have also attracted more attention relative to other functions. In this regard, various miRNA profiling studies have shown expression of miRNAs during development of a variety of holometabolous (Aravin et al., 2003; Behura et al., 2011; Behura and Whitfield, 2010; Mukherjee and Vilcinskas, 2014; Rao et al., 2012; Skalsky et al., 2010; Yu et al., 2008) and hemimetabolous insects (Chen et al., 2012;

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Cristino et al., 2011). Compared to other insects, miRNA functions have been better characterized in *Drosophila*. Following miRNA identification in *Drosophila*, their roles have been characterized in different physiological pathways of the insect. The *let-7* complex (*let-7-C*), for example, is a polycistronic locus encoding three conserved miRNAs including let-7, miR-100, and miR-125 that are importantly involved in the fly metamorphosis and development (Sokol et al., 2008). The *Drosophila* mutants, with *let-7-C* deletion, were morphologically normal, however, they were not able to fly or move as much as the wild type flies due to disruption of their muscle development (Sokol et al., 2008). Lack of *let-7-C* in *Drosophila* was also shown to negatively affect metamorphosis and wing development in this insect. The mutant flies showed smaller wings compared to the wild type flies due to the smaller size of cells in the mutant flies (Caygill and Johnston, 2008). Follow up studies showed that expression of the *let-7-C* locus in *Drosophila* is induced by the molting hormone, 20-hydroxyecdysone, which controls the ecdysis and metamorphosis of insects and other arthropods (Chawla and Sokol, 2012). These findings, among others, revealed the important functional role of miRNAs in insects.

The cotton bollworm, *Helicoverpa armigera* (Lepidoptera; Noctuidae) is a notorious polyphagous and cosmopolitan insect pest responsible for heavy economic losses to agriculture in the world (Bidari et al., 2018; Karimi et al., 2012; Safuraie-Parizi et al., 2014). We previously showed that the RNAi pathway in this insect plays a role in insect-bacteria interactions (Baradaran et al., 2019). In this study, we characterized the core miRNA pathway genes and transcriptionally analyzed their expression during the development of this insect. By suppression of the miRNA pathway, we showed that this pathway is involved in the development of *H. armigera*.

2. Material and method

2.1. Insect culture

Different developmental stages of *H. armigera* were obtained from a colony at the insect rearing laboratory of Tarbiat Modares University, which was already collected from their natural habitats. They were maintained at optimum growth conditions (25 ± 2 °C, 65 ± 5% relative humidity and a photoperiod of 16:8 h [Light: Dark]).

2.2. Characterization of miRNA core genes in H. armigera

We considered mRNAs from ESTs and transcriptome sequences extracted from the National Centre for Biotechnology Information (NCBI) to identify and characterize miRNA core genes (*Drosha*, *Pasha*, *Loquacious, Exportin-5, Dicer-1, and Argonaute-1*) in *H. armigera*, Basic Local Alignment Search Tool (BLAST) was used to find homologous sequences, and sequence alignments were performed using clustal Omega (Remmert et al., 2011). Protein domains were analyzed by SMART 4.0 program (Letunic and Bork, 2018) and phylogenetic trees were constructed by the neighbor-joining (NJ) method based on amino acid sequences using MEGA 6 software (Tamura et al., 2013). Bootstrap sampling was reiterated 1000 times.

2.3. RNA isolation and expression analysis of genes and miRNAs

To analyze gene expression level, total RNA samples from each stage and extracted gut, fat body and the rest of the body from the 4th instar larvae were extracted using Trizol solution according to the manufacturer's instructions (Molecular Research Center). All the samples were then treated with DNase I (Promega) to remove possible DNA contamination. The RNA concentrations were measured by using a spectrophotometer (Epoch, BioTek) and a total of 2 µg total RNA was reverse transcribed with MMLV-reverse transcriptase enzyme (Promega) at 42 °C for 1 h using an oligo(dT) primer. Transcript levels of *Drosha*, *Pasha*, *Loquacious, Exportin-5, Dicer-1 and Argonaute-1* were analyzed by RT-qPCR using gene-specific primers (Table 1), utilizing RPL27 as a reference gene. For each experiment, three biological replicates were analyzed by using a Mic-qPCR cycler (BMS). RT-qPCR was carried out according to the following parameters: one cycle at 95 °C for 15 min, 40 cycles at 95 °C for 15 s, 57 °C for 30 s, 73 °C for 30 s with a final cycle at 95 °C for 1 min.

Table 1
Primers used in this study.

Primer name	Forward sequence	Reverse sequence
Dcr1_qPCR	AGGAGGTTCATGCGTTTCGT	TGTGGGTACCGTCTTCGTTG
Exp5_qPCR	GCCGGTCTAGCTCAACAACT	GTATGAGCGAGCGTGAGTGA
Ago1_qPCR	TTGATGTTTCTGCAACCGCC	TGCGAGTCTGTCAATGGCTT
Drosha_qPCR	CTGCACCCTGAGATGTGGTT	CTGCGTCGGCACACATTTAG
Pasha_qPCR	ACAAGGCTCGACACAACGAA	GTTAGAGCCCGAATGGGTGG
Loqs_qPCR	GCAATGTGCCTGAGACTGGA	GCATGGTACGAAGGTGGAGG
RPL27_qPCR	GAAGCCAGGTAAAGTGGTGCT	GTGTCCGTAGGGCTTGTCTG
Dcr1_RNAi	GGAGAGCCATAGACGGAGGA	GTGTCCTCGTTGATCTTGGGT
T7 sequence	TAATACGACTCACTATAG	

The expression levels of let-7 and miR-184 were analyzed using stem-loop RT-PCR. The cDNA samples were synthesized using the specific stem-loop primers and the expression levels of the miRNAs were assessed by qPCR using miRNA-specific forward and universal reverse primes (Chen et al., 2005).

2.4. Gene silencing

RNA interference using double-stranded RNA (dsRNA) was used to silence *Dicer-1*. DNA fragments of *Dicer-1* and *GFP* were amplified by PCR with specific primers that contained T7 promoter sequences (Table 1) at their 5′ end for *in vitro* RNA synthesis. dsRNA was then produced and purified for each fragment using the MEGAScript T7 kit according to the manufacturer's instructions (Ambion). Synthesis and quality were analyzed by running dsRNAs on an agarose gel and the concentration of RNA was determined by measuring absorbance at 260 nm. For gene silencing, larvae were injected twice with dsRNA (2 µg per injection) within a 48 h interval. Control larvae were injected with dsGFP. To assess if silencing of *Dicer-1* affected miRNA in the injected insects, the expression of let-7 and miR-184 was investigated as previously stated.

2.5. Statistical analysis

The expression levels of *Pasha*, *Drosha*, *Loquacious*, *Exportin-5, Dicer-1 and Argonaute1* were analyzed by analysis of variance (ANOVA) followed by Tukey multiple comparisons. Expression analyses of the *Dicer-1* gene in larvae after dsGFP or dsDicer-1 were analyzed by *Z*-test. Data of qPCR were analyzed with the $2_{T}^{-\Delta\Delta C}$ method (Livak and Schmittgen, 2001). Graphs were created with GraphPad Prism (version 7) software.

3. Results

3.1. The miRNA core genes of H. armigera are structurally and phylogenetically similar to those of other lepidopterans

BLAST analyses of Pasha, Drosha, Loquacious and Exportin-5 amino acid sequences exhibited high levels of similarity to their homologs in *Spodoptera litura* (with 99% identity for Pasha, 97% identity for Drosha and Loquacious, and 100% identity for Exportin-5). Conserved domain analysis with SMART program showed that *H. armigera* Pasha contains two double-stranded RNA binding motifs (DSRM) (Fig. 1A). *H. armigera* Drosha protein contains two ribonuclease III C-terminal domains (RIBOc), one DSRM, one PHA03307 and one DUF1777 domain (Fig. 1B). Loquacious possess three DSRM domains (Fig. 1C). The Exportin-5 protein contains one Exportin-1-like protein domain (Xpo1)

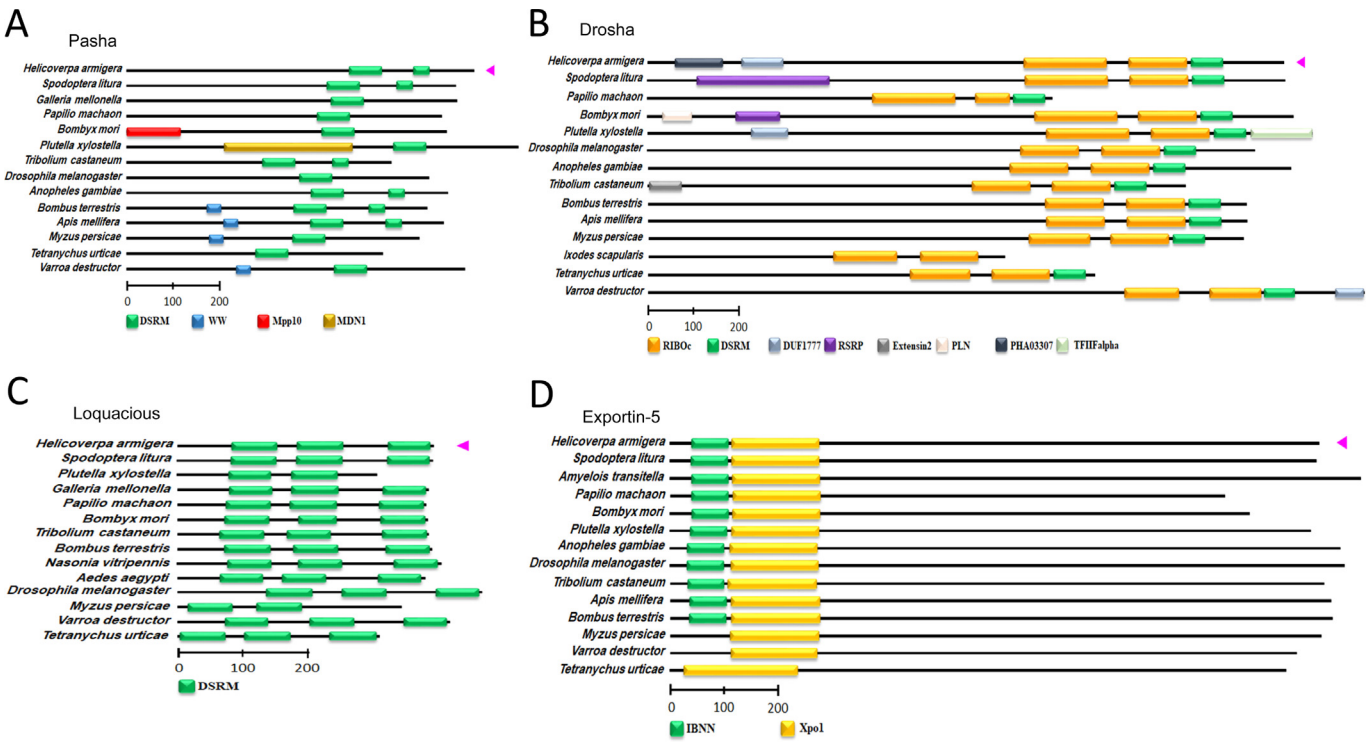

Fig. 1. Domain organization analysis of Pasha, Drosha, Loquacious and Exportin-5 proteins. Schematic depiction of domain organization of Pasha (A), Drosha (B), Loquacious (C) and Exportin-5 (D) proteins in different insects. RIBOc: ribonuclease III C-terminal domains; DSRM: double-stranded RNA binding motifs; Xpo1: Exportin1-like protein domain; IBNN: Importin-beta N-terminal domain; DUF1777; PHA03307.

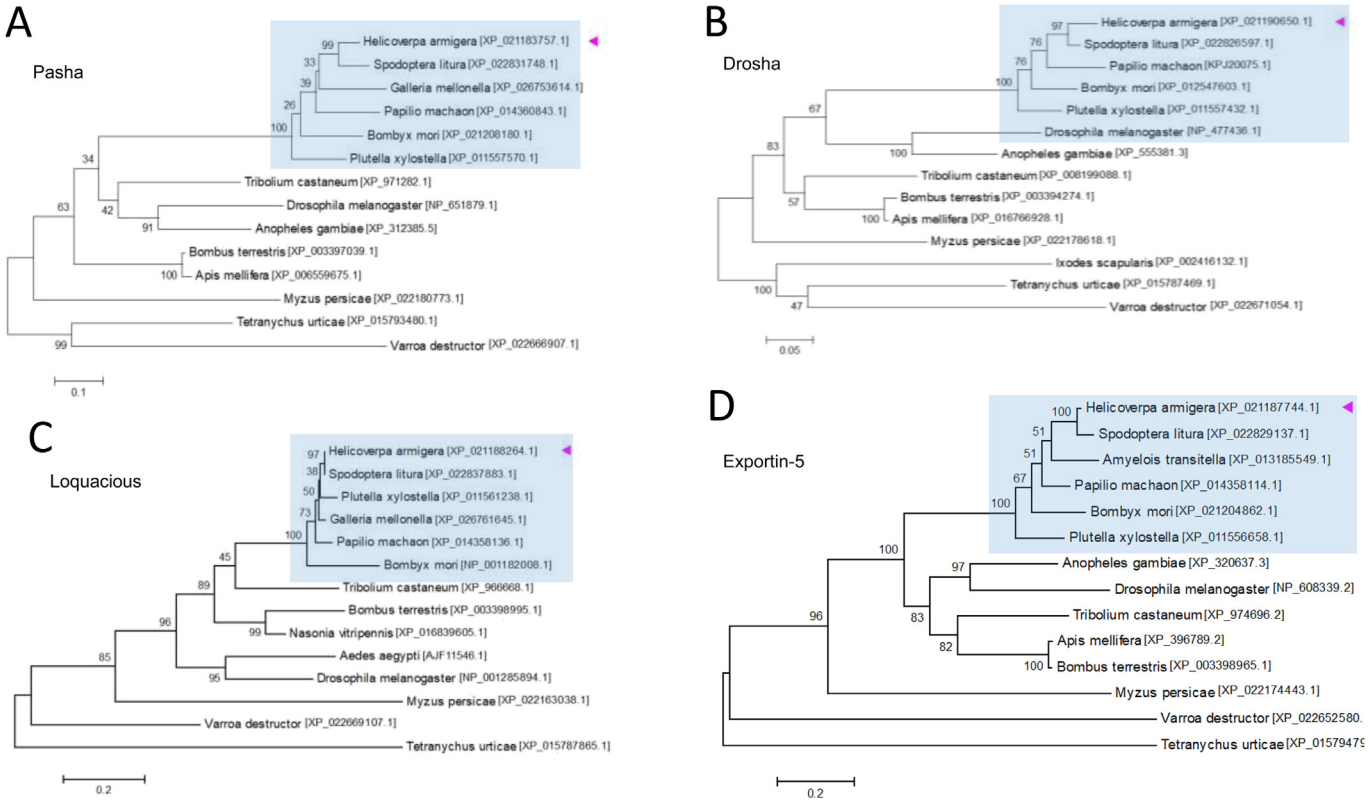

Fig. 2. Phylogenetic analysis of Pasha, Drosha, Loquacious and Exportin-5 proteins in different insects. Evolutionary relationships between Pasha (A), Drosha (B), Loquacious (C) and Exportin-5 (D) proteins in different insect species were drawn using the neighbor-joining method. *H. armigera* proteins are pointed out by arrows, and their clades are shaded.

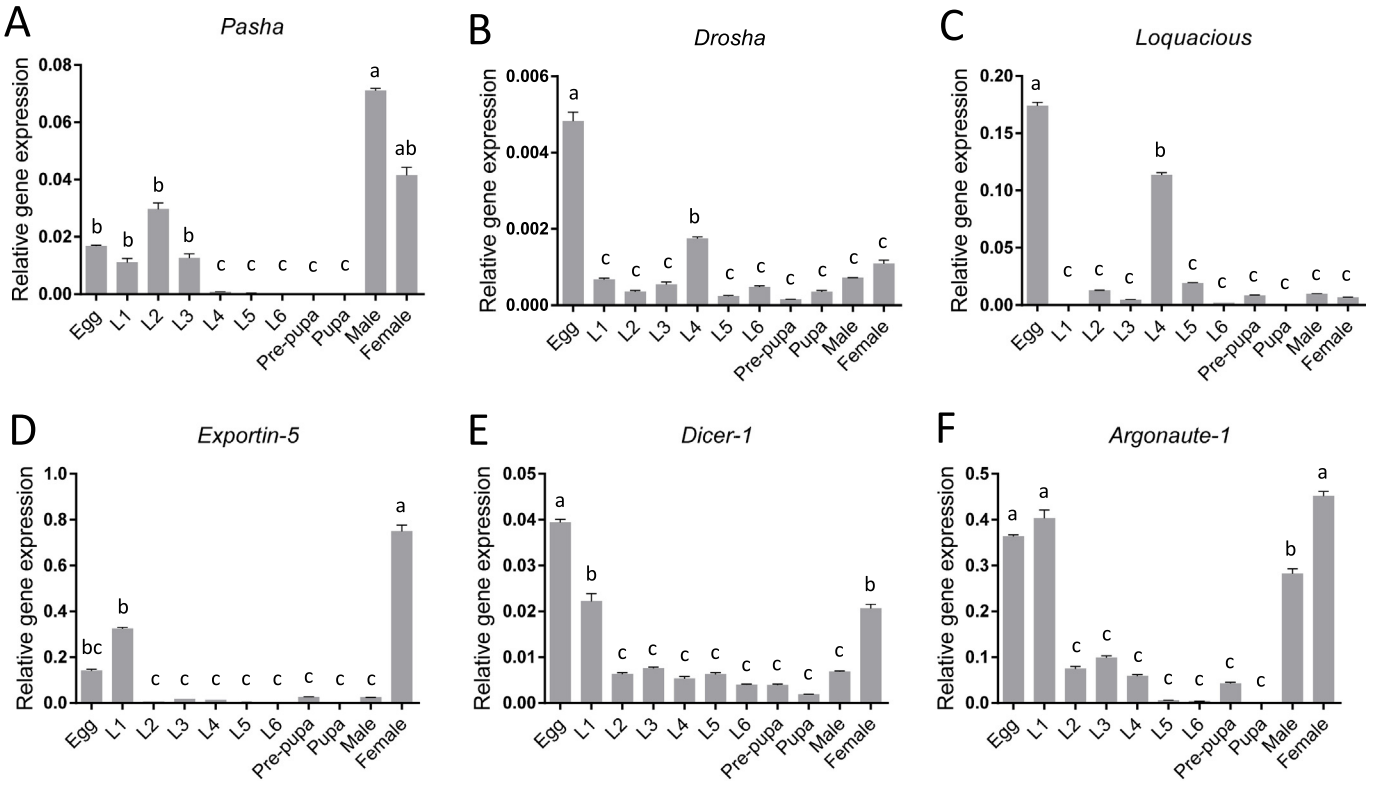

Fig. 3. Dynamic expression of miRNA pathway core genes during development of *H. armigera*. The expression levels of *Pasha* (A), *Drosha* (B), *Loquacious* (C), *Exportin-5* (D), Dicer-1 (E) and *Argonaute-1* (F) were quantified using RT-qPCR; *RPL-27* was used as the reference gene. Data are reported as means ± SE of three independent biological replications (different letters means significant differences, $P < 0.01$; analysis of variance followed by Tukey multiple comparisons).

and one Importin-beta N-terminal domain (IBNN) (Fig. 1D). In order to examine the phylogenetic relationship of Pasha, Drosha, Loquacious and Exportin-5 families, we constructed phylogenetic trees of these proteins in *H. armigera* along with those of other insect species (Fig. 2A–D). In all the phylogenetic trees, *H. armigera* and *S. frugiperda* homologous proteins were placed together in one clade with high bootstrap values and as sister group to other lepidopterans. All the lepidopteran proteins together formed a distinct clade in the phylogenetic trees indicated by shading in Fig. 2A–D.

3.2. The miRNA core genes are differentially expressed during developmental stages and in different tissues of H. armigera

Gene expression analyses indicated that all the major genes of the miRNA pathway are expressed in *H. armigera.* The results showed stage-specific expression of the miRNA core genes in all the developmental stages. Overall, the expression levels of the miRNA core genes in the eggs, 1st instar larvae and adults were significantly higher than the other stages (Fig. 3A–F). Mid to late larval stages, pre-pupa and pupa showed low levels of miRNA core gene expression levels relative to the other stages. The transcript levels of most miRNA pathway core genes in females were higher than males, in particular *Exportin-5* and *Dicer-1* (Fig. 3A–F). Also, our results showed that miRNA core genes were expressed in different tissues including the insect gut and fat body (Fig. 4A–F). The levels of gene expression were almost similar in the gut and fat body with the exception of *Drosha* and *loquacious* that were expressed in the gut more than the fat body (Fig. 4 B, C).

RNAi of Dicer-1 resulted in down-regulation of the cellular miRNAs, let-7 and miR-184.

Specific dsRNAs targeting the coding region of *Dicer-1* were synthesized and injected into the hemolymph of the 5th instar larva, while dsGFP was injected into the control larvae. RT-qPCR results revealed specific silencing of *Dicer-1* in *H. armigera* following dsDicer-1 injection (Fig. 5A). To determine if *Dicer-1* silencing affects cellular miRNAs, the expression levels of let-7 and miR-184 were analyzed and found that both miRNAs were significantly down-regulated following *Dicer-1* silencing (Fig. 5B and C).

3.3. Silencing of Dicer-1 negatively affected H. armigera

Suppression of *Dicer-1* gene expression by RNAi led to increased mortality of the larvae compared to controls and lower rates of pre-pupa and pupa formation (Fig. 6A). Also, *Dicer-1* silencing resulted in lower pupal weight and longer pupal period (Fig. 6B and C) and morphological aberrations in the adults particularly in the wings (Fig. 6D). The adults with deformed wings were not able to fly.

4. Discussion

miRNAs play important roles in different physiological processes in insects through regulation of gene expression (Asgari, 2012). Each miRNA can potentially regulate hundreds of transcripts and it has been estimated that about 30–75% of different transcripts are regulated by miRNAs (Bartel, 2009). In this study, we characterized the miRNA pathway in *H. armigera* and transcriptionally analyzed the core genes involved in this pathway.

Protein domains comparisons showed that Pasha, Drosha, Loquacious, and Exportin-5 have the typical architecture of their respective protein families and share high similarities with their homologs in other lepidopteran insects. Phylogenetic analyses also indicated high similarities between homologous proteins in lepidopteran insects. These results suggest that the functions of these proteins in different insects are most likely the same. Considering their important functions in the miRNA biogenesis pathway, the core genes are indirectly involved in the regulation of gene expression by generating miRNAs.

The expression analyses revealed that the core miRNA machinery

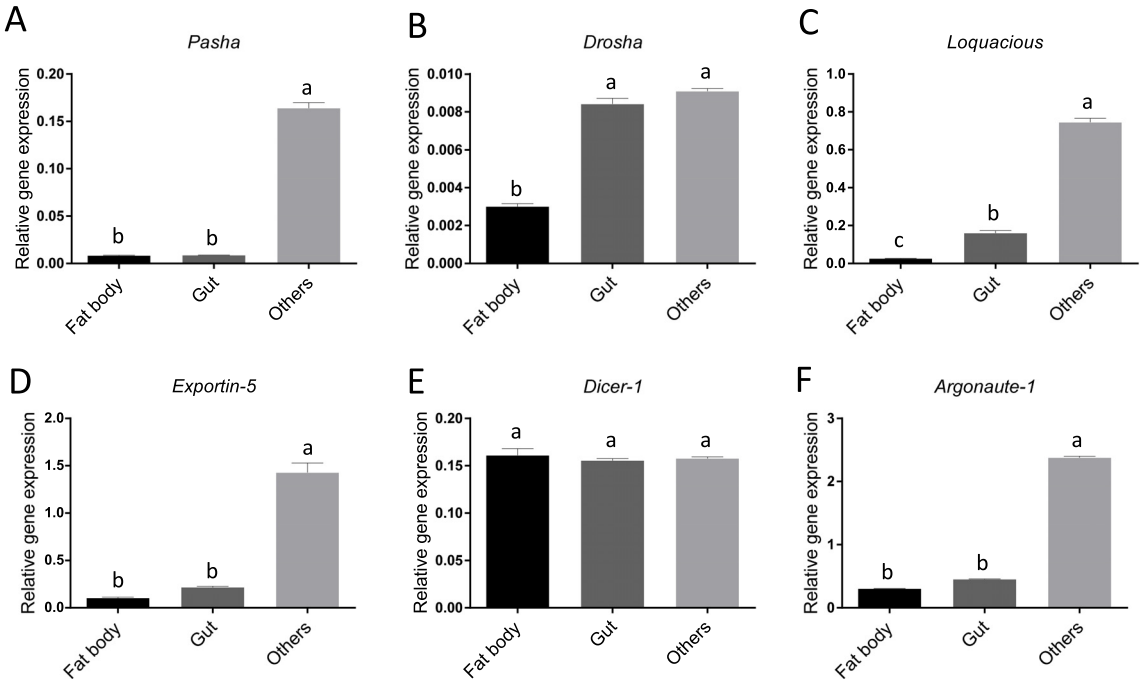

Fig. 4. Expression of miRNA pathway core genes in different tissues of *H. armigera*. The expression levels of *Pasha* (A), *Drosha* (B), *Loquacious* (C), *Exportin-5* (D), Dicer-1 (E) and *Argonaute-1* (F) in the fat body, gut and the rest of insect larvae were quantified using RT-qPCR; *RPL-27* was used as the reference gene. Data are reported as means ± SE of three independent biological replications (different letters means significant differences, $P < 0.01$; analysis of variance followed by Tukey multiple comparisons).

genes express in different tissues (e.g. fat body and midgut) of *H. armigera*. The gut and fat body are both metabolically active tissues with many genes involved in vital physiological processes of the insect, such as digestion and immunity. Therefore, the expression results suggest that the miRNA pathway functions and most likely regulate gene expressions in these tissues through regulating miRNA biogenesis. These core genes were also expressed during development of *H. armigera* particularly in the egg, 1st instar larvae, and adults that highlights their stage-specific function in the insect. In *S. littura,* it has also been reported that *Dicer-1*, *Argonate1,* and *Loquacious* are expressed in midgut and fat body and differentially expressed during developmental stages with highest expression level in the adults (Gong et al., 2015). Our

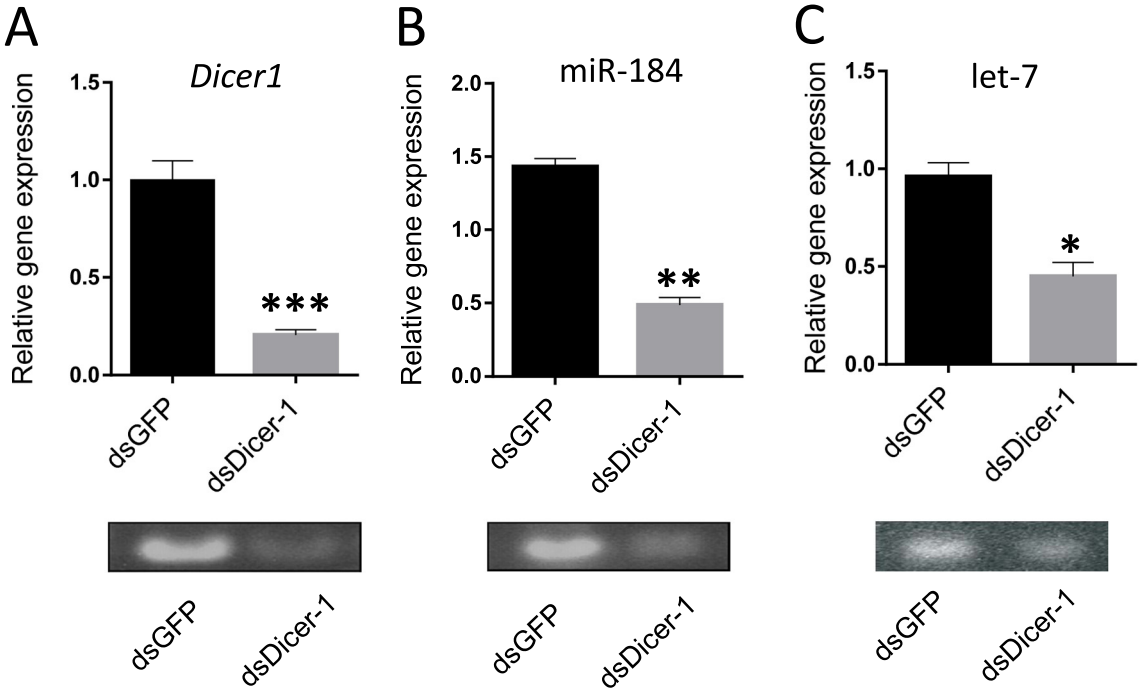

Fig. 5. RNAi of *dicer-1* and its effect on the expression levels of miR-184 and let-7. Quantitative (RT-qPCR) and semi-quantitative (gel electrophoresis) expression analyses of *Dicer-1* (A), miR-184 (B) and let-7 (C) in *H. armigera* larvae after dsGFP or dsDicer-1 injection confirmed significant reduction in the transcript levels of the gene; *RPL-32* was used as the reference gene. Data are reported as means ± SE of three independent biological replications. Asterisks indicate significant differences. (** $p < 0.01$, *** $p < 0.001$; analysis of variance followed by Tukey multiple comparisons).

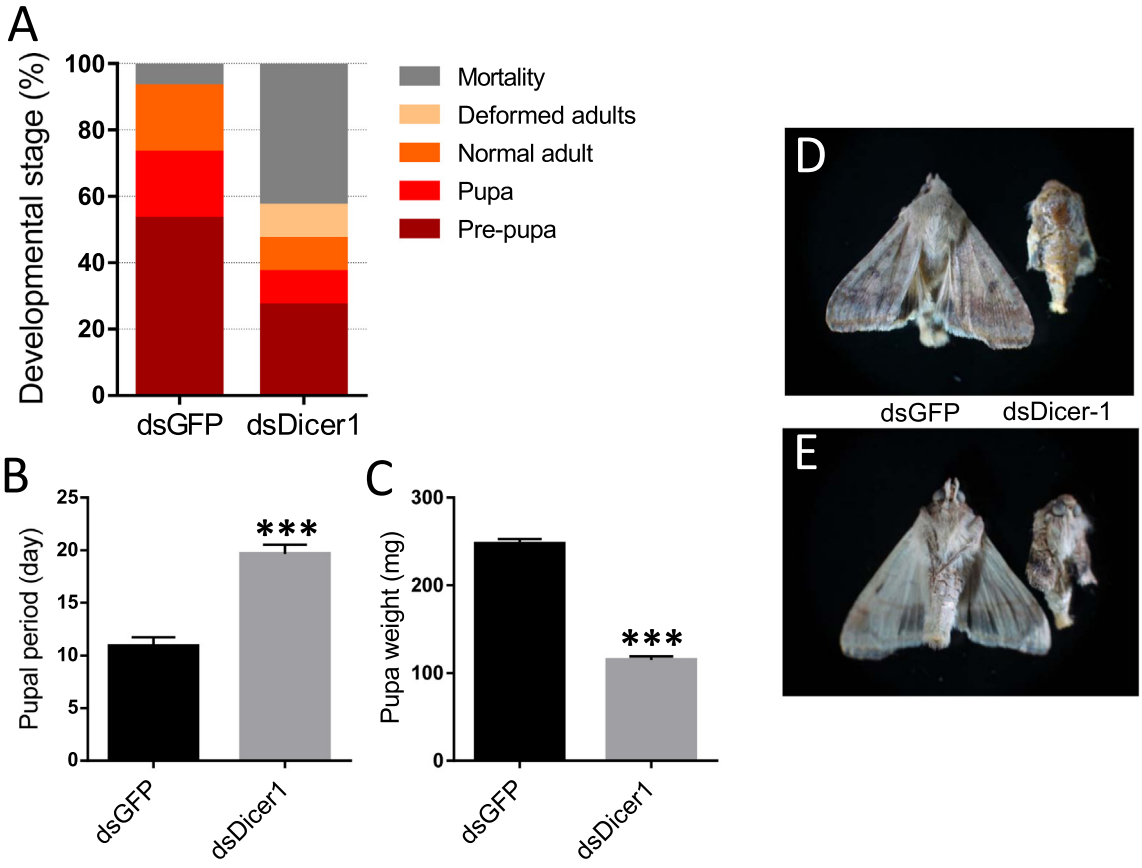

Fig. 6. RNAi of *dicer-1* affected development of *H. armigera*. dsDicer-1 or dsGFP were injected into 5th instar larvae and the subsequent developmental stages including the number of pre-pupa, pupa, normal and deformed adults (A), pupal period (B), pupal weight (C) and morphology of emerged adults (D, E) were compared.

results also showed that the miRNA core genes were highly expressed in females relative to males revealing gender-specific expression of this pathway in *H. armigera*. Differential miRNA levels in different sexes has also been reported in red flour beetle *Tribolium castaneum* (Freitak et al., 2012). These findings indicate the important role of the miRNA pathway during development of both sexes of these insects.

Disruption of the miRNA biogenesis resulted in deregulation of miRNAs. *Dicer-1*, as an important core gene, engages in the pathway by cleaving the pre-miRNA in the cytoplasm and generates approximately 22 nt double-stranded miRNA. Having an important function, *Dicer-1* is an attractive target for silencing of the miRNA pathway in different studies (Hussain et al., 2013; Mehrabadi et al., 2013). In this study, we also silenced *Dicer-1* to suppress the miRNA pathway and evaluated its consequences on the cellular miRNA expression and insect biology. Transcript-knock down of *Dicer-1* resulted in down-regulation of let-7 and miR-184 that confirmed the essential role of *Dicer-1* in the canonical miRNA pathway in *H. armigera*. We also determined the role of the miRNA pathway on the development of *H. armigera* and found that *Dicer-1* RNAi resulted in mortality, retarded development, and morphological aberrations in adults revealing the importance of miRNAs in the insect's development.

The *Dicer-1 D. melanogaster* mutant showed defects in embryogenesis (Lee et al., 2004) and olfactory neuron morphogenesis (Berdnik et al., 2008). The miRNAs let-7 and miR-184 are among the highly expressed miRNAs in *H. armigera* and other holometabolous insects that are associated with insect development (Ling et al., 2014; Zhang et al., 2018). let-7 has been shown to target regulatory genes in the ecdysone pathway in *Bombyx mori* and reduced let-7 expression resulted in the developmental arrest in this insect (Ling et al., 2014). In *D. melanogaster*, let-7 suppression caused inconsistencies in the production and destruction of muscle, and major changes in adult insect behavior, including flying power and reproduction (Sokol et al., 2008). miR-184 is dynamically expressed during the development of *Drosophila* and is involved in the regulation of oogenesis and embryogenesis (Aboobaker et al., 2005; Iovino et al., 2009). Loss of miR-184 resulted in lack of egg production and defects in early embryogenesis (Iovino et al., 2009), while overexpression of miR-184 and let-7 positively affected metabolism and longevity in *Drosophila* (Gendron and Pletcher, 2017). The functions of a few miRNAs have been reported in *H. armigera* such as the regulation of chitinase (Agrawal et al., 2013), ecdysone receptor (Jayachandran et al., 2013a) and digestive proteases genes (Jayachandran et al., 2013b; Lomate et al., 2014). Deregulation of these miRNAs resulted in mortality, deformation of pupa and adults and reduced fecundity in *H. armigera* (Agrawal et al., 2013; Jayachandran et al., 2013b) that is consistent with our results. Similar effects have been also reported as a result of suppression of the miRNA pathway in hemimetabolous insects. In the beetle *Tribolium castaneum, Dicer-1* RNAi resulted in an occasional wing expansion defect, highlighting the role of miRNAs in wing development (Tomoyasu et al., 2008). Suppression of *Dicer-1* in the last instar cockroach *B. germanica* impaired metamorphosis to the adult stage that resulted in insects retaining nymphoid features (Gomez-Orte and Belles, 2009). The miR-2 family was shown to regulate *B. germanica* metamorphosis and development through the juvenile hormone signaling pathway (Lozano et al., 2015). Further, silencing of *Dicer-1* in the migratory locust, *Locusta migratoria*, resulted in reduced miRNA contents, disrupted molting and caused high mortality (Wang et al., 2013).

5. Conclusion

In this study, we transcriptionally analyzed the core miRNA genes including *Pasha, Drosha, Loquacious, Exportin-5, Dicer-1 and Argonaute-1* during development and in tissues of *H. armigera.* Silencing *Dicer-1* reduced the miRNA content in the larvae and led to retarded development, mortality and morphological defects. These results reveal the essential role of miRNAs in the physiological processes and development of *H. armigera* and could potentially be utilized in novel pest control measures. For instance, silencing the miRNA pathway core genes using specific dsRNAs to the genes delivered to insects through genetically modified plants or formulated with nanoparticles could be employed to disrupt the development of the pest leading to pest population suppression. However, designing RNAi probes that are species-specific or are at least specific to agricultural pests could be challenging due to the conservation of those genes.

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