ORIGINAL ARTICLE

MicroRNA-dependent development revealed by RNA interference-mediated gene silencing of LmDicer1 in the migratory locust

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Abstract MicroRNAs (miRNAs) are small noncoding RNAs, which participate in many biological processes. The small RNA transcriptome in the migratory locust has been characterized and 50 conserved miRNA families and 185 potential locust-specific miRNA family candidates have been identified using high-throughput sequencing. However, it is unclear whether miRNAs influence a wide variety of locusts’ biological processes, such as growth or development. In insects, Dicer1 ribonuclease transforms miRNA precursors into mature miRNAs. Thus, using systemic RNA interference (RNAi) to silence the expression of LmDicer1 in the migratory locust, Locusta migratoria, we reduced miRNA contents in the locust and disrupted two types of molt (nymph–nymph, and nymph–adult). The RNAi of LmDicer1 also resulted in a high mortality in L. migratora. Our study revealed that LmDicer1 was essential for miRNA regulation and development of L. migratoria. These results further support our notion that LmDicer1 could serve as an excellent target for developing novel strategies for controlling this important insect pest.

Key words development, dicer1, Locusta migratoria, microRNA

Introduction

MicroRNAs (miRNAs), 18- to 25-nucleotide-long non-coding RNA, play a critical role in many biological processes by binding to the 3′-untranslated region (3′-UTR) of target mRNAs (He & Hannon, 2004; Morin et al., 2008). Dicer ribonucleases are important in the biogenesis of miRNAs as they are involved in the production of mature miRNAs from miRNA precursors (pre-miRNAs). The loss of Dicer1 in Drosophila melanogaster results in embryogenesis defects (Lee et al., 2004) and disruption of olfactory neuron morphogenesis (Berdnik et al., 2008). RNAi of Dicer1 during metamorphosis of the beetle Tribolium castaneum, results in a mild morphological phenotype with only occasional wing expansion defects (Tomoyasu et al., 2008). Moreover, depletion of Dicer1 in the penultimate nymphal instar of the cockroach Blattella germanica results in reduced levels of mature miRNAs in the last instar nymph and severely impaired metamorphosis after the next molt (Gomez-Orte & Belles, 2009), which suggests that there may be important differences concerning the role of miRNAs in holometabolan and hemimetabolan metamorphosis. However, to date there has been little research on dicer1 in L. migratoria.

In a previous study, Wei et al. (2009) characterized the small RNA transcriptome in the migratory locust and identified 50 conserved miRNA families and 185 potential locust-specific miRNA family candidates, using high-throughput sequencing. However, the role of miRNAs during the developmental processes of locusts
remains unclear, and whether or not miRNAs associate with the biological processes of locusts, such as development or metamorphosis remains unexplored.

To examine the functions of locust miRNAs in developmental processes, here we silenced the expression of dicer1 in the locust by using RNA interference (RNAi) in the nymphs to observe the phenotypes during locust development. The results might contribute to elucidating the molecular regulating mechanism underlying miRNA-dependent development in the migratory locust.

**Materials and methods**

**Insects**

Locusts used in experiments were from the same stock at the Institute of Zoology, CAS, China. The insects were reared under a 14:10 h light:dark photo regime at 30 ± 2°C and fed on fresh wheat seedlings and bran (Kang et al., 2004).

**Cloning and sequencing of Dicer1**

The Dicer1 sequences were systematically searched in the locust transcriptome database (Chen et al., 2010). A fragment of 4,475 nucleotides similar to known insects’ Dicer1 sequences was obtained, which was used to design specific primers for rapid amplification of complementary DNA (cDNA) ends (RACE) experiments to generate the full-length cDNA sequence of the locust Dicer1.

Total RNA was isolated from locusts using Trizol reagent (Promega, Madison, WI, USA). The 1.5 μg RNA was used to synthesize the first-strand cDNA using M-MLV Reverse Transcriptase (Promega) and an oligo(dT)18 primer. Pairs of gene-specific primers were used to confirm the 4,475 bp cDNA fragment from the locust transcriptome database. For amplification of 3′-end and 5′-end cDNA sequence, SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) was applied, according to the manufacturer’s protocol.

Polymerase Chain Reaction (PCR) for confirming the fragment of Dicer1 searched from the locust transcriptome database was performed for 35 cycles using the following conditions: denaturing at 94°C for 1 min, annealing at 50–60°C (depending on the primers) for 1 min and extension at 72°C for 1–2 min based on the product size, followed by a final extension of 10 min at 72°C using Taq DNA Polymerase (TaKaRa, Dalian, China). For RACE-PCR, advantage® 2 Polymerase (Clontech) was applied to amplify 3′-end and 5′-end cDNA fragments. Amplified PCR fragments from each reaction were purified using Gel Mini Purification Kit (Tiangen, Beijing, China), and the purified fragment was subcloned into pGEM-T easy Vector (Promega) for sequencing from both directions.

**Phylogenetic analysis of the LmDicer1 sequence**

The amino acid sequences of LmDicer1 were translated from the cDNA sequences based on the standard codon table. For the phylogenetic analysis, we collected full-length homolog sequences of Dicer1 from other insect species on National Center for Biotechnology Information (NCBI) GenBank database. Three crustaceans (Phylum: Arthropoda), Litopenaeus vannamei, Penaeus monodon and Marsupenaeus japonicas, were used as outgroups. Deduced amino acid sequences were aligned using ClustalW with default parameters (Larkin et al., 2007). A neighbor-joining (NJ) tree was constructed using MEGA4 to infer orthology relationships among Dicer1s from different insect species (Tamura et al., 2007).

**Assays of quantitative PCR (qPCR) for expression analysis of LmDicer1 mRNA and miRNA**

Total RNA enriched for small RNAs from fourth instar nymphs on different days was isolated using the mirVana miRNA Isolation Kit (Ambion, Carlsbad, CA, USA). Oligo(dT)-primed cDNA was prepared using M-MLV Reverse Transcriptase (Promega). The first-strand cDNA of miRNA was synthesized using a miRNA first-strand cDNA synthesis kit (Ambion). The sequences of miR-8 and let-7 were used as forward primers, whereas the reverse primers were the adaptor sequences from the kit. Quantitative PCR of miRNAs and mRNAs were performed by using SYBR Green miRNA expression and gene expression assays, respectively, according to the manufacturer’s instructions (Tiangen) on a real-time PCR system (Roche, Mannheim, Germany: LightCycler® 480 System). PCR data was analyzed by using the 2−ΔΔCt method of relative quantification. For miRNA expression analysis, U6 was used as the endogenous control; and for LmDicer1 gene expression analysis, RP49 was used as the endogenous control. Dissociation curves were determined for each miRNA and mRNA to confirm a unique amplification. Statistical analysis of relative expression results was carried out with SPSS software (SPSS Inc., Chicago, IL, USA).

**Functional analysis of LmDicer1 by RNAi**

RNAi was performed by injecting double-stranded RNA (dsRNA) into nymphs. Templates for in vitro
Table 1  Primers used to full length cDNA verification, qPCR analysis and dsRNA synthesis of LmDicer1 and cDNA synthesis, qPCR analysis of miR-8, let-7.

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Primer name</th>
<th>Primer sequence (5′–3′)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-length of Dicer1 verification</td>
<td>Dicer1-F1</td>
<td>ACAAATCTTTACTCTGTCGCTAA</td>
<td>751</td>
</tr>
<tr>
<td></td>
<td>Dicer1-R1</td>
<td>GCATCATCTTGCCAGGACT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dicer1-F2</td>
<td>GGAAGTGCCCTCGCAAGATGA</td>
<td>1078</td>
</tr>
<tr>
<td></td>
<td>Dicer1-R2</td>
<td>GTTGTGATCTGCTGTGCTTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dicer1-F3</td>
<td>TTTTGACCCTGTATGACCT</td>
<td>1479</td>
</tr>
<tr>
<td></td>
<td>Dicer1-R3</td>
<td>ATGTAATCTGCGAACCCTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dicer1-F4</td>
<td>TTTTGACGAGGATTCGGAGACT</td>
<td>616</td>
</tr>
<tr>
<td></td>
<td>Dicer1-R4</td>
<td>AAGGGCCTGAGGATGGAAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dicer1-F5</td>
<td>AGAGGCTGAGGATGGAAC</td>
<td>800</td>
</tr>
<tr>
<td></td>
<td>Dicer1-R5</td>
<td>AGAGGCTGAGGATGGAAC</td>
<td></td>
</tr>
<tr>
<td>5′ RACE-R</td>
<td></td>
<td></td>
<td>1274</td>
</tr>
<tr>
<td>5′ RACE adapter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3′ RACE-F</td>
<td></td>
<td></td>
<td>1666</td>
</tr>
<tr>
<td>3′ RACE adapter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>qPCR of Dicer1</td>
<td>Dicer-QF</td>
<td>TGGACTGAGGAAGACTCTTCCT</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>Dicer-QR</td>
<td>CTGGTGAGGCTTTACAGATGCTTA</td>
<td></td>
</tr>
<tr>
<td>dsRNA synthesis of Dicer1</td>
<td>Dicer-IF</td>
<td>taatacgactcactataggtTTTGGCAGGATTCGGAGACT</td>
<td>365</td>
</tr>
<tr>
<td></td>
<td>Dicer-IR</td>
<td>taatacgactcactataggtTCCAATCTACGGGTATCCATA</td>
<td></td>
</tr>
<tr>
<td>cDNA synthesis and qPCR of miRNA</td>
<td>miR-8RT</td>
<td>CTCAACTGCTGAGGATTCGGAGACT</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>miR-8QF</td>
<td>CAGTTGAGACATCGTTACCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-8QR</td>
<td>ACACCTCGCTGATTTACACATGCTAGGTA</td>
<td>63</td>
</tr>
<tr>
<td>let-7RT</td>
<td></td>
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<td></td>
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<tr>
<td>let-7QF</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>let-7QF</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| cDNA, complementary DNA; qPCR, quantitative polymerase chain reaction; dsRNA, double-stranded RNA; RACE, rapid amplification of cDNA ends; F, forward primer; R, reverse primer. The T7 polymerase promoter sequences are written in lowercase letters.

transcription reactions were prepared by PCR amplification from plasmid DNA of the cDNA clone of LmDicer1 using the T7 primer pairs (shown in Table 1). dsRNA was synthesized using T7 RiboMAX™ Express RNAi System (Promega) following the manufacturer’s instructions. The synthesized dsRNAs were dissolved in nuclease-free water and adjusted to 10 μg/μL. The newly emerged fourth instar nymphs were used for injection experiments. The 20 μg of LmDicer1 dsRNA was injected into the abdomen between the second and third abdominal segments using a manual microinjector. Control nymphs were injected with equivalent volumes of dsGFP (green fluorescent protein). Totally 52 nymphs were injected with dsRNA of LmDicer1 and the 55 control nymphs were injected with dsGFP. The nymphs were incubated and observed carefully after the injection. We chose 4-day-old fifth instar nymphs to assess the effects of the RNAi treatment on LmDicer1 mRNA levels by using reverse transcription PCR (RT-PCR) and qPCR, one biological replication contained five nymphs, and four replications were prepared in this experiment.

Results

Analyses of cDNA and deduced amino acid sequences of LmDicer1

We got a fragment of Dicer cDNA by bioinformatics from the locust transcriptome database. Subsequently, 5′ and 3′ RACE gave a full-length sequence of 6 975

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nucleotides, which encoded a protein of 2,325 amino acids with a predicted molecular mass of 216.2 kDa (Fig. 1A). A ScanProsite (http://prosite.expasy.org/scanprosite/) search revealed that the L. migratoria Dicer sequence has two amino-terminal helicase domains, a PAZ (Piwi/Argonaute/Zwille) domain, two RNase III domains and two dsRNA binding domains (Fig. 1A), which is a typical organization of a Dicer protein (Carmell & Hannon, 2004) and similar to B. germanica Dicer proteins 1 (Gomez-Orte & Belles, 2009). The phylogenetic analysis indicated that it was a Dicer1 orthologue, supported by high bootstrap values (Fig. 1B). Furthermore, the L. migratoria Dicer amino acid sequence shows 45% identity with D. melanogaster Dicer1 and only 25% with D. melanogaster Dicer2. Our phylogenetic analyses, domain architecture and sequence alignment support that this L. migratoria Dicer sequence belongs to Dicer1, and we called it LmDicer1. The full-length cDNA sequence of LmDicer1 was submitted to NCBI database with accession number JQ900305.

**RNAi of Dicer1 depletes miRNAs**

To silence Dicer1 expression in L. migratoria by RNAi, a dsRNA of LmDicer1 was synthesized in vitro and injected into the fourth instar nymphs of L. migratoria. Expression of LmDicer1 showed few variations during the fourth instar nymph (Fig. 2A), thus we chose randomly freshly emerged fourth instar nymphs to inject dsDicer1. The silence efficiency of LmDicer1 mRNA was assessed on day 4 of the fifth instar nymph. Significantly lower levels of LmDicer1 transcripts were detected by both RT-PCR and qPCR. Specifically, the LmDicer1 transcript level was reduced by about 45% in the nymphs injected with LmDicer1 dsRNA compared with the control insects injected with dsGFP (Fig. 2B). Then we tried to assess whether RNAi of LmDicer1 impairs miRNA maturation, taking miR-8 and let-7 as examples (Fig. 2C). MiR-8 showed a practically invariant expression during the whole post-embryonic development, whereas let-7 was characteristically up-regulated in the transition from nymph to adult stages (unpubl. data). Total RNA enriched for small RNAs was extracted on day 4 of the fifth instar nymphs, which had been treated in the newly emerged fourth instar with dsGFP or dsLmDicer1, respectively. Quantitative RT-PCR analysis showed that miR-8 and let-7 levels were significantly repressed when treated with dsLmDicer1 compared with controls treated with dsGFP (Fig. 2C).

**Depletion of Dicer1 and miRNAs impairs development**

We carefully observed locust development after RNAi of LmDicer1, the result showed that, in a total of 52 fourth instar nymphs injected with LmDicer1 dsRNA, 12 died when insects molted to the next stage, most of them (40 out of 52, i.e., 76.9%) survived to the fifth instar. Among the 40 survivors, 21 died and 19 survived when molting to the adult. Thus, the total mortality of the nymphs from the fourth instar to the adult was 63.5%. In contrast, the total mortality in the control during the same time span was only 5.5% (3 out of 55) (Table 2, Fig. 3J).

The nymphs treated with dsGFP (n=55), smoothly molted to the fifth instar nymphs (Fig. 3A,B,J and Table 2) and then to adults (52 out of 55) (Fig. 3C,J), with normal morphology. In contrast, the nymphs (12 out of 52) treated with dsLmDicer1 failed molting to the next instar nymphs with abnormal morphology; part of the insect body was strongly emboweled by old cuticles and there was unsuccessful molting and death (Fig. 3D,E and Table 2). The remaining nymphs (40, i.e., 76.9%) molted to the fifth instar nymphs morphologically similar to...
controls and underwent a subsequent molt. Consequently, insects injected with dsLmDicer1 molted abnormally to the adult stage. They could not completely peel off the exuviae and died (19, i.e., 36.5%), but revealed adult features (adult general shape, long wings and genital region well-formed) (Fig. 3F,G,J and Table 2). Some locusts (19, i.e., 36.5%) showing a successful molt were similar to controls in morphology, but the morphology of the wings were severely shortened and twisted (Fig. 3H,I and Table 2). All these results clearly show important biological functions of LmDicer1 during the development of L. migratoria.

**Discussion**

The migratory locust is a typical hemimetabolous insect that undergoes an incomplete metamorphosis, bypassing the pupal stage (i.e., egg, nymph and adult). The locust has also been serving as an important model system in the study of development, neuroscience, physiology,
evolution, as well as others (He et al., 2004; Simpson & Sword, 2008; He et al., 2006). Dicer1 is a key enzyme involved in miRNA biosynthesis (Jacek et al., 2010). Studies on dicer1 in very different groups of insects are important for better understanding the characteristics and functions of miRNA in development. In this paper, we reported, for the first time, a full-length cDNA encoding dicer1 from locusts. The deduced amino acid sequence of the LmDicer1 cDNA shows a close phylogenetic relationship with those from other insect species. Moreover, depletion of Dicer1 in the fourth instar nymph of the migratory locust resulted in reduced levels of mature miRNAs, abnormal molt during development, and severely impaired metamorphosis when locusts molt to adults. The results therefore suggest that Dicer1 and miRNAs are crucial for modulating locust molting and development, and we presume that miRNAs act by disrupting translation and promoting mRNA decay of genes related to molting, the gene products of which give antimolting properties.

In Drosophila, let-7 and miR-125 mutants exhibit a pleiotropic phenotype arising during metamorphosis. The abrupt gene is a target of let-7, and its repression in muscle is essential for the timing of neuromuscular junction maturation during metamorphosis (Caygill & Johnston, 2008). MiR-1 specifically expressed in the mesoderm and its muscle cell derivatives, played an important role in the development of muscles and the heart, and regulated Notch signaling (Kwon et al., 2005; Sokol & Ambros, 2005). MiR-7 regulated the Notch signaling pathway and ensured the accurate differentiation of sense organs (Pek et al., 2009). The mutant of miR-278 can lead to defective energy homeostasis and relatively small insulin production (Teleman et al., 2006). In Caenorhabditis elegans, lin-4 is required to downregulate target genes in early larval stages, whereas let-7 acts in the last larval stage, just prior to the transition to adulthood, and loss of either miRNA alters the timing of decisions affecting cell fates (Moss, 2007). Our results indicate that in the locust, RNAi of LmDicer derepresses expression of miRNA miR-8 and let-7 and causes profound changes in development of locusts, such as defects that affect the molt of nymph to nymph and nymph to adult. However, we cannot definitely say which mature miRNA contributes to the effects of disrupting perfect molting, and how miRNAs regulate the development or metamorphosis of migratory locusts needs to be further investigated. The precise molecular mechanisms that underlie post-transcriptional repression by miRNAs mediating development of locusts still remain largely unknown.

The migratory locust is an important agricultural pest, which causes hundreds of millions of dollars worth of damage every year. Chemical insecticides have been applied frequently for many years to control locusts, inevitably leading to insecticide resistance (Ma et al., 2004; Yang et al., 2008). Insect control strategies by using a novel mode of action would be valuable for managing the emergence of insect resistance. A great success has recently been achieved by using the RNAi to silence essential genes for controlling growth and development of insects (Baum et al., 2007; Mao et al., 2007). Our findings in the hemimetabolous migratory locust showed some differences concerning the role of dicer1 with the results from holometabolan and hemimetabolan metamorphosis insects such as T. castaneum and B. germanica (Tomoyasu et al., 2008; Gomez-Orte & Belles, 2009). In L. migratoria, using RNAi to silence the expression of Dicer1, we depleted miRNA contents in the locust and disrupted both types of molt (nymph–nymph and nymph–adult). The RNAi of LmDicer1 also resulted in a high mortality in L. migratoria. Our study revealed that LmDicer1 is essential for the growth and development of L. migratoria. These results further support our notion that LmDicer1 could serve as an excellent target for developing novel strategies for controlling this important insect pest.

Table 2  Summary of the RNA interference experiments carried out on fourth instar nymphs of L. migratoria.

<table>
<thead>
<tr>
<th>dsRNA</th>
<th>n</th>
<th>Fourth instar nymphs</th>
<th>Fifth instar nymphs</th>
<th>Adult with twisted wings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Abnormal molting death</td>
<td>No phenotypic death</td>
<td>Abnormal molting death</td>
</tr>
<tr>
<td>dsGFP</td>
<td>55</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>dsDicer1</td>
<td>52</td>
<td>11</td>
<td>1</td>
<td>19</td>
</tr>
</tbody>
</table>

†Total number of fourth instar nymphs used for dsRNA injection.

‡See the text for a complete description of the phenotypes.

dsRNA, double-stranded RNA; GFP, green fluorescent protein.

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miRNA-dependent development in locust

Fig. 3 Effect of dsLmDicer1 injected into fourth instar nymphs on development of Locusta migratoria. (A, B and C) normal fourth, fifth instar nymph and normal adult; (D and E) insects failed to detach the old cuticle during molting to fifth instar nymph; (F and G) insects injected with dsLmDicer1 molted imperfectly to adult; (H and I) adults with the wings not well stretched; (J) survivorships of the locusts at fourth, fifth instar and adult following the LmDicer1 double-stranded RNA or dsGFP (green fluorescent protein) injection at the fourth instar stage.

Acknowledgments

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Disclosure

The authors declare no conflicts of interest.

Reference


