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Identification of microRNA and their potential role in regulation diapause termination in seven spot ladybird beetle, *Coccinella septempunctata*

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Abstract

Diapause is an alternative development stage in seven spot ladybird beetle, *Coccinella septempunctata*. However, the regulatory mechanism governing the initiation, maintenance, and termination of diapause in the seven-spot ladybird have not been extensively studied. MicroRNAs (miRNAs), a type of non-coding RNA, might be involved in diapause regulation and related physiological processes. The objective of this study is to investigate the potential involvement of miRNAs in diapause termination in *C. septempunctata*. High-throughput sequencing was used to identify miRNAs associated with diapause termination in *C. septempunctata*. A total of 769 miRNAs were identified, potentially implicated in diapause termination, including 673 evolutionarily conserved miRNA and 96 putatively novel-miRNAs. Among these, two evolutionarily conserved miRNAs, aae-miR-305-5P and tca-miR-277-5P, exhibited differential abundance during diapause termination compared to diapause. aae-miR-305-5P was overexpressed in diapause termination ladybird beetle and may be responsible for silencing the expression of candidate genes in peroxisome pathway associated with diapause termination. Conversely, tca-miR-277-5P was under-expressed in diapause termination and may promote the expression of genes related to the longevity regulating pathway, thereby increase the lifetime, a characteristic feature of diapause termination. In addition, a putatively novel-miRNA (unconservative_c62764) was overexpressed in diapause termination ladybird beetle, potentially contributing to the decreased expression of genes related to Wnt signaling pathway during diapause termination. These findings highlighting the significant roles of microRNAs in pathway such as longevity regulation, perisome function, and Wnt signaling, which may regulate diapause termination in *C. septempunctata*. This study might help us to unveil the miRNA involvement in gene expression regulation of diapause termination in insects.

Keywords *Coccinella septempunctata*, Diapause termination, microRNA, High-throughput sequencing, RNAi

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Introduction

Diapause is a phenotypically plastic, alternative developmental pathway that enabling them to survival in unfavorable environmental conditions and synchronize their life cycles with seasonal changes (Denlinger 2002; Hahn and Denlinger 2011). By entering a dormant state, insects conserve energy and resources, enduring optimal timing for growth, reproduction, and population regulation (Hand et al. 2016). Additionally, diapause provides protection against predators and parasites, enhancing insect survival and reproduction success in various environments (Wheeler 2003). Diapause can be classified into three primary phases: diapause induction or preparation, diapause maintenance, and diapause termination (Denlinger and Armbruster 2014). Diapause is triggered or indicated to be triggered by alterations in gene expression, leading to nutrient sequestration, metabolism suppression, slowing or halting of development, and increased tolerance to environmental stresses (Denlinger 2002, 2008; Hahn and Denlinger 2011; Denlinger and Armbruster 2014; Fan et al. 2023; Anna et al. 2023). Environmental cues such as temperature and photoperiods also play key roles in regulating diapause maintenance, ensuring survival and reproductive success in variable conditions. Diapause termination in insects often involves the activation of specific signaling pathways, hormonal changes, and environmental cues, which trigger the resumptions of metabolic activity, developmental processes, and reproductive maturation. Additionally, diapause termination may be facilitated by the degradation of dormancy-inducing factors and the upregulation of genes associated with growth and reproduction, ultimately allowing the insect to transition from a dormant state to an active one in response to favorable environmental conditions (Li et al. 2023).

The seven-spot ladybird beetle, *Coccinella septempunctata* L. (Coleoptera: Coccinellidae), represents is a beneficial arthropod predator and extensively employed as a biological control agent globally (Singh et al. 2004; Xiao et al. 2016; Yu et al. 2014). Both larvae and adults of *C. septempunctata* are known for their efficacy in preying on aphids that infest various crops, therefore, the seven-spot ladybird beetle was selected as an effective natural enemy in an integrated pest management (IPM) programs targeting aphids infestation (Xiao et al. 2016). With the advancement of artificial diet rearing technique for *C. septempunctata* (Simelane et al. 2004), it has become feasible to release sufficient number of these beetles into fields to combat heavy aphid infectations simultaneously. Cooling has proven to be a simple effective method for storing adults during the production process (Denlinger 2008). However, low environmental temperature (< 18 °C) easily induced prolonged diapause in *C. septempunctata,* and diapause termination does not occur immediately under normal environmental conditions. The lengthy duration (approximately 2 weeks) required for diapause termination poses a constraint on the artificial rearing of *C. septempunctata*. Thus, it is imperative to elucidate the molecular mechanism of diapause termination in *C. septempunctata*.

MicroRNA (miRNA) is a type of non-coding single-stranded RNA molecule with a length of about 21–24 nucleotides. Its primary function is to regulate gene expression by targeting mRNA sequences in the 5′ untranslation region (5′ UTR), the coding sequence (CDS) or the 3′ UTR of the target mRNA (Asgari 2013; Mattick 2009). In insects, a diverse array of miRNAs have been identified, playing crucial roles in various biological processes, such as embryonic development, tissue differentiation, cell morphogenesis, metabolism, stress resistance, and immunity (Leung and Sharp 2010; Legeai et al. 2010; Mukherjee and Vilcinskas 2014; Yu et al. 2009; Freitak et al. 2012; Hussain and Asgari 2014; Zhang et al. 2015; Rahimpour et al. 2019; De Lella Ezcurra et al. 2016; Duan et al. 2022). Several studies have demonstrated that miRNAs were involved in diapause regulations (Reynolds et al. 2017). For examples, small RNAs have been shown to regulate pupal diapause in *Sarcophaga bullata* (Reynolds et al. 2013) and diapause termination in *Helicoverpa zea* (Reynolds et al. 2019). In *S. bullata*, two evolutionarily conserved miRNAs were significantly up-regulated and eight miRNAs were significantly down-regulated after diapause induction (Reynolds et al. 2017). Similarly, in *Aedes albopictus*, the expression levels of seven miRNAs have significant changed during diapause in larvae (Batz et al. 2017). In *Bombyx mori*, two miRNAs (bmo-miR-3384-3p and bmo-miR-2761-3p) are closely related to diapause regulation (Fan 2016). In the context of diapause termination, miR-71 initiates diapause termination by inhibiting the expression of insulin receptor pathway gene in *Caenorhabditis elegans* (Ling et al. 2017). Base on this inference, miRNAs play a significant role in regulating diapause in *C. septempunctata*.

The previous studies demonstrated that the adults of *C. septempunctata* display a typical reproductive diapause, which markedly extends life span, but significantly decrease fecundity and hatching rate (Wang 2012; Li et al. 2023). In this study, the high-throughput sequencing was used to investigate the role of miRNA in the diapause termination process in *C. septempunctata*; aiming to: (1) identify differentially abundant miRNAs; (2) identify the potential target genes of these miRNAs; (3) reveal miRNA targeted diapause termination-related gene functions. Our study holds scientific significance by shedding light on the potential molecular mechanism underlying diapause termination in *C. septempunctata*.

Material and method

Insect rearing

Adults of *C. septempunctata* were collected from experimental wheat fields (39° 95′ N, 116° 28′ E) of the Beijing Academy of Agriculture and Forestry Sciences (BAAFS), Beijing, China, May 2013. The ladybird beetles were reared on the soybean aphids, *Aphis glycines* (Hemiotera: Aphididae) at 25 ± 2 °C; 65% RH; 12D:12L photoperiod, in the Laboratory of Natural Enemies Research, Institute of Plant Protection, BAAFS (L-100, Suntech, Beijing, China). All *C. septempunctata* were maintained in custom-built cages (50 cm × 50 cm × 60 cm; 45-micron mesh screen on aluminium frames) with 40 pairs of *C. septempunctata* adults to one cage and fed with *A. glycines* daily on fresh soybean.

Sampling

The diapause and diapause termination *C. sempunctata* were collected based on previous study (Wang 2012). The newly emerged ladybirds (2-day) were paired and placed in small cage (diameter: 6.0 cm; high: 2.5 cm) supplied enough *A. glycines* daily. These small cages were placed in versatile environmental test chamber (Sanyo, MLR-351H) under diapause condition at 18 °C, 70% RH and 10L:14D photoperiod. The ladybirds which did not lay eggs at 40 days after treatment were considered as diapause. For the diapause termination, ladybirds were transferred into other versatile environmental test chamber under diapause termination condition at 25 °C, 70% RH and 14L:10D photoperiod. The criterion of diapause termination is that diapause ladybirds begin to lay eggs.

RNA extraction and illumina sequencing

Small RNA libraries were constructed from total RNA isolated from diapause and diapause termination females, and three replicates per treatment using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of each RNA sample was assessed using Nanodrop ONE spectrophotometer (ThermoFisher, Waltham, MA, USA). Six libraries were constructed and Illumina sequencing were conducted by Baimaike company (Beijing, China). The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v4-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina platform (HiSep2500) and single-end reads were generated.

miRNA identification and annotation

Fastq formatted raw data (raw reads) were cleaned by removing reads containing adapter, reads containing poly-N and low-quality reads from raw data. After cleaning, all reads were further trimmed by removing the sequences smaller than 18 nt or longer than 30 nt. Q20, Q30, GC-content and sequence duplication level of the trimmed data were calculated. All the downstream analyses were based on the high-quality sequences.

Using Bowtie tools software, the trimmed reads were compared with Silva, GtRNAdb, Rfam (Gardner et al. 2009) and Repbase (Jurka et al. 2005) database sequence alignment to filter ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA) and other ncRNA and repeats, respectively. The remaining reads were used to blast known miRNA and novel miRNA predicted by comparing with genome and miRBase. Randfold tools soft was used to identify novel miRNA secondary structure prediction.

In the conserved miRNA identification, the remaining sequences were blasted with the known precursor and mature miRNA sequence from Arthropods in miRBase database (v21), and the sequences which were identical with known miRNA were considered as conserved miRNA (Kozomara and Griffiths-Jones 2013). miRDeep2 was used to identify the novel miRNA (Batz et al. 2017; Friedlander et al. 2012).

Analysis of differentially abundance miRNAs

Differential abundance analysis of miRNAs was performed using the DESeq2 R package (1.10.1). DESeq2 provide statistical routines for determining differential expression in digital miRNA expression data using a model based on the negative binomial distribution. The resulting *P* values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate (FDR). The differential abundance of miRNA with $\log_2^{(FC)} \geq 1$; FDR ≤ 0.05 found by DESeq2 were assigned as differentially expressed.

Target prediction of differentially abundance miRNAs

Two target prediction algorithms miRanda (Betel et al. 2008; Enright et al. 2003) (http://www.microrna.org/microrna/getDownloads.do) and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/welcome.html) (Rehmsmeier et al.2004; Kruger and Rehmsmeter 2006) were used to predict the target gene of differentially abundance miRNAs based on the sequence information of corresponding species of conserved and novel miRNAs. Furthermore, functional annotation of target gene was predicted using BLAST software based on the following databases: Nr (NCBI non-redundant protein sequences) (Deng et al. 2006); KOG/COG (Clusters of Orthologous Groups of Proteins) (Koonin et al. 2004); Swiss-Prot (A manually annotated and reviewed protein

sequence database) (Apweiler et al. 2004); KEGG (KEGG Ortholog database) (Kanehisa et al. 2004); GO (Gene Ontology) (Ashburner et al. 2000).

Enrichment analysis of GO and KEGG pathway

Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was conducted by the top GO software based Wallenius non-central hyper-geometric distribution. KOBAS software was used to test the statistical enrichment of differential expression genes in KEGG pathways (Mao et al. 2005).

Quantitative reverse-transcription PCR

Three miRNAs (aae-miR-305-5P, tca-miR-277-5P and unconservative_c62764) and their target genes (*CsACX*, *CsAC2* and *CsSIAH1*) were analyzed by RT-qPCR to validate our RNA-seq data. Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription of miRNAs was carried out using the miScriptIIRT Kit (Qiagen, Dusseldorf, Germany) according to manufacturer's protocols for using the HiSpec buffer, which specifically transcribes mature miRNAs. Reverse transcription of target genes was carried out using the PrimeScript™ RT reagent Kit (Takara, Dalian, China) according to manufacturer's protocols.

Relative abundance of miRNAs in diapause and diapause termination groups were measured using Applied Biosystems® Real-time PCR Instrument (ABI Laboratories, Hercules, CA, USA) along with the miScript SYBR Green PCR Kit (Qiagen), which used a combination of one universal primer and one primer that was designed to detect miRNA sequence (Table 1). Relative abundance of target genes in diapause and diapause termination groups was also measured using Applied Biosystems® Real-time PCR Instrument along with the SYBR *Premix Ex Taq* II Kit (Takara). The optimized quantitative PCR program consisted of an initial denaturation at 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s. Dissociation curve was obtained to verify amplification specificity, in which the samples were cooled to 60 °C after denaturing, increasing 0.5 °C/10 s for each cycle with a total of 70 cycles until reaching 95 °C to denature the double-stranded DNA. The relative abundance of miRNAs and their target genes were normalized to U6 and Tubulin as internal reference, respectively.

Suppression of miRNAs expression and effect on target expression

The inhibitors of aae-miR-305-5P, tca-miR-277-5P and unconservative_c62764 were designed and synthesized by tsingke company (Beijing, China) (Table 2). The fourth instar larvae of *C. septempunctata* were selected for injections of miRNA inhibitors at 300 ng/larva. DEPC water was injected as control. Each experiment

Table 2 Sequence information of miRNA inhibitors in *C. septempunctata*

Name	Sequence
aae-miR-305-5P-inhibitor	CCAGAGCACCUGAUGAAGUACAAU
tca-miR-277-5P-inhibitor	GUGUAAACGCGCAUCUGGCACG
Unconservative_c62764-inhibitor	UGUGAACCACUCUACGAACAAG

Table 1 Primers used to analyze abundance of miRNAs and target genes in *C. septempunctata*

Primer Name	Sequence (5′-3′)	Tm (℃)	Product size (bp)
Reverse transcription quantitative PCR (RT-qPCR) for miRNA analysis			
aae-miR-305-5P-F	TGTACTTCATCAGGTGCTCTGGA	62.0	
tca-miR-277-5P-F	TGCCAGATGCGCGTTTACAC	62.0	
Unconservative_c62764-F	CTTGTTCGTAGAGTGGTTCACA	62.0	
U6-F	TCGGTTAGTACTTGGATGGGA	62.0	
Universal primer	GAATCGAGCACCAGTTACGC	62.0	
Reverse transcription quantitative PCR (RT-qPCR) for target gene analysis			
CsACX(Q)-F	CTGATACGAACGAACTACCAA	55.9	130
CsACX(Q)-R	ATTGATTTCCACACAGACCAC	57.3	
CsAC2(Q)-F	AGCTGGAGACCTTGTACCAT	60.7	145
CsAC2(Q)-R	CTGATATTGTCCAGCATGTCCT	58.5	
CsSIAH1(Q)-F	ATTCTTCGAGCAAGATGTCCAC	58.6	156
CsSIAH1(Q)-R	ATCGACCACACATTGTATGACC	58.4	
Tubulin-F	ACAGGTTTCAAAGTGGGTATCA	60.0	102
Tubulin-R	GGTGGTGTTTGACAACATGC	60.0	

was replicated three times, with a minimum of 40 insects per replicate. The injected insects were reared under standard rearing conditions. RT-qPCR was used to monitor changes in the transcript levels of target genes at 2 and 4 days after the injection. Four insects were collected from each time point and pooled as a sample for total RNA extraction and RT-qPCR analysis.

Statistical analysis

Relative miRNA and target genes abundance were evaluated in three replicates for each group with three technical replicates for each miRNA. $2^{-\Delta\Delta CT}$ method was used to calculate the abundance of miRNA and their target genes. Statistical analysis of the abundance of miRNA and their target genes were performed via SPSS 26.0 software (SPSS, Chicago, IL, USA). All data in this research were converted logarithmically before using One-Way ANOVA.

Results

RNA libraries and read mapping

All microRNA reads were submitted to the National Center for Biotechnology Information (NCBI) Short Read Archive with the accession numbers (PRJNA665787). Three diapause and three diapause termination libraries were sequenced, and 78.11 M raw data was obtained, including 39.34 M in diapause groups and 38.76 M in diapause termination groups. A total 69.71 M clean reads were generated after trimming and filtering out low quality sequences (Supplementary Table 1). The typical length of miRNA is 18–26 nt, and the maximum miRNA is 22 nt (Fig. 1).

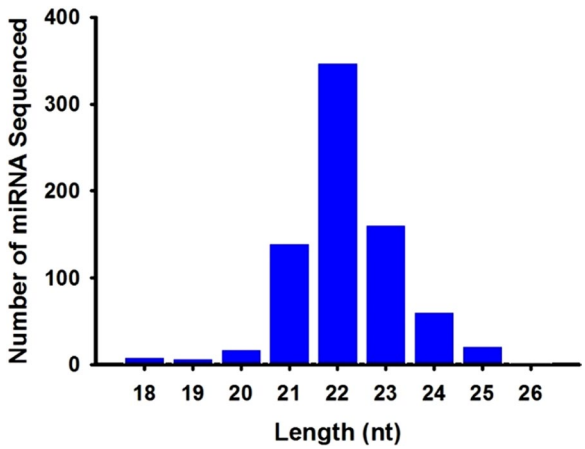

Fig. 1 Abundance of each size of small RNA sequenced based on nucleotide (nt) length

Discovery of miRNAs in diapause and diapause termination

Mappable reads with 18–26 nt were clustered into families of unique sequences that were mapped to sequence database miRBase (v21). After clustering these sequences, 769 miRNAs were identified, including 673 evolutionarily conserved miRNA. The number of evolutionarily conserved miRNA in each library was ranged from 633 to 653 (Table 3).

The transcript sites of miRNA are located in intron, intergenic region and the reverse sequence of coding sequence. The precursor of miRNA has symbolic hairpin structure and the formation of mature miRNA was obtained by Dicer cutting. Based on miRNA biological characteristics, sequence information miRNA precursor and the structure energy of miRNA precursor, the miRDeep 2 software was used to identify putatively novel miRNAs based on the scores of Bayesian model. In total, 96 putatively novel miRNAs were identified in libraries of diapause and diapause termination. The number of putatively novel miRNAs in each library was ranged from 73 to 89 (Table 3).

Differences in miRNA abundance related to diapause and diapause termination

The miRNAs were tested for differential abundance between diapause and diapause termination. We found two evolutionarily conserved miRNAs with differential abundance between diapause and diapause termination [differential abundance ratio ≥ 2.0 and False Discovery Rate (FDR) ≤ 0.05]. aae-miR-305-5P was over-expressed and tca-miR-277-5P was under-expressed in diapause termination group as compared with diapause (Table 4). In addition, 8 putatively novel miRNAs also have the differential abundances between diapause and diapause termination. All of the putative novel miRNAs were over-expressed in diapause termination comparing to

Table 3 Summary of evolutionarily conserved and putatively novel miRNAs in *C. septempunctata*

Sample	Evolutionarily conserved-miRNAs	Putatively novel-miRNAs	Total
Diapause 1	634	73	707
Diapause 2	653	78	731
Diapause 3	638	82	720
Diapause termination 1	651	82	733
Diapause termination 2	648	89	737
Diapause termination 3	633	75	708
Total	673	96	769

Evolutionarily conserved-miRNAs: number of known miRNAs; Putatively novel-miRNAs: number of newly predicted miRNAs; Total: total miRNA number

Table 4 Evolutionarily conserved-miRNAs with significantly difference in diapause relative to diapause termination

miRNA		Log_2^{-FC}	*P*-value	FDR	Predicted gene target	
					Number	Name
Up-regulated miRNAs	aae-miR-305-5P	1.143	0.0034	0.031	5	c138002.graph_c0; c68846.graph_c0; 68930.graph_c0; c73425.graph_c0; 86362.graph_c0
Down-regulated miRNAs	tca-miR-277-5P	− 1.504	0.0058	0.038	21	c100051.graph_c0; c108321.graph_c0; c112655.graph_c0; c120562.graph_c0; c124057.graph_c0; c128276.graph_c0; c142027.graph_c0; c20284.graph_c0; c3019.graph_c0; c39199.graph_c0; c56931.graph_c0; c62240.graph_c0; c70523.graph_c3; c73464.graph_c1; c73894.graph_c0; c74074.graph_c0; c74337.graph_c0; c74793.graph_c0; c75203.graph_c0; c7960.graph_c0; c91339.graph_c0

diapause. The differential abundance ratios were ranged from 1.824 to 5.461(Table 5).

miRNA target prediction and characterization

MicroRNA target prediction was characterized the potential downstream effects of regulation by differential abundance miRNAs using target prediction algorithms of miRanda and RNAhybrid. In total, 975 putative target genes were identified corresponding to 624 evolutionarily conserved miRNAs. In addition, 1030 putative target genes were identified corresponding to 84 putatively novel miRNAs (Table 6).

Gene Ontology (GO) was used to predict the biological processes, cellular component and molecular function. Biological processes likely regulated by differentially expressed miRNA include biological regulation, response to stimulus and signaling and so on. Cellular component likely regulated by differentially expressed miRNA include synapse, cell junction and so on. Molecular function likely regulated by differentially expressed miRNA include molecular transducer activity and receptor activity (Fig. 2). TopGO software was used to identify diapause termination related miRNAs. These miRNAs might regulate target genes expressions related to diapause termination of *C. septemtunctata*. Gene Ontology defined process to be enriched in comparisons of diapause termination to diapause transcriptomes of *C. septemtunctata*. 11 GO pathway were identified in biological process, including: cellular biosynthetic process ($P=0.00035$); organic substance biosynthetic process ($P=0.00035$); cellular macromolecular complex assembly ($P=0.00907$); cellular macromolecular biosynthetic process ($P=0.01025$) and so on (Supplementary Table 2). 10 GO pathway were identified in cellular component, including: intracellular membrane-bounded organelle ($P=0.00092$), cytoplasmic part ($P=0.00766$); mitochondrial part ($P=0.00916$); mitochondrial membrane ($P=0.01309$) and so on (Supplementary Table 3). However, only 1 GO pathway DNA binding ($P=0.045$) was identified in molecular function (Supplementary Table 4).

Five KEGG pathways with a probability of being regulated by diapause termination, like longevity regulating pathway, peroxisome pathway, AGR-RAGE signalling pathway, Wnt signaling pathway and neuroactive ligand-receptor interaction (Table 7). Furthermore, some notable diapause termination—relevant miRNA were identified. For example, tca-miR-277-5p regulates the gene encoding adenylate cyclase 2 in longevity regulating pathway; aae-miR-305-5p regulates the gene encoding acyl-CoA oxidase in peroxisome pathway, and unconservative_c62764 regulates the gene encoding E3 ubiquitin-protein ligase in Wnt signaling pathway.

Difference in miRNA abundance related in diapause and diapause termination

RT-qPCR was used to measure abundance of three candidate miRNAs (aae-miR-305-5P, tca-miR-277-5 and unconservative _c62764) identified with RNA-Seq that showed significant changed in diapause termination as compared with their diapause counterparts. Our results showed that aae-miR-305-5P and unconservative _c62764 were significantly increased in diapause termination groups as compared with diapause. tca-miR-277-5P was significantly reduced in diapause termination as compared with diapause (Fig. 3). The change tendency of these candidate miRNAs wase accordance with RNA-Seq results. In addition, the developmental expression pattern of these three candidate miRNAs were also evaluated using RT-qPCR. The highest expression levels of these three miRNAs were occurred in adult stage. And the relative low expression levels of these three miRNAs were occurred in early development stage (egg and first instar larvae) (Fig. 4).

Suppression of miRNA and its effect on the expression level of their target gene

To verify the relationship between miRNAs and predicted targets with RNA-seq, we performed the experiment to measure the relative transcript levels of target genes by inhibiting the expression of miRNAs.

Table 5 Relatively novel-miRNAs with significantly difference in diapause relative to diapause termination

miRNA			Log_2^{-FC}	*P*-value	FDR	Predicted gene target	
						Number	Name
Upregulated miRNAs	1	Unconservative_c109349.graph_c0_3147	2.287	0.0007	0.013	36	c100918.graph_c0; c122727.graph_c0; c22255.graph_c0; c24368.graph_c0; c26111.graph_c0; c38564.graph_c0; c42692.graph_c0; c45321.graph_c0; c49291.graph_c0; c56544.graph_c0; c57397.graph_c0; c58389.graph_c0; c60294.graph_c0; c61421.graph_c0; c62109.graph_c1; c62670.graph_c0; c62701.graph_c0; c64308.graph_c0; c64806.graph_c0; c65659.graph_c0; c66413.graph_c0; c68695.graph_c1; c69480.graph_c0; c70635.graph_c0; c71472.graph_c0; c71733.graph_c0; c71980.graph_c0; c72272.graph_c2; c72295.graph_c0; c72403.graph_c0; c73620.graph_c1; c77955.graph_c0; c80859.graph_c0; c85089.graph_c0; c895.graph_c0; c90.graph_c1
	2	Unconservative_c59627.graph_c0_55355	3.804	2.095	0.0001	4	c128860.graph_c0; c138281.graph_c0; c46803.graph_c0; c70966.graph_c1;
	3	Unconservative_c61557.graph_c0_64036	3.959	0.0073	0.046	1	c66384.graph_c0
	4	Unconservative_c62764.graph_c0_70947	1.824	0.0021	0.031	21	c109394.graph_c0; c3645.graph_c0; c46456.graph_c0; c50507.graph_c0; c51971.graph_c0; c60713.graph_c0; c62764.graph_c0; c62939.graph_c1; c64982.graph_c0; c66210.graph_c0; c66303.graph_c0; c66386.graph_c0; c67488.graph_c0; c68654.graph_c1; c70594.graph_c4; c70636.graph_c0; c72137.graph_c0; c72812.graph_c0; c74233.graph_c1; c74294.graph_c0; c74300.graph_c2
	5	Unconservative_c64080.graph_c0_79989	1.841	0.00016	0.0031	6	c120625.graph_c0; c40394.graph_c0; c46456.graph_c0; c60713.graph_c0; c64080.graph_c0; c74300.graph_c2;
	6	Unconservative_c68303.graph_c0_135085	2.193	0.0042	0.035	1	c68303.graph_c0
	7	Unconservative_c68600.graph_c1_141125	2.132	0.0043	0.0347	5	c70307.graph_c0; c70406.graph_c0; c70932.graph_c0; c72165.graph_c0; c74669.graph_c0;
	8	Unconservative_c71287.graph_c1_203528	5.461	0.00023	0.0043	5	c122889.graph_c0; c65746.graph_c0; c73163.graph_c0; c738.graph_c0; c8424.graph_c0;

Our results revealed a significant association between miRNA and target genes. Specifically, injection of aae-miR-305-5P-inhibitor and tca-miR-277-5P-inhibitor into fourth instar larvae led to a significant elevation in the expression of target gene *CsACX* and *CsAC2* compared to injection with DEPC water, respectively. After injection of aae-miR-305-5P inhibitor for 2 days, the expression level of its target gene *CsACX* significantly increased by 28.13-fold. In addition, following injection of tca-miR-277-5P for 2 days, the expression level of its target gene *CsAC2* significantly increased by 29.4-fold. Conversely, injection of unconservative_c62764-inhibitor resulted in reduced expression of *CsSIAHI* in fourth instar larvae of *C. septempunctata*. Injection of unconservative_c62764-inhibitor for 2 days, the expression level of its target gene *CsSIAHI* significantly decreased by 68.5% (Fig. 5).

Discussion

Diapause is a programmed dormancy mechanism that allows organism to endure predictable periods of unfavourable conditions by temporarily halting development

Table 6 Summary of predicated target gene of miRNA in *C. septempunctata*

Types	All-miRNA	miRNA with Target	Target-gene
Evolutionarily conserved-miRNAs	673	624	975
Putatively novel-miRNAs	96	84	1030
Total	769	708	1946

Types: miRNA Types; Known-miRNA: known miRNA; Novel-miRNA: newly predicted miRNA; All-miRNA: total number of miRNAs; miRNA with Target: predicted number of miRNAs for target genes; Target-gene: the predicted number of target genes

and reducing metabolism (Denlinger 2002; Kostal et al. 2009). Understanding the molecular underpinnings of diapause is critical for predicting changes in the geographic and seasonal distributions of insects. Although the well-established adaptive significance of diapause, the molecular mechanism underlaying this phenomenon remained elusive. Identification of the pathways involved in diapause termination in the natural enemy (*Coccinella septempunctata, Harmonia axyridis* and so on) will provide a foundation for exploring rapid diapause termination methods. Some reports suggest the possibility that miRNA may have a significant role in establishing and maintaining multiple aspects of insect diapause (Reynolds et al. 2017). In the current study, we used the natural enemy *C. septempunctata* that were allowed to diapause termination under natural conditions, to investigate the involvement of miRNA regulation in diapause termination.

Diapause represents a graded, dynamic developmental trajectory, a precise definition of diapause termination has been elusive (Ragland et al. 2011). Kostal defines

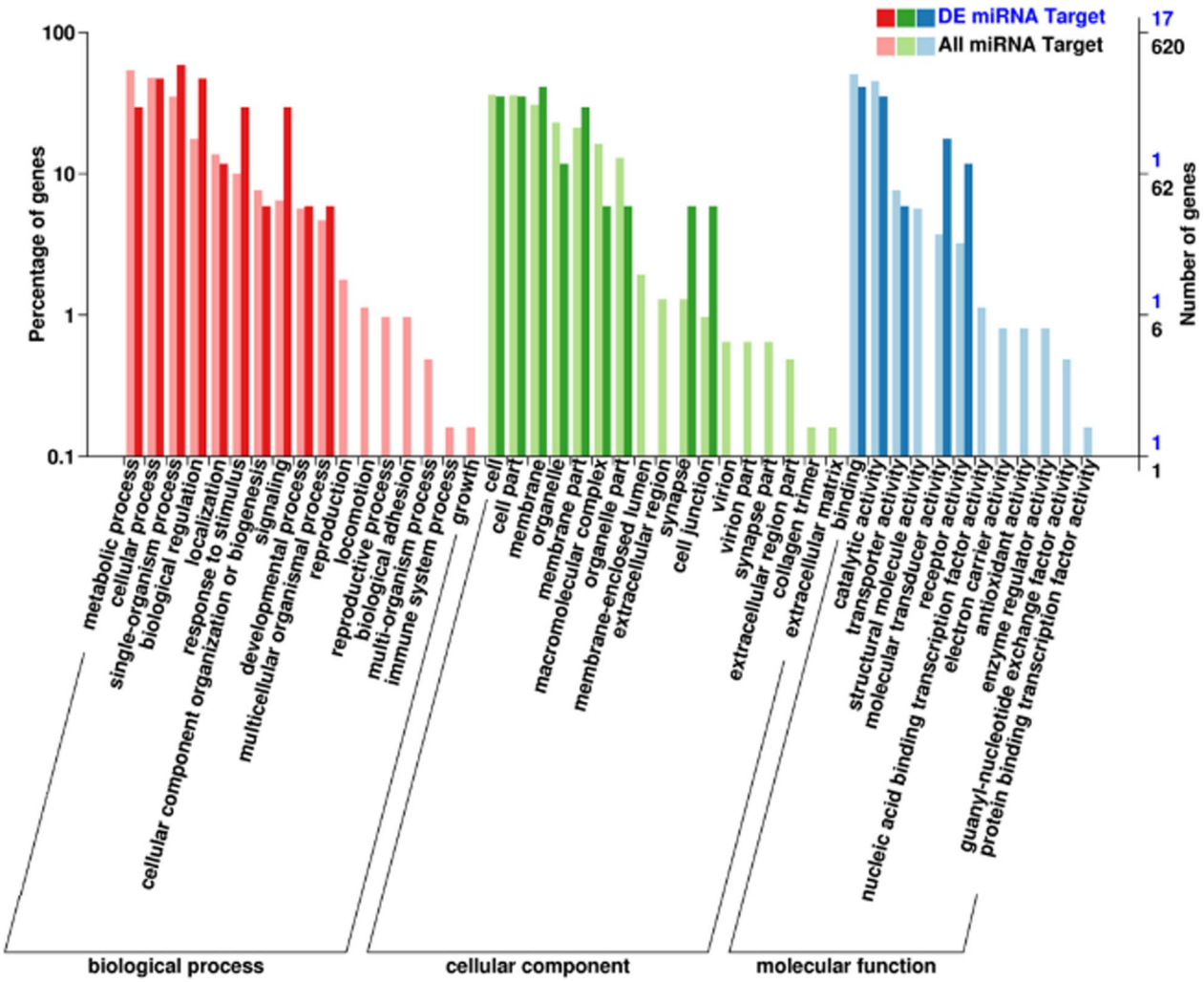

Fig. 2 Gene Ontology annotation of differentially expressed miRNA target genes of *C. septempuncata* (level2)

Table 7 KOBAS predicted targets of miRNAs that were differentially expressed in diapause relative to diapause termination

KEGG pathway	miRNA regulator	Predicted gene targets	
		Number	Gene ID
Longevity regulating pathway-multiple species (ko04213)	tca-miR-277-5p	1	Adenylate cyclase 2 c74793.graph_c0
Peroxisome (ko04146)	aae-miR-305-5p	2	acyl-CoA oxidase c73425.graph_c0
	unconservative_c59627.graph_c0_55355		2-hydroxyacyl-CoA lyase 2 c138281.graph_c0
AGE-RAGE signaling pathway in diabetic complications (ko04933)	unconservative_c68600.graph_c1_141125	2	p38 MAP kinase c70406.graph_c0
	unconservative_c109349.graph_c0_3147		Ras-related C3 botulinum toxin substrate 1 c71733.graph_c0
Wnt signaling pathway (ko04310)	unconservative_c62764.graph_c0_70947	1	E3 ubiquitin-protein ligase SIAH1 c67488.graph_c0
Neuroactive ligand-receptor interaction (ko04080)	unconservative_c109349.graph_c0_3147	1	gamma-aminobutyric acid receptor subunit beta c72403.graph_c0

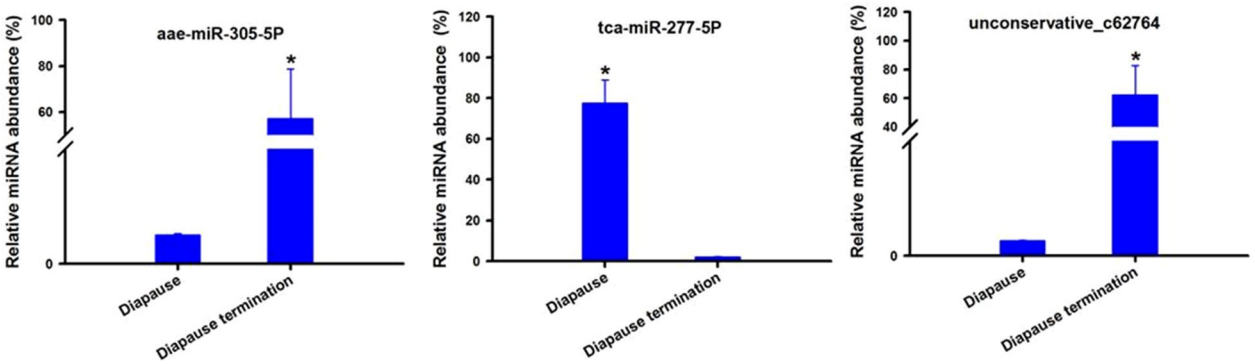

Fig. 3 Relative abundance of candidate miRNAs and their predicted targets in diapause termination and diapause status of *C. septempunctata* as determined by RT-qPCR, respectively. Asterisk above the standard error bars indicate significant differences based on ANOVA followed by T-test ($P < 0.05$). U6 and Tubulin were used as an internal reference gene to normalize the differences of miRNA and target genes, respectively

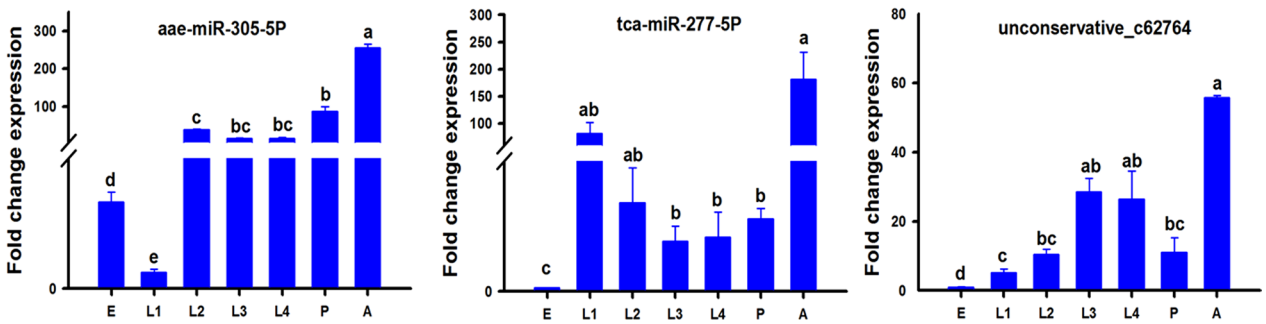

Fig. 4 Relative abundance of candidate miRNAs at different development stages of *C. septempunctata* as determined by RT-qPCR. E represent 1-day eggs; L1, L2, L3 and L4 represent first, second, third and fourth instar larvae; Pre-present 1-day pupae; and A represent 1-day old adults, respectively. Each developmental stage was analyzed with three biological samples and each sample was run with three technical replicates. Different letters above the standard error bars indicate significant differences based on ANOVA followed by Tukey's HSD multiple comparison test ($P < 0.05$). U6 was used as an internal reference gene to normalize the differences among the samples. Fold of each candidate miRNAs expression were calculated based on the 1- day eggs expressions in the developmental stage

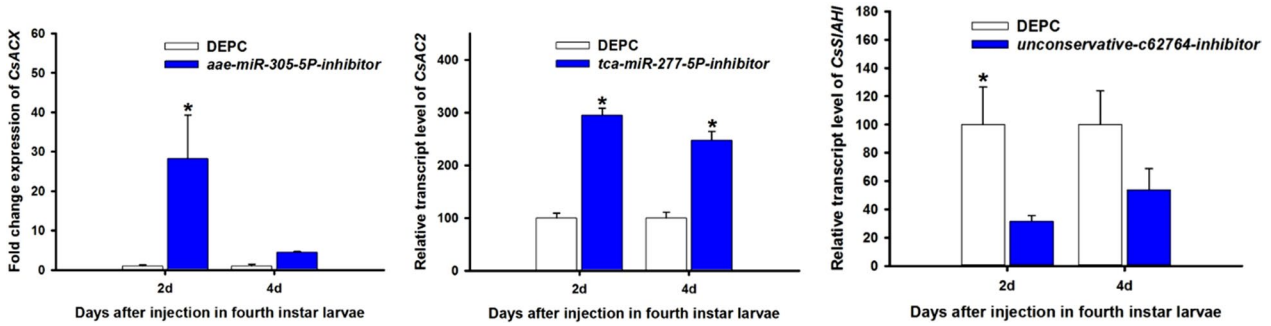

Fig. 5 Relative transcript level of predicted targets after injected with miRNA-inhibitors in fourth instar larvae of *C. septempunctata* respectively. Asterisk above the standard error bars in-dicate significant differences based on ANOVA followed by T-test ($P < 0.05$)

diapause termination as a phase during which a diapausing individual become potentiated for release from metabolic and developmental suppression, ultimately leading to the resumption of direct development (Kostal 2006). The previous study had demonstrated that the typical characteristic of diapause in *C. septempunctata* is reproductive stagnation (Wang 2012). Similar to initiation, diapause termination can be stimulated directly by an environmental cue. In our study, the diapause *C. septempunctata* transfer into diapause termination status can be stimulated by natural token cues of higher temperature and long photoperiods. The typical characteristic of diapause termination in *C. septempunctata* is the reinitiation of reproduction.

The characteristic of miRNA in diapause relative to diapause termination

Establishing shifts in miRNA abundance related to diapause termination is a critical initial step in understanding how these small RNAs may regulate diapause termination (Denlinger and Armbruster 2014). To our knowledge, miRNA abundance has most frequently been negatively corrected with the expression of its target genes. Activation of development and metabolism is a common feature of diapause termination in insects (Fan et al. 2023; Anna et al. 2023), thus, we anticipated that the majority of differentially expressed miRNAs would be under-expressed during diapause termination in conjunction with the large-scale increase in gene expression. Using next-generation sequencing, we identified 769 miRNAs in a total of three diapause and diapause termination replicates. Two evolutionarily conserved miRNAs were differentially abundant in diapause relative to diapause termination (Tables 3 and 4). One miRNA aae-miR-305-5P belong to 305 family and was up-regulated in diapause terminate process in *C. septempunctata*. Furthermore, one evolutionarily conserved miRNA tca-miR-277-5p belong to 277 family that was down-regulated in diapause termination in *C. septempunctata*.

Due to the limited number of experimentally verified miRNA targets or functions in insect species, the functional relevance of differentially expressed miRNAs must be inferred from published studies on other animals. In the previous reports, the function of miR-305 family are regulates intestinal stem cell (ISC) proliferation and differentiation, an integral part of gut remodelling during metamorphosis. In *D. melanogaster*, ISC proliferation and differentiation are regulated by miR-305 through the targeting of genes in the insulin and Notch signalling pathways, especially insulin receptor (InR) and phosphatidyl-inositol-3-kinase (pi3K) (Foronda et al. 2014; Parthasarathy and Palli 2008). In the previous report, miR-305-5P has no significant change even 48 h post-diapause in *S. bullata* (Reynolds 2017). However, in diapause termination of *Helicoverpa zea* study, miR-305-5P was significantly up-regulated 8d post injected diapause hormone to terminate diapause (Reynolds et al. 2019). Our results are consistent with the finding in *H. zea*, indicating that miR-305-5P was up-regulated during diapause termination in *C. septempunctata* compared to their diapause counterparts (Table 3). This finding suggests that miR-305-5P may play a conserved role in regulating diapause termination across insect species. Furthermore, previous studies have high lighting the multifaceted roles of miR-277 in lipid metabolism and reproduction by targeting insulin-like peptides in *Aedes aegypti* (Ling et al. 2017), control branched-chain amino acid catabolism and affects lifespan in *Drosophila melanogaster* (Esslinger et al. 2013), control metamorphosis in *Helicoverpa armigera* (Shen et al. 2020). In addition, the miR-277 family has been implicated in the relationship of diapause processes, such as: miR-277-3P was under-expressed in diapausing pupae in *S. bullata* (Reynolds 2017), and miR-277-3P was decreased after diapause termination in *H. zea* by injecting three chemical diapause

terminators ecdysone, diapause hormone and diapause hormone analogue, respectively (Reynolds et al. 2019). In our study, we observed a significant decrease in another member of the miR-277 family, tca-miR-277-5P was significantly decreased in diapause termination group as compared with diapause counterparts in *C. septempunctata* (Table 3). In addition, eight putatively novel miRNAs also showed significant changes in abundance following diapause termination compared with their diapause counterparts (Table 4). These findings provide evidence of shifts in miRNA abundance associated with diapause termination in *C. septempunctata*, further underscoring the intricate regulatory network governing this critical physiological transition.

The characteristic of miRNA target in diapause relative to diapause termination

The mechanism of diapause termination can act at various stages along an ontogenetic trajectory. Although specific mechanism may vary among species, the neuroendocrine system is a crucial regulator of diapause termination across insects. During diapause termination, organism exit the metabolic depression characteristic of diapause, leading to dramatic changes in the expression pattern of metabolic and cell development gene (Ragland et al. 2011). Integration of our miRNA data with *C. septempunctata* transcriptome analysis using KOBAS software suggests that pathways such as the longevity regulating pathway, peroxisome, AGR-RAGE signalling pathway in diabetic complications, Wnt signal pathway and neuroactive ligand-receptor interaction may be regulated by miRNAs during diapause termination in *C. septempunctata* (Table 6). A previous study in *Rhagoletis pomonella* demonstrated that three KEGG categories: tyrosine metabolism, biosynthesis of steroids and Wnt signaling were significantly differently expressed during diapause termination (Ragland et al. 2011). Our finding further supported the importance of Wnt signaling pathway in diapause termination. Genes within the Wnt signaling pathway have experimentally confirmed roles in insect development, particularly in controlling the cell cycle and transitions from cell cycle arrest to active proliferation (Reya and Clevers 2005). The up-regulation of Wnt pathway genes provides additional evidence that preparation for the re-initiation of development occurs prior to the initial release from metabolic depression during diapause. However, our results showed that one putatively novel miRNA unconservative_c62764 was up-regulated in diapause termination as compared with diapause counterparts. One possible explanation for this observation is that some miRNAs may positively regulate their target rather than inhibit their expression (Vasudevan et al. 2007). Additionally, our study also provide evidence that post-transcriptional regulation of peroxisome and longevity regulating pathway-related genes by aae-miR-305-5P and tca-miR-277-5P could be involved in diapause terminate process in *C. septempunctata*, respectively.

In summary, our study provide evidence that changes in miRNA abundance occur in response to diapause termination in *C. septempunctata*. Further studies that manipulate abundance of miRNAs will allow us to assess their role in the diapause termination. miRNA and predicate target gene highlights likely candidates, but ultimately additional observations of protein abundance and activation followed by functional manipulation (RNAi and CRISPR-Cas9 manipulation) will be needed to confirm the role of candidate genes and pathway for diapause termination in *C. septempunctata*.

Supplementary Information

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Supplementary Material 1.

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Author contributions
Conceived and designed the experiments: MW, JL, LZ, SW, DX; Per-formed the experiments: MW, JL; Analyzed the data: MW, JZ, DX; Con-tributed reagents/materials/analysis tools: SW, DX.; Wrote the paper: MW, SW, DX.; Contributed with revisions: MW, LZ, SW, DX.

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Availability of data and materials
The data set used/analyzed during the current study is available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declared that they have no competing interest in connection with the evaluated manuscript.

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