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Research

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Long Noncoding RNA lncR17454 Regulates Metamorphosis of Silkworm Through let-7 miRNA Cluster

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Abstract

A number of long noncoding RNAs (lncRNAs) have been identified in silkworm, but little is known about their functions. Recent study showed that the let-7 miRNA cluster (contains let-7, miR-2795, and miR-100) was transcribed from the last exon of lncRNA lncR17454 in silkworm. To investigate the functional role of lncR17454, dsRNAs of lncR17454 were injected into the hemolymph of 1-d-old third-instar larvae of *Bombyx mori*, repression of lncR17454 led to molting arrestment during the larval–larval and larval–pupal transition of silkworm, which was consistent to the result as let-7 knockdown in other studies. The expression level of mature let-7, miR-100, and miR-2795 decreased 40%, 36%, and 40%, respectively, while the mRNA level of two predicted target genes of let-7, the *Broad Complex isoform 2* (*BR-C-Z2*) and the BTB-Zinc finger transcription repression factor gene *Abrupt* (*Ab*), increased significantly after lncR17454 knockdown. In contrast, when adding the 20-Hydroxyecdysone (20E) to silkworm BmN4 cell lines, the expression level of lncR17454 and let-7 cluster all increased significantly, but the expression of *Abrupt*, the predicted target gene of let-7, was repressed. Dual-luciferase reporter assays confirmed *Abrupt* was the real target of let-7. Here we found that the lncRNA lncR17454 can play regulator roles in the metamorphosis of silkworm through let-7 miRNA cluster and the ecdysone signaling pathway, which will provide new clues for lepidopteran pest control.

Key words: silkworm, long noncoding RNA, miRNA, metamorphosis, ecdysone signaling pathway

A large part of eukaryote genomic loci can be transcribed into long noncoding RNAs (lncRNAs) with length more than 200 nt (Li and Liu 2019). lncRNAs can regulate gene expression in multiple levels (Caygill and Johnston 2008, Chen et al. 2020), such as epigenetic regulation of chromatin (Gendrel and Heard 2014, Samata and Akhtar 2018), transcriptional (Huarte et al. 2010, Orom et al. 2010, Hung et al. 2011, Bonasio and Shiekhattar 2014), posttranscriptional (Yoon et al. 2014, Maniati et al. 2019), and translational regulation (Cai et al. 2018), as well as having effects on protein transportation or location (Wang and Chang 2011, Li et al. 2019). Moreover, some lncRNAs function as a decoy/sponge or precursor of microRNAs (miRNAs; Liu et al. 2014, Yoon et al. 2014, Lu et al. 2016, Thomson and Dinger 2016). Such as H19, the oncofetal lncRNA is the precursor RNA of miR-675 (Cai and Cullen 2007, Tsang et al. 2010). The association of H19 with tumorigenesis and invasion is assumed to be owed to the regulation of its integrated carcinogenic miR-675 (Vennin et al. 2015, Schwarzenbach 2016).

The silkworm *Bombyx mori* is a holometabolous insect that has been domesticated and used for silk production (Mita 2009, Yu et al. 2011). A large number of noncoding RNAs (ncRNAs) were found to be involved in silkworm development (Li et al. 2011, Wu et al. 2016, Zhou et al. 2018, Xu et al. 2019, Chen et al. 2020). miRNAs also have pivotal effects on silkworm metamorphosis, let-7 can regulate the expression of orphan nuclear receptor *FTZ-F1* and the ecdysone-induced protein 74EF isoform A (*Eip74EFA*, *E74A*), knockdown of let-7 lead to developmental arrest during the larval–larval and larval–pupal transition (Ling et al. 2014).

Recent study showed that the let-7 miRNA cluster (contains let-7, miR-2795, and miR-100) was transcribed from the last exon of lncRNA lncR17454 (GenBank no. GITK01005596.1). To further

study the functional role of lncR17454, dsRNAs were injected into the hemolymph of *B. mori* larvae. We found lncR17454 can play regulator roles in the metamorphosis of silkworm through let-7 miRNA cluster.

Materials and Methods

Silkworm Rearing and Tissue Collection

The silkworm strain *Dazao P50* was reared at 25°C, humidity 60%, with a 16:8 (L:D) h photoperiod, and fed with fresh mulberry leaves. Tissues of brain, testis, ovary, malpighian tubule, ventral nerve cord, cuticle, hemolymph, fat body, midgut, and the silk gland were dissected from 3-d-old fifth-instar larvae. The whole body of silkworm with the content of midgut removing was collected from the 2-d-old first-instar larvae, 2-d-old second-instar larvae, 2-d-old third-instar larvae, 2-d-old fourth-instar larvae, 3-d-old fifth-instar larvae, and the molting stages of the first- to the fourth-instar larvae, as well as the sexed tissues of pupa (female and male) from the first to the ninth day of pupation, and the virgin male and female moths. All samples were frozen immediately in liquid nitrogen. RNAs were extracted with TRIzol (Thermo Fisher, Waltham, MA) method.

Quantitative Real-Time RT-PCR Analysis

Total RNA was extracted from different tissues and developmental stages of silkworm with the TRIzol (Thermo Fisher) method. The first strand of cDNA was synthesized with 2 μg RNA by a PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) with oligo $d(T)_{15}$. The primers used for lncRNAs and protein-coding genes are listed in Table 1. Quantitative real-time PCR (qRT-PCR) was conducted using the SYBR Green FS Universal SYBR Green Master mix (Roche, Cornwall, United Kingdom) on the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The PCR procedure was as follows: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s with 40 cycles. *actinA3* was used as an internal control (housekeeping gene) for lncRNAs and protein-coding genes. Reverse transcription and qRT-PCR of miRNAs were performed using the All-in-One miRNA qRT-PCR Detection Kit (GeneCopoeia, Rockville, MD). U6 was used as an internal control. The primers used are listed in Table 1. The $2^{-\Delta Ct\Delta Ct}$ method (Livak and Schmittgen 2001) was used to calculate the relative expression of mRNAs from the Cts obtained in the PCR quantification (Ct is the cycle threshold, which indicates the number of cycles experienced when the fluorescent signal in each reaction tube reaches a set threshold). ΔCt represents the average Ct value of the sample minus the internal control. ΔCtΔCt represents the average Ct value of sample minus control sample. Three independent experiments were conducted and each sample had three repeats.

Knocking Down of lncRNA in Silkworm

To knocking down the expression of lncR17454, dsRNAs (designed with nucleotides at positions 500 to 822 of lncR17454 in the first exon) were synthesized following the instruction of MEGAscript RNAi Kit (Thermo Fisher) through T7 promoter (the primers are listed in Table 1). Four-microgram dsRNA of lncR17454 was injected into the hemolymph of the 1-d-old third-instar larvae to knockdown it; EGFP was used as an internal control. Silkworms were reared separately on fresh mulberry leaves after injection. The ones with phenotype of molting arrest were dissected with the content of midgut removing. RNA was extracted with the TRIzol (Thermo Fisher) method. Quantitative real-time PCR was performed to determine the

Table 1. Primer set used in the experiments

Name	Forward primer (5′–3′)	Reverse primer (5′–3′)	GenBank ID
FTZF1	ATGCGTCGCCGAAAGAGCCT	ATTGCGACCACCGCGCATAC	D10953.1
E74A	GCACAAGAACAAGCCAGACA	GTCGATCTCGACGATGTCCT	DQ471939.1
LDH	AACTGGAAGGAGCTTCACGA	TGCTCGCCCTTGAGATAAGT	EU000385.1
Abrupt	TTTTAACGCTCTGCGATTTGA	CAGGGTGCTGGCAAGGATTTT	XM_004930967
E75A	GGGTTCCACTACGGCGTCCACT	ACACGACCGAATCGCACAGCA	NM_001112609.1
BR-C-Z2	AAGACGTGGCGTACACAGAC	TCAGGAATGAGGACAAGCTG	NM_001111334.1
EcR	GCTGGTCTGATAACGGTGGCT	CAAGGATTCCGGCGACATAAC	NM_001043866
Abrupt-3UTR	CCGCTCGAGtttttgcttattggtaacaa	ATAAGAATGCGGCCGttaatgaaacaagtatattt	XM_004930967
actinA3	ATTTACTAAGGTGTGCTCG	CCAGTTAGTGACGATTCC	NM_001126254
U6	CTAAAATTGGAACGATACAG		AY649381.1
miR-2795	CAAGTTTGGTGATACGCGGGCGC		NR_107378.1
let-7	TGAGGTAGTAGGTTGTATAGT		NR_107232.1
miR-100	AACCCGTAGATCCGAACTTGTG		NR_107306.1
let-7 mutant	actttatcatttaatTATAGT		
lncR17454	GCCATTTGGACTACGCGAGT	TGGGGCGTTTTAACGGTG	GITK01005596.1
lncR17454-dsRNA-T7	GAAATTAATACGACTCACTATAGGCGCGAAAGTGTTTGCT	GAAATTAATACGACTCACTATAGGCTGCTAGACATGGCCT	GITK01005596.1
EGFP-dsRNA-T7	GAAATTAATACGACTCACTATAGGACGTAAACGGCCACAAGTTC	GAAATTAATACGACTCACTATAGGTGTTCTGCTGGTAGTGGTCG	MH070103.1
lncR17454-PROX1	GGGGTACCTAATCTCAGTAAAATAGTGG	CCAAGCTTCTAGACAAAGTGGAGTTT	
lncR17454-PROX2	GGGGTACCGGGAGAAAGAGCAGGAGAAT	CAAGCTTATGGTCGCCACAACGACT	

expression of lncRNAs, miRNAs, and protein-coding genes. *actinA3* was used as internal controls for protein-coding genes and lncRNAs; U6 was used as the internal control for miRNAs. Three independent experiments were conducted, and, each sample had 10 individuals.

BmN4 Cell Culture and 20-Hydroxyecdysone Treatment

BmN4 cells were cultured in 10% fetal bovine serum (FBS)-containing (Thermo Fisher) Grace medium (Thermo Fisher) at 28°C to reach 70–80% confluency in 6-well plate. 20-Hydroxyecdysone (20E) was added into the medium with a final concentration of 5 μM. Cells were collected after 48-h treatment; RNAs were extracted with TRIzol (Thermo Fisher) method.

Dual-Luciferase Reporter Assay to Detect the Interaction of miRNAs and Their Target Genes

The targets of miR-2834 were predicted by Targetscan (Lewis et al. 2003) and RNAHybrid (Kruger and Rehmsmeier 2006). Besides *FTZ-F1*, *E74*, Broad Complex isoform 2 (*BR-C-Z2*) *BWK-1-like* (leukemiarelated gene), *BmLDH* (lactate dehydrogenase) (Ling et al. 2014) and *pyruvate carboxylase* (Wang et al. 2020), the predicted target of let-7 in other studies, the seed sequence of let-7 also matched the 3′ UTR of BTB-zinc finger regulatory gene *Abrupt* (*Ab*) well. So the wild-type 3′ UTR of *Ab* with a length of 221 bp was cloned from BmN4 cell lines and inserted into the pmirGlo vector (Promega, Madison, WI) with XhoI and NotI (Thermo Fisher) between the firefly luciferase ORF and SV40 poly(A). The mammalian HEK293T cells were maintained in DMEM medium (Sartorius, Kibbutz Beit-Haemek, Israel) containing 10% FBS (Thermo Fisher) at a temperature of 37°C with CO_2 concentration of 5% to reach 70–80% confluency. Cells were transfected with 100 nm miRNA mimics and 100 ng recombinant pmirGlo[*Abrupt*] per well through X-tremeGENE siRNA Transfection Reagent (Roche). The experiment was designed with five groups, which were normal cells, cells transfected with pmirGlo[*Abrupt*] vector, cells cotransfected with pmirGlo[*Abrupt*] and NC mimics, cells cotransfected with pmirGlo[*Abrupt*] and *let-7* mimics, cells cotransfected with pmirGlo[*Abrupt*] and *let-7* mutant mimics (sequence of mimics was list in Table 1). After 48-h transfection, the cells were lysed with cell lysate, 20 μl cell lysate of each sample was put into 96-well plate which contains the substrate of luciferase, the fluorescence value of samples was detected by dual-luciferase reporter assay kit (Promega). The ratio of Firefly luciferase activity (F) to Renilla luciferase activity (R) was calculated. Data analysis was performed using one-way analysis of variance (ANOVA) and Turkey's test. Experiments were conducted with three biological repetitions and three replications for each sample.

Activity Detection of Promoters With Luciferase Assay

Genomic DNA was extracted from the BmN4 cell lines with DNAzol method (Molecular Research Center, Cincinnati, OH). A proximal transcriptional start site (TSS) 697-bp upstream of mir-100 precursor (named PROX1), and a proximal TSS 663-bp upstream of let-7 precursor (named PROX2) were cloned from genomic DNA and inserted into the pGL3-Enhancer vector (Promega) with KpnI and HindIII (Thermo Fisher) upstream of the firefly luciferase ORF and SV40 poly(A), respectively. HEK293T cells were cultured in 10% FBS (Thermo Fisher)-containing DMEM medium (Sartorius, Kibbutz Beit-Haemek, Israel) at 37°C to reach 70–80% confluency in 24-well plates. Cells were transfected with 2 μg recombinant vector pGL3-Enhancer-PROX1 or pGL3-Enhancer-PROX2 with FuGENE HD Transfection Reagent (Promega), pGL3-Enhancer vector was used as an internal control. After 48-h transfection, the cells were lysed with cell lysate, 100 μl cell lysate of each sample was put into 96-well plate which contains the substrate of luciferase. The fluorescence value of the sample was detected by Steady-Glo Luciferase Assay System (Promega). Three independent experiments were conducted and each sample had three repeats. Data analysis was performed using one-way ANOVA and Turkey's test.

Data Analysis

All of the experiments were conducted in three independent triplicates, and each sample had three repeats. The data were processed by the software SPSS Statistics 22.0, exhibited with mean and standard error in the graphs. Significance analysis was conducted with one-way ANOVA and Student's *t*-test.

Results

Expression of lncR17454 and let-7 miRNA Cluster

Recent study showed that the let-7 miRNA cluster, containing let-7, miR-2795, and miR-100, was transcribed from the last exon of lncRNA lncR17454 in silkworm *B. mori* (Fig. 1A), which indicated that lncR17454 might act as precursor of let-7 miRNA cluster. miR-2795 was adjacent to miR-100 with 156-bp distance, but far away from let-7 with distance of 2,151 bp (Fig. 1A).

To elucidate whether lncR17454 acts as the precursor of let-7 miRNA cluster, expression of lncR17454, let-7, miR-100, and miR-2795 were detected during the different tissues and developmental stages of silkworm by qRT-PCR. Besides miR-2795 (Fig. 2G), the other two miRNAs had consistent expression patterns with lncR17454 during larval development, the expression level of them was lower at the first- and second-instar larvae, then increased from the third-instar larva and peaked at the fifth-instar larvae (Fig. 2A, C, and E).

During pupal development, lncR17454 had the lowest expression level at the second day of prepupation, and the highest expression level at the fifth day of pupa in both the males and females (Fig. 2B), which was consistent to that of miR-100 and miR-2795 in the females (Fig. 2F and H). During male pupation, miR-100 and miR-2795 both had the lowest expression level at the second day of prepupation, but peaked at the virgin moth and the first day of prepupation, respectively (Fig. 2F and H). The rock-bottom of let-7 appeared at the first day of pupa, and the peak was at the first day of prepupation in the females, but in the males, which appeared at the second and the first day of prepupation, respectively (Fig. 2D).

Comparing to the relatively consistent expression patterns between lncR17454 and let-7 miRNA cluster at different developmental stages, they showed irrelevant expression trends in different tissues of silkworm. lncR17454 was accumulated more in the brain and cuticle, but let-7 had highest expression level in the fat body and midgut (Fig. 1B). The expression level of miR-100 was highest in the fat body and silk gland, while miR-2795 was highly concentrated in the hemolymph (Fig. 1B). This indicated that although in the same miRNA cluster, the three miRNAs might transcribe from different promoters and play different roles in various tissues of silkworm. To investigate whether there are different promoters generating the three miRNAs, a proximal TSS 697-bp upstream of mir-100 (named PROX1), and another proximal TSS 663-bp upstream of let-7 precursor (named PROX2) were inserted into the pGL3-Enhancer

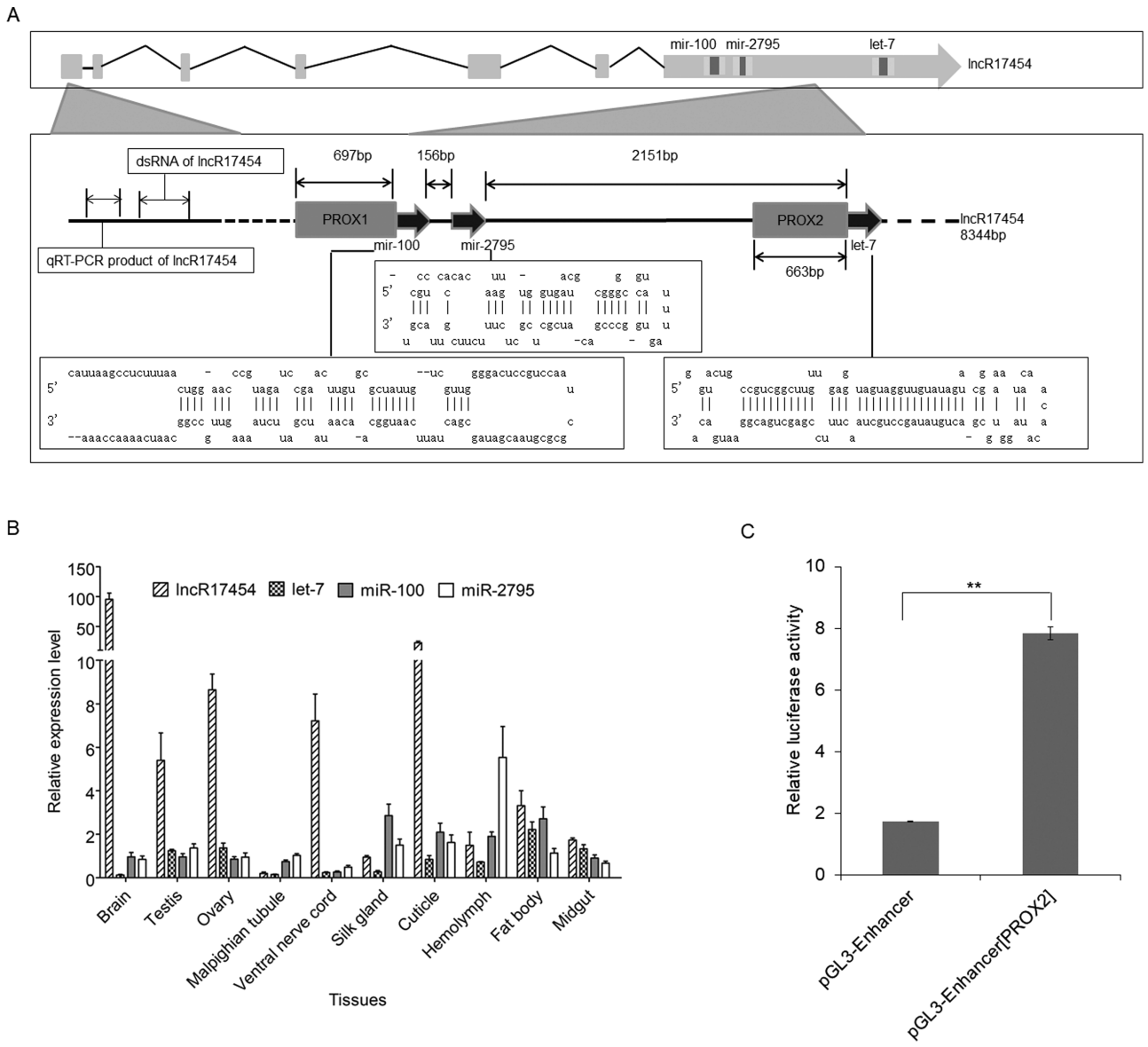


Fig. 1. (A) Genome location of lncR17454 and let-7 miRNA cluster. Yellow blocks represented the exons of lncR17454, green blocks represented the proximal regions that cloned from genomic DNA for promoter activity testing, purple bases represented the mature sequences of miRNAs. (B) Relative expression of lncR17454 and let-7 miRNA cluster in different tissues of the 3-d-old fifth-instar larva. (C) Activity detection of promoters with luciferase assay. pGL3 represents HEK293 cells that were transfected with the vector of pGL3-Enhancer, pGL3-Enhancer [PROX2] represents the HEK293 cells that were transfected with pGL3-Enhancer [PROX2], PROX2 is the proximal TSS 663 bp upstream of let-7 precursor. Three independent experiments were conducted and each experiment had three repeats. Significance analysis was conducted with ANOVA and Student's *t*-test. The data were from three independent experiments (means ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$).

vector. Luciferase assay was used to detect the activity of promoters. The luciferase activity increased 4.5 times after pGL3-Enhancer-PROX2 transfection compared with the negative control, suggesting that there might be independent transcriptional factors upstream of let-7. But no increased luciferase activity was detected after pGL3-Enhancer-PROX1 transfection (Fig. 1C).

Knocking Down lncR17454 Arrest the Molting of Silkworm

To gain insight into the function of lncR17454, dsRNAs of which were injected into the hemolymph of 1-d-old third-instar larvae. The molting process of silkworm was arrested after lncR17454 knockdown, one silkworm died with skin being not got rid of the posterior proleg after 24 h dsRNA injection (Fig. 3A), the undigested food was detained in the midgut. Thirteen percent of the silkworms died during the molt from the fourth to the fifth instar, with unshed skins, soft bodies, shapeless excrement, and undigested food detained in the midguts (Fig. 3A and B). During pupation, the silkworms produced apparently normal cocoons, but could not pupate successfully, with a percentage of 10%. Interesting, we found that one silkworm could molt from the third instar to the fourth instar after lncR17454 retard, but its body length increased less than 1 cm in the following 7 d before it died (Fig. 3C). In totally, 48% of silkworms could not molt successfully with lncR17454 knockdown (Fig. 3D), which indicated that repression of lncR17454 retarded the silkworm molting process, which was similar to that knocking down of let-7 as Ling *et al*. described (Ling et al. 2014).

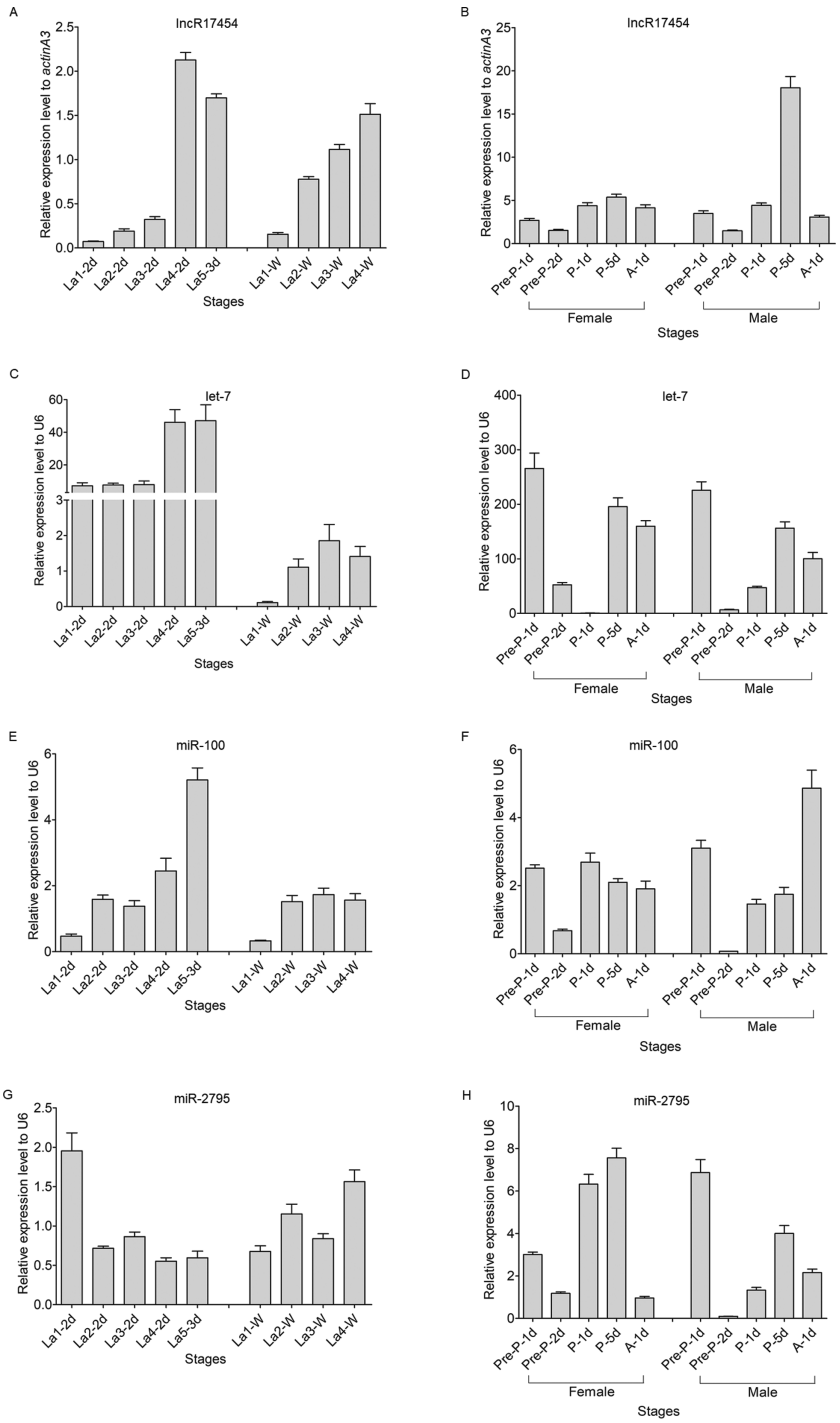
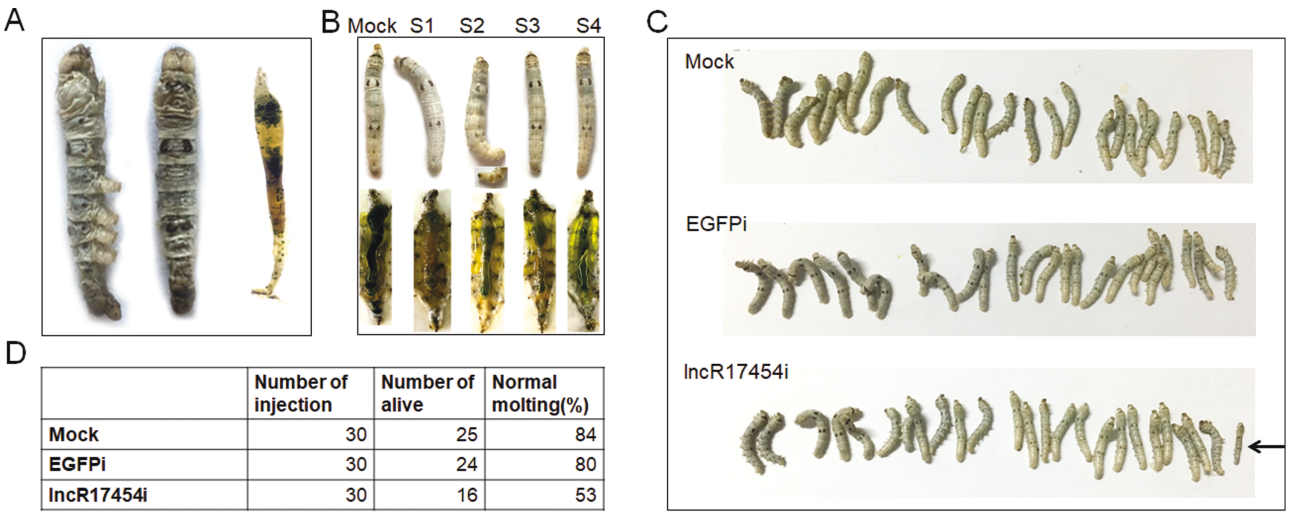


Fig. 2. Relative expression level of lncR17454 (A and B), let-7 (C and D), miR-100 (E and F), and miR-2795 (G and H) at different developmental stages of silkworm. La1-2d, La2-2d, La3-2d, and La4-2d represent the 2-d-old first-, second-, third-, and fourth-instar larvae, respectively. La5-3d represented the 3-d-old fifth-instar larvae. Pre-P-1d and Pre-P-2d represented the first and second day of pre-pupa, respectively. P-1d, P-5d represented the first day and the fifth day of pupa. A-1d means the virgin moth. *actinA3* and U6 were used as the internal control of lncRNA and miRNAs, respectively. Three independent experiments were conducted and each sample had three repeats. Significance analysis was conducted with ANOVA and Student's *t*-test.

To explore whether lncR17454 play roles in the molting process of silkworm through being as the precursor of let-7 miRNA cluster, the expression of lncR17454, let-7, miR-2795, and miR-100, as well as target genes of let-7 were studied by qRT-PCR. Results showed that the expression level of lncR17454 decreased 80% after dsRNA injection, while the expression of mature let-7, miR-100, and miR-2795 decreased 40%, 36%, and 40%, respectively (Fig. 4A), suggesting that knockdown of lncR17454 inhibited the expression of let-7 cluster in silkworm. The expression of predicted target genes of let-7 in silkworm and *Drosophila*, such as *E74A*, *BR-C-Z2*, *E75A*, *FTZF1*, *EcR* as well as *Abrupt*, was also studied. Results showed that the mRNA level of *Abrupt* and *BR-C-Z2* increased 2.7 times and 25 times after lncR17454 knockdown, but the expression of *E74A*, *E75A*, *FTZF1*, and *EcR* was all decreased, especially *E74A*



D

	Number of injection	Number of alive	Normal molting(%)
Mock	30	25	84
EGFPi	30	24	80
lncR17454i	30	16	53

Fig. 3. (A) Abnormal phenotype of silkworm during the molting from the fourth-instar larvae to the fifth-instar larvae (undigested food was detained in the midgut) after injection of lncR17454 dsRNAs. (B) Anatomical structure of silkworm after injection of lncR17454 dsRNAs showing changes from abnormal to death. S1–S4 represented the abnormal phenotype of midgut during dying process. (C) Phenotype of silkworm after injection of lncR17454 dsRNAs during molting stage. Arrow means the retarded silkworm with body length being increased less than 1 cm within 7 d. (D) Statistic numbers of silkworms that were injected with dsRNA of lncR17454 or EGFP. Mock means the normal silkworms. Three independent experiments were conducted and each sample had 10 individuals.

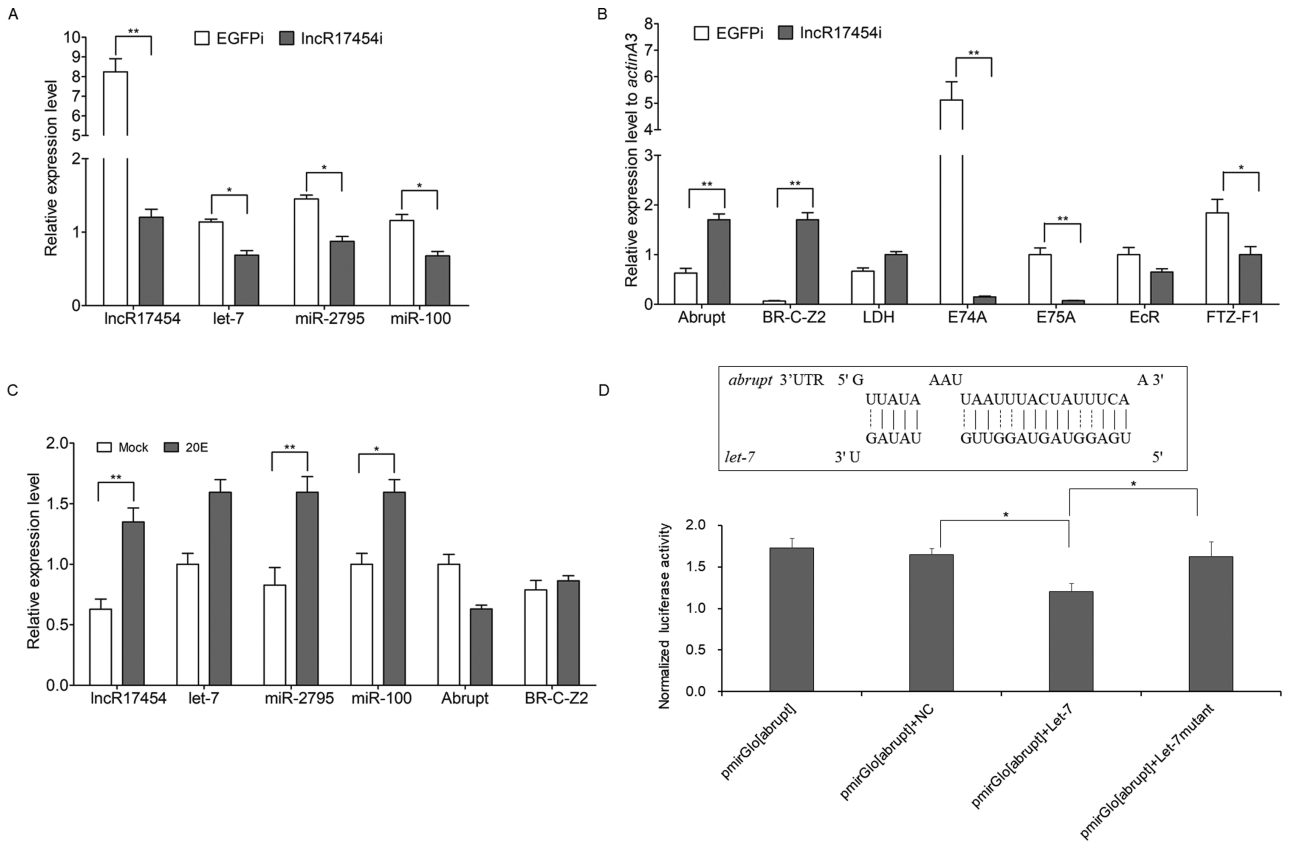


Fig. 4. (A) Expression of lncR17454 and let-7 miRNA cluster in silkworm after injection of lncR17454 dsRNAs. (B) Expression of target genes of let-7 after lncR17454 knockdown. (C) Relative expression of lncR17454, let-7 miRNA cluster, and *Abrupt* gene after 20E addition in BmN4 cells. *actinA3* and *U6* were used as the internal controls for lncRNAs and miRNAs, respectively. (D) Predicted interaction of *let-7* and the 3′UTR of *Abrupt* as well as the verification of miRNA target genes by luciferase activity analysis. Three independent experiments were conducted and each sample had three repeats. Significance analysis was conducted with ANOVA and Student's *t*-test. The data were from three independent experiments (means ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$).

and *E75A* (Fig. 4B), which was little different to what Ling et al. found (Ling et al. 2014).

20E Induce the Expression of lncR17454

The molting process of silkworm is stimulated by ecdysone. To determine whether lncR17454 can be induced by ecdysone, 20E was added to BmN4 cell lines of silkworm. The expression of lncR17454 was induced by 53%. While the expression level of mature let-7, miR-2795, and miR-100 all increased, with a percentage of 37%, 48%, and 44%, respectively (Fig. 4C). When we detected the expression of *Abrupt* and *BR-C-Z2*, the expression of *Abrupt* was decreased significantly, with a percentage of 37%, but the expression change of *BR-C-Z2* was not significant (Fig. 4C), indicated that *Abrupt* might be the real target of let-7.

Abrupt Was the Real Target of let-7

To study whether *Abrupt* was the real target of let-7 in silkworm, dual-luciferase reporter assay was used to elucidate the interaction of let-7 and *Abrupt* in vitro. The 221 bp of 3′ UTR of *Abrupt* was inserted in the pmiGlO vector downstream of the firefly luciferase gene. Mimics or mutant mimics of let-7 were transfected with pmiGlO[*Abrupt*], respectively. Result showed that the luciferase activity decreased significantly when cotransfected with pmiGlO[*Abrupt*] and let-7 miRNA mimics compared with the negative control ($P < 0.05$, Fig. 4D), indicated that *Abrupt* was the real target of let-7.

Discussion

miRNAs play pivotal roles in metamorphosis of silkworm (Liu et al. 2007, 2018; Jiang et al. 2013; Ling et al. 2014), knockdown of let-7 results in molting arrest during larval-to-larval and larval-to-pupal transition of silkworm (Ling et al. 2014). let-7 is a conserved and essential component of the heterochronic pathway involving temporal developmental changes (Bussing et al. 2008, Hertel et al. 2012, Pasquinelli et al. 2000). In silkworm, expression of let-7 is coincided with the pulse of ecdysone, increased after the third larval molt, particularly at the end of each larval instar, and peaked after pupation (Liu et al. 2007). let-7 always origin from the same miRNA cluster which includes let-7, miR-100, and miR-125 (an ortholog of *C. elegans* lin-4) on the same primary transcript in most insect species. In *Drosophila*, let-7, miR-125, and miR-100 are expressed as one primary, poly-adenylated RNA *let-7 Complex* (*let-7C*), with length of 2,449 nt and three exons (Caygill and Johnston 2008, Sokol et al. 2008, Chawla and Sokol 2012). Flies with *let-7-C* knockout appeared molting arrest at the very end of metamorphosis, the one which can enclosed to adults displayed chronic defects in function, including severely reduced motility, flight, and fertility, as well as clear juvenile features in their neuromusculature (Caygill and Johnston 2008, Sokol et al. 2008). The component of let-7 miRNA cluster in *B. mori* was an exception, from which the precursor of miR-125 is instead of miR-2795 (Ling et al. 2014). let-7 miRNA cluster was transcribed from the last exon of lncRNA lncR17454 (Wang et al. 2020), indicating that lncR17454 might also function as *let-7C* that in *Drosophila*. lncRNA lncR17454 had more expression in brain, nerve tissues, and epidermis of silkworm, knocking down of lncR17454 lead to the molting arrest during larval–larval and larval–pupal transition, showing the similar result that let-7 was inhibited in silkworm (Ling et al. 2014). Further analysis showed that expression of let-7, miR-100, and miR-2795 wasall decreased after lncR17454 knockdown. The expression of *Abrupt*, target gene of let-7, was increased significantly after lncR17454 repression. The reverse effect was detected when the expression of lncR17454 and let-7 was induced by 20E in BmN4 cell lines. With luciferase reporter assay, we found that *Abrupt* was another real target of let-7. The BTB-zinc finger (BTB-ZF) domain protein Abrupt (Ab) is a transcription factor, which plays roles in animal survival, epithelial cell fate, neuron differentiation, and neuronal identity switch; *Ab* can be posttranscriptionally repressed by the steroid-induced miRNA let-7 in *Drosophila* (Kucherenko et al. 2012, Bonasio and Shiekhattar 2014, Yoon et al. 2014). Abrupt was the negative regulator of steroid hormone signaling during the oogenesis of *Drosophila*, ecdysone signaling controls the start of border cell migration, initiation of which requires removal of Abrupt in border cells, the reduction of Abrupt is triggered by Jak/Stat signaling and reinforced by ecdysone signaling, increasing levels of 20E result in a pulse of high ecdysone receptor expression and reduced Abrupt levels (Godt and Tepass 2009). In our result, we found that lncR17454 was the precursor of let-7 miRNA cluster, knockdown of lncR17454 reduced the expression of let-7 cluster, but induced accumulation of let-7 target gene *Abrupt*, the negative regulator of ecdysone signaling pathway, which leads to the molting arrest of silkworm. Here lncR17454 was knocked down with dsRNAs designed in the first exon, the expression of the last-exon-origin let-7 miRNA cluster, including let-7, miR-100, and miR-2795, all decreased, which might be the reason that differential expression pattern of *E74A* and *FTZ-F1* exhibited after lncR17454 knockdown, or only let-7 knockdown as Ling et al. found (Ling et al. 2014). Meanwhile, the usage of different tissues and stages of silkworm might also lead the different results. As well, the offtarget effect of miRNAs also needs to be considered.

In this study, we found lncRNA lncR17454 can play regulator roles in the metamorphosis of silkworm through let-7 miRNA cluster and the ecdysone signaling pathway. The ecdysone-lncR17454-*let-7*-*Ab* axis might response earlier in the ecdysone signaling pathway, ecdysone induce the expression of lncR17454, precursor of let-7 miRNA cluster, which result in the accumulation of let-7, miR-2795, and miR-100, then followed the reduction of *Abrupt*, the barrier of ecdysone signaling, and changed of expression of downstream genes. These results added new factor of lncRNA to the ecdysone signaling pathway, and provided new clues for further study of lnRNAs and lepidopteran pest control.

Author Contributions

YF: Visualization, Investigation, Data curation, Writing-Original draft preparation. YW: Visualization, Investigation, Data curation. QH: Software, Validation. CZ, XL: Validation. YK: Conceptualization, Supervision, Reviewing and Editing. DL: Conceptualization, Methodology, Software, Writing, Supervision, Funding acquisition.

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