Long Noncoding RNA IncR17454 Regulates Metamorphosis of Silkworm Through let-7 miRNA Cluster

Yu Fu,1 Yi Wang,1 Qunxia Huang,1 Chenyue Zhao,1 Xinmei Li,1 Yunchao Kan,1,2,3 and Dandan Li1,3,*

1Henan Key Laboratory of Insect Biology in Funiu Mountain, Henan International Joint Laboratory of Insect Biology, College of Life Science and Agricultural Engineering, Nanyang Normal University, 1638 Wolong Road, Nanyang, Henan 473061, China, 2School of Life Science and Technology, Henan Institute of Science and Technology, 90 East of Hualan Avenue, Xinxiang, Henan 453003, China, and 3Corresponding authors, e-mail: lidannytc@126.com; yckan1974@nynu.edu.cn

†These authors contributed equally to this work.

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 IncR17454 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 (Huart 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 IncR17454 (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 (dT)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 (GeneCopeia, Rockville, MD). U₆ was used as an internal control. The primers used are listed in Table 1. The 2⁻ΔΔCt method (Livak and Schmittgen 2001) was used to calculate the relative expression of miRNAs 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. ΔΔCt 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  | ATGCGTCGCCGAAAAGACCT        | ATTCGCCACCACCGCCGACATC      | D10953.1         |
| E74A   | GCACAAGAACAGCCAGAC          | GTCGATCCTCGAGCTGCTCTC       | DQ471939.1       |
| LDH    | AACTGAAGGAGGCTTCAGA         | TGCTCGCCCTTGGAGAATGT        | EU00385.1        |
| Abrupt | TTTTACGCTCTGGATTTGGTT       | CGAGGTGGTGGCAAGGATTCTT      | XM_004930967     |
| Abrupt-3UTR | ACCGTTGAGGCTCGCAAGGTCT | ACAGCGCCCGAATCCGACAGCA      | NM_001112609.1   |
| E75A   | GGGTTCATAACGGCGCTTCACT     | TCGAAGTGGAGCAGCTGCA         | NM_001111334.1   |
| BR-C-Z2| AAGACGTCGCGGTCAAGACAG      | TCGAAGTGGAGCAGCTGCA         | NM_001111334.1   |
| EcR    | GTCGTCTGGATAAGGGTGCT        | CAAGGATTCCCGCGACATAAC       | NM_001043866     |
| Abrupt-3UTR | CCGCTCGAGGTTGGATGAAAGC      | ATAGGAATGCAGGTGTTGGATTCTTG | XM_004930967     |
| actinA₃| ATTTACTAAGGTGTGGCT          | CCAATTAGGCGAGATGTTGC        | NM_001126524.1   |
| U₆     | CTAATAAGGTGGAGAAGCCATACAG  | ATAGGAATGCAGGTGTTGGATTCTTG | AY649381.1       |
| miR-2795| CAGTTTTGGTGGATACCCGGCGGCC  | CCAATTAGGCGAGATGTTGC        | NR_107378.1      |
| let-7  | TGAGGATGCTAGGTTGTAATGT      | CCAATTAGGCGAGATGTTGC        | NR_107232.1      |
| let-7 mutant | acttattatattatattatattat | CCAATTAGGCGAGATGTTGC        | NR_107306.1      |
| lncR17454-PROX1 | GCCATTTGGGACTACCCCGAGT | TGGGGGCTTTTAAACCGTG | GIK01005596.1    |
| lncR17454-PROX2 | GCCGGAGAGAAAAATCGGCTCAGC | GAAATTCACAGCTTACATATAGGCTG | GIK01005596.1    |

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...
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 CO2 concentration of 5% to reach 70–80% confluency. Cells were transfected with 100 nm mRNA 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 lystate, 20 μl cell lystate 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 lystate, 100 μl cell lystate 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 IncR17454 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 IncR17454 in silkworm B. mori (Fig. 1A), which indicated that IncR17454 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 IncR17454 acts as the precursor of let-7 miRNA cluster, expression of IncR17454, 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 IncR17454 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).

**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 lystate, 100 μl cell lystate 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 IncR17454 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 IncR17454 in silkworm B. mori (Fig. 1A), which indicated that IncR17454 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 IncR17454 acts as the precursor of let-7 miRNA cluster, expression of IncR17454, 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 IncR17454 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, IncR17454 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 IncR17454 and let-7 miRNA cluster at different developmental stages, they showed irrelevant expression trends in different tissues of silkworm. IncR17454 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
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%. Interestingly, 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).
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.
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 IncR17454 dsRNAs. (B) Anatomical structure of silkworm after injection of IncR17454 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 IncR17454 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 IncR17454 or EGFP. Mock means the normal silkworms. Three independent experiments were conducted and each sample had 10 individuals.

Fig. 4. (A) Expression of IncR17454 and let-7 miRNA cluster in silkworm after injection of IncR17454 dsRNAs. (B) Expression of target genes of let-7 after IncR17454 knockdown. (C) Relative expression of IncR17454, 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 IncR17454
The molting process of silkworm is stimulated by ecdysone. To determine whether IncR17454 can be induced by ecdysone, 20E was added to BmN4 cell lines of silkworm. The expression of IncR17454 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 IncRNA IncR17454 (Wang et al. 2020), indicating that IncR17454 might also function as let-7C that in Drosophila. IncRNA IncR17454 had more expression in brain, nerve tissues, and epidermis of silkworm, knocking down of IncR17454 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 was all decreased after IncR17454 knockdown. The expression of Abrupt, target gene of let-7, was increased significantly after IncR17454 repression. The reverse effect was detected when the expression of IncR17454 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 IncR17454 was the precursor of let-7 miRNA cluster, knockdown of IncR17454 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 IncR17454 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 IncR17454 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 IncRNA IncR17454 can play regulator roles in the metamorphosis of silkworm through let-7 miRNA cluster and the ecdysone signaling pathway. The ecdysone-IncR17454-let-7-Ab axis might response earlier in the ecdysone signaling pathway, ecdysone induce the expression of IncR17454, 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 IncRNA to the ecdysone signaling pathway, and provided new clues for further study of IncRNAs 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.

Acknowledgments
We thank Dr. Muwang Li for providing the B. mori Dazao P50 strain. This research was supported by the Natural Science Foundation of Henan province (212300410063), National Natural Science Foundation of China (31970480), Young Elite Scientist Sponsorship Program by China Association for Science and Technology (YESS 20150026).

References Cited
Bonasio, R., and R. Shiekhattar. 2014. Regulation of transcription by long noncoding RNAs. Annu. Rev. Genet. 48: 433–455.
Bussing, I., F. J. Slack, and H. Grosshans. 2008. let-7 microRNAs in development, stem cells and cancer. Trends Mol. Med. 14: 400–409.
Cai, X., and B. R. Cullen. 2007. The imprinted H19 noncoding RNA is a primary microRNA precursor. RNA. 13: 313–316.
