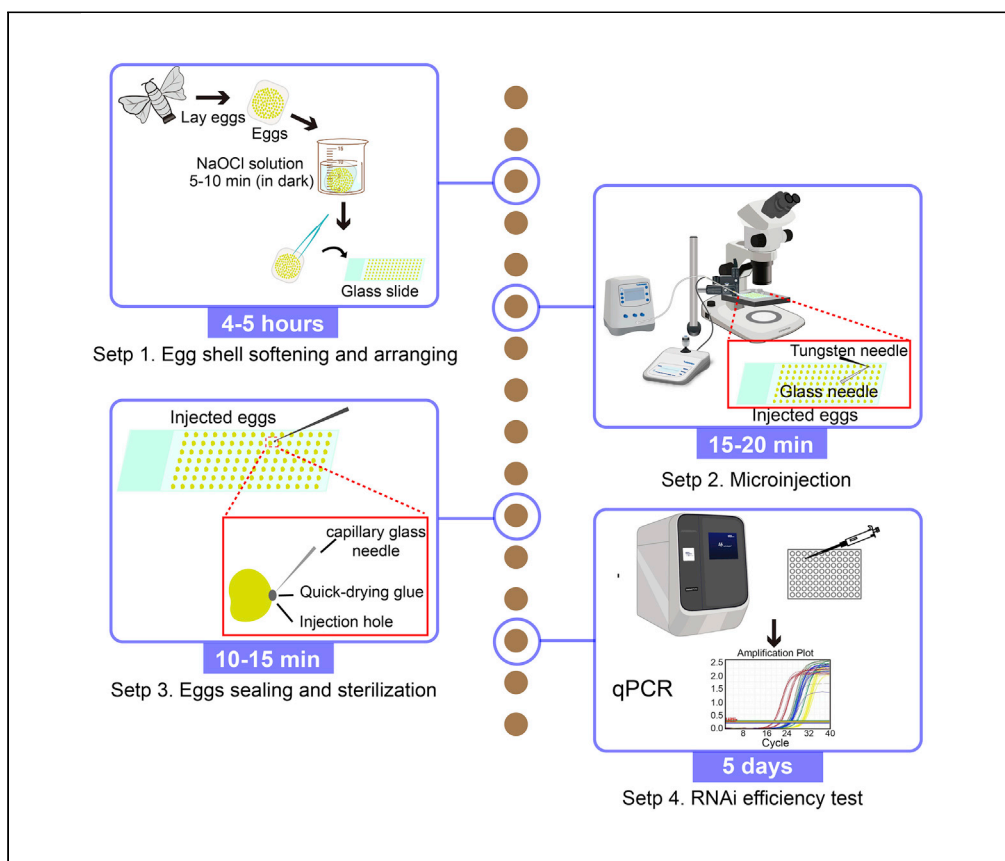


Protocol

Knock down of target genes by RNA interference in the embryos of lepidopteran insect, *Bombyx mori*



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Highlights

Establishment of a microinjection platform for lepidopteran embryos

Silkworm embryos at different stages are sensitive to RNAi

Mixed injection of dsRNA of different genes can knock down target genes

RNA interference (RNAi) is a technique used for posttranscriptional gene silencing, but lepidopteran insects are not sensitive to RNAi. Here, we present a protocol for knocking down the expression level of target genes by RNAi in *Bombyx mori* embryos. We describe the preparation of double-stranded RNAs (dsRNAs) of target genes, followed by microinjection of embryos at different developmental stages, with single or mixed dsRNA. Finally, we use RT-qPCR to verify RNAi efficiency.

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Protocol

Knock down of target genes by RNA interference in the embryos of lepidopteran insect, *Bombyx mori*Guanfeng Xu,^{1,2,3,*} Yulin Tian,^{1,2} Yuling Peng,^{1,2} and Sichun Zheng^{1,2,4,*}

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SUMMARY

RNA interference (RNAi) is a technique used for posttranscriptional gene silencing, but lepidopteran insects are not sensitive to RNAi. Here, we present a protocol for knocking down the expression level of target genes by RNAi in *Bombyx mori* embryos. We describe the preparation of double-stranded RNAs (dsRNAs) of target genes, followed by microinjection of embryos at different developmental stages, with single or mixed dsRNA. Finally, we use RT-qPCR to verify RNAi efficiency.

For complete details on the use and execution of this protocol, please refer to Xu et al. (2021)

BEFORE YOU BEGIN

Preparation of healthy unmated adults of lepidopteran insects

⌚ Timing: 2–2.5 weeks

1. The one-day-old fifth instar *B. mori* larvae (P50 strain) were obtained from the Research and Development Center of the Sericulture Research Institute of the Academy of Agricultural Sciences of Guangdong Province, China.
2. Rear *B. mori* larvae on fresh mulberry leaves at 25°C and 70% humidity under a photoperiod of 12 h light:12 h darkness.
3. Separate the silkworm cocoon in a plastic box (163 mm × 276 mm × 56 mm) with 15 small compartments to prevent the moths from mating until pupae becoming moths.

Preparation of double-stranded RNAs of target genes

⌚ Timing: 1–2 days

4. Extract total RNA at a final concentration of 1000 ng/μL from *B. mori* embryos using Eastep® Super Total RNA Extraction Kit (Promega, Beijing, China) following the manufacturer's protocol (www.promega.com.cn/resources/protocols/technical-manuals/eastep-protocols/rna-extraction-kit-ls1040/).
5. Synthesize cDNAs using HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, Nanjin, China) following the manufacturer's protocol (<https://www.vazyme.com/product/91.html>).



6. Amplify the 300–400 bp unique fragments of the ORFs of genes of interest as the templates for synthesizing gene-specific dsRNAs. To benchmark our protocol, we focus on DNA methyltransferase 1 (*Dnmt1*) and methyl-CpG binding domain protein2/3 (*MBD2/3*) (Figure 1). Amplify using the specific primers referring to [key resources table](#).
 - a. Produce templates of PCR products. A T7 RNA polymerase promoter can be added to target DNA sequence using PCR by including the T7 promoter sequence at 5'-end of the amplification primers (Figure 2).
 - b. Run PCR reaction.
PCR reaction master mix:

Reagent	Amount
DNA template	2 μ L
2 x Taq Master Mix (Dye Plus)	10 μ L
Primer 1	0.5 μ L
Primer 2	0.5 μ L
ddH ₂ O	7 μ L

PCR cycling conditions:

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	10 s	34 cycles
Annealing	55°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	10 min	1
Hold	12°C	Forever	

- c. Examine PCR products using agarose gel electrophoresis before transcription to verify whether a PCR product of the expected size is generated.
- d. Collect and purify the PCR product.
 - i. Add 2.5 volumes of 95% ethanol into the PCR product.
 - ii. Turn upside down 15 times to mix well and place the tube in a refrigerator at -20°C for 30 min.
 - iii. Spin at the 20,000 \times g in a centrifuge (5427R, Eppendorf, Hamburg, Germany) at 4°C for 10 min.

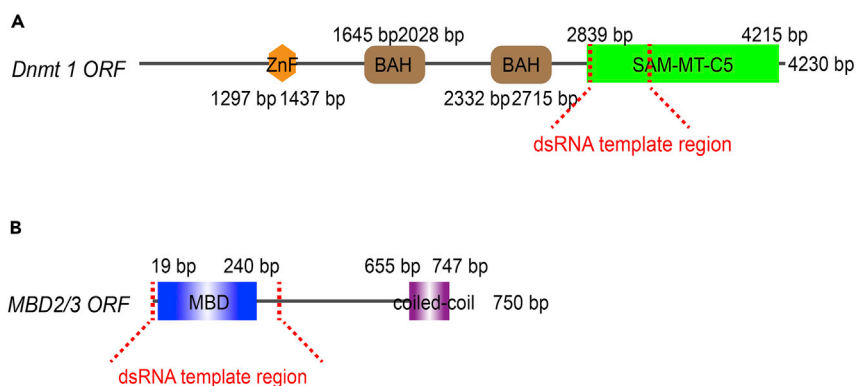


Figure 1. Schematic of ORF regions of *Dnmt1* and *MBD2/3* genes

Schematic of ORF regions of *Dnmt1* (A) and *MBD2/3* (B) genes which are selected as the templates for the synthesis of double-strand RNAs.

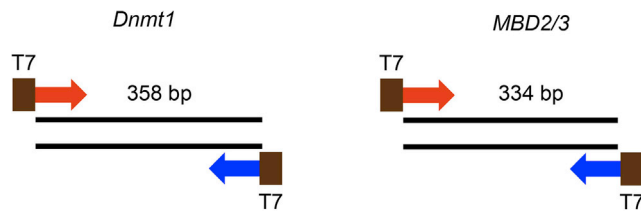


Figure 2. The PCR reactions with a T7 promoter to generate templates for dsRNA synthesis

- iv. Carefully aspirate supernatant using a pipette and wash the pellet with cold 75% ethanol by slowly turning the tube upside down for 15–20 times.
 - v. After removing ethanol, air-dry for 10–15 min to fully evaporate the remaining ethanol until the white DNA precipitate becomes translucent, and resuspend the DNA sample in 30–50 μ L Nuclease-Free Water. Store at -20°C .
7. Synthesize dsRNA according to the instruction of T7 RiboMAXTM Express RNAi System (www.promega.com.cn/products/rna-analysis/rna-interference/t7-ribomax-express-rnai-system/?catNum=P1700#protocols) (Promega, Wisconsin, USA). Use agarose gel electrophoresis to verify whether dsRNA is a single band with exact length.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
<i>Bombyx mori</i> (P50, non-diapause)	Research and Development Center of the Sericulture Research Institute of the Academy of Agricultural Sciences of Guangdong Province	N/A
Oligonucleotides		
Primers used in this protocol (see Table 1 in “materials and equipment”).	TSINGKE Biological technology	N/A
Chemicals, peptides, and recombinant proteins		
Ethanol	Nanjing Reagent	C0691510226
Nuclease-Free Water	Thermo Fisher Scientific	AM9938
Agarose	TSINGKE Biological technology	TSJ001
Tris	Sigma-Aldrich	T1503
EDTA Na ₂ 2H ₂ O	Sigma-Aldrich	E5134
NaOCl solution	Sigma-Aldrich	239305
RNAex Pro Reagent	Accurate Biotechnology	AG21102
Chloroform	Guangzhou Chemical Reagent Factory	N/A
iso-Propyl alcohol	Guangzhou Chemical Reagent Factory	N/A
M-MLV RT and 5x M-MLV RTase Buffer	Takara Bio	2641A
DNase I RNase-free 10x DNase I buffer	Takara Bio	D2215
Formaldehyde solution	Sigma-Aldrich	47083-U
Critical commercial assays		
Eastep® Super Total RNA Extraction Kit	Promega	LS1040
HiScript II 1st Strand cDNA Synthesis Kit	Vazyme	R211-01
Hieff qPCR SYBR Green Master Mix	YESEN	11202ES08
2 x Taq Master Mix (Dye Plus)	Vazyme	P112-01
T7 RiboMAX TM Express RNAi System	Promega	P1700
Software and algorithms		
GraphPad Prism 6	GraphPad Software Inc	RRID: SCR_002798 https://www.graphpad.com/scientific-software/prism/

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Rnase-free pipette tips	Thermo Fisher Scientific	AM12665
Rnase-free pipette tips	Thermo Fisher Scientific	AM12650
Rnase-free pipette tips	Thermo Fisher Scientific	AM12635
Rnase-free PCR tube	Thermo Fisher Scientific	AM12225
Rnase-free EP tube	Thermo Fisher Scientific	AM12400
Glass capillaries (0.02 inches in inner diameter, 0.04 inches in outer diameter, 7 inches in length)	Drummond Scientific	#3-000-203-G/X
Microscope slide	Sail Brand	7105
Microloader	Eppendorf	I157654M
Quick-drying glue	Kuosen chemical technology	N/A
Eppendorf FemtoJet 4i and 4r micro autoinjector	Eppendorf	N/A
QuantStudio 6 Flex fluorescence quantitative PCR instrument	Thermo Fisher Scientific	N/A
Powerpac basic	BIO-RAD	N/A
Horizontal electrophoresis tank	BIO-RAD	N/A
Chemidoc touch imaging system	BIO-RAD	N/A
NANODROP 2000 spectrophotometer	Thermo Fisher Scientific	N/A
Refrigerated centrifuges (5427R)	Eppendorf	N/A
Optical microscope (SZX2)	Olympus	N/A
Needle holder	Olympus	N/A
Tungsten needle	Olympus	N/A
Tweezer	Anex	N/A
pn-31 needle puller	Narishige	N/A

MATERIALS AND EQUIPMENT

50 × TAE Buffer

Reagent	Final concentration	Amount
Tris	2 M	242 g
EDTA Na2 2H2O	100 mM	37.2 g
MilliQ water	N/A	Up to 1 L
Total	N/A	1 L

Store at 25°C for 30 days.

75% Ethanol

Reagent	Amount
Ethanol	750 mL
RNase-free water	250 mL
Total	1 L

Store at 25°C for 30 days.

STEP-BY-STEP METHOD DETAILS

Softening and arrangement of silkworm eggs

⌚ Timing: 4–5 h

In this step, the embryos will be prepared for injection, including softening of the eggshells and arranging the eggs onto a glass slide.

Table 1. Primers used in this protocol

Primer	Sequence
Cloning primer at the 5'- end of <i>BmDnmt1</i>	ATCAAGCTGGAGTTGCAGAATG
Cloning primer at the 3'- end of <i>BmDnmt1</i>	TTAAAGGCCACAAAGTTACGAA
Cloning primer at the 5'- end of <i>BmDnmt1</i> including the T7 promoter sequence	TAATACGACTCACTATAGGATCAAGCTGGAGTTGCAGAATG
Cloning primer at the 3'- end of <i>BmDnmt1</i> including the T7 promoter sequence	TAATACGACTCACTATAGGTTAAAGGCCACAAAGTTACGAA
Cloning primer at the 5'- end of <i>BmMBD2/3</i>	ATGAACATATCTATCGAAAGAAAAC
Cloning primer at the 3'- end of <i>BmMBD2/3</i>	GTTTGAATATTGATGCTGTTTGA
Cloning primer at the 5'- end of <i>BmMBD2/3</i> including the T7 promoter sequence	TAATACGACTCACTATAGGATGAACATATCTATCGAAAGAAAAC
Cloning primer at the 3'- end of <i>BmMBD2/3</i> including the T7 promoter sequence	TAATACGACTCACTATAGGGTTTGAATATTGATGCTGTTTGA
Cloning primer at the 5'- end of <i>GFP</i>	TACGGCGTGCAGTGCTTCAGCC
Cloning primer at the 3'- end of <i>GFP</i>	GTGCTCAGGTAGTGGTTGTCGG
Cloning primer at the 5'- end of <i>GFP</i> including the T7 promoter sequence	TAATACGACTCACTATAGGTACGGCGTGCAGTGCTTCAGCC
Cloning primer at the 3'- end of <i>GFP</i> including the T7 promoter sequence	TAATACGACTCACTATAGGGTGTCTCAGGTAGTGGTTGTCGG
RT-qPCR primer at the 5'- end of <i>RP49</i>	CAGGCGGTTCAAGGGTCAATAC
RT-qPCR primer at the 3'- end of <i>RP49</i>	TACGGAATCCATTTGGGAGCAT

1. Separate female and male moths after mating for four hours. Place female moths on a clean paper and lay eggs in dark. The oviposition time was recorded.

△ **CRITICAL:** If there are large number of silkworms, you can put the mating female silkworms in a refrigerator at 4°C, and take them out when they are needed to lay eggs. They can be stored at 4°C for up to one week.

2. Collect eggs every 30 min, soak the paper with silkworm eggs in 20% NaOCl solution for 5–10 min to soften egg shells.

△ **CRITICAL:** NaOCl solution is easy to decompose when it is exposed to light, therefore, the softening process should be carried out in dark.

3. Take out the eggs soaked in 20% NaOCl solution and rinse them in clean water for several times.
 - a. Carefully absorb the water stain on the surface of the silkworm eggs with absorbent paper, and then use tweezers to gently place the silkworm eggs neatly on a glass slide coated with glue (Kuosen chemical technology, Hangzhou, China).
 - b. Eggs (100–120) are arranged in 8–10 rows and 10–12 columns on each slide (Figure 3).

△ **CRITICAL:** Arrange the "D"-like silkworm eggs in the direction from the raised side to the right to make the needle on the right easy to enter the egg.

Microinjection

⌚ **Timing:** 15–20 min

In this step, embryos are injected at different developmental stages or with different injection methods (dsRNA for single gene or mixed dsRNA for two genes).

4. After the paste on the slide is dried, place the slide on the stage of the optical microscope (SZX2, Olympus, Tokyo, Japan).
5. Pull capillary glass needle using pn-31 needle puller (NARISHIGE, Tokyo, Japan) under the conditions of parameter: Heater (98°C), Magnet sub (25) and Magnet main (65).
6. Draw 4–6 μL of the prepared dsRNA (5 μg/μL) solution (RNase-free water) with a capillary glass needle.

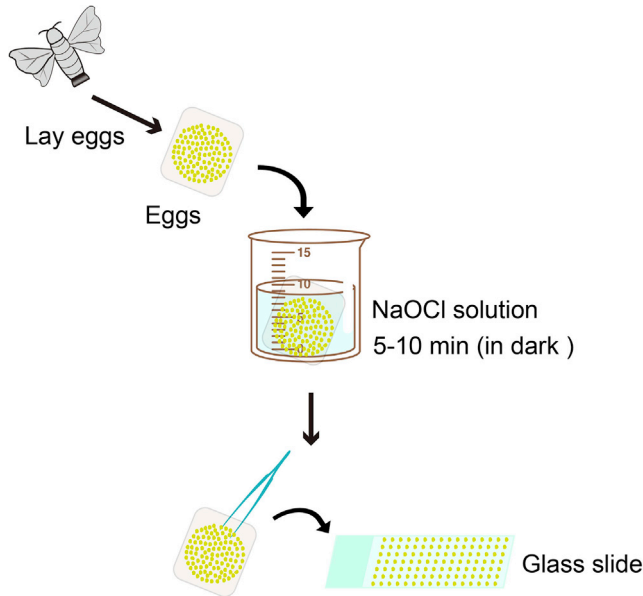


Figure 3. Schematic of silkworm egg softening and arrangement

- Set up two groups of RNAi experiments. In one group, to detect the effect of injected dsRNAs on the RNAi efficiency, load dsRNA of one gene and a dsRNA mixture of two genes (*dsDnmt1* and *dsMBD2/3*) into the capillary glass needle with sealed pointed end, respectively, using a microloader (Eppendorf, Hamburg, Germany) and inject the egg at fertilized egg stage. *dsGFP* is used as a control for injection (Figure 4A).
- In the other group, to detect the RNAi effect in different development stages, separately inject *dsDnmt1* or *dsMBD2/3* into embryos at different developmental stages (Figure 4B).

△ **CRITICAL:** Avoid bubbles in the capillary glass needle when adding dsRNA.

- Assemble the dsRNA-filled capillary glass needle into a needle holder, and install a tungsten needle in another needle holder.

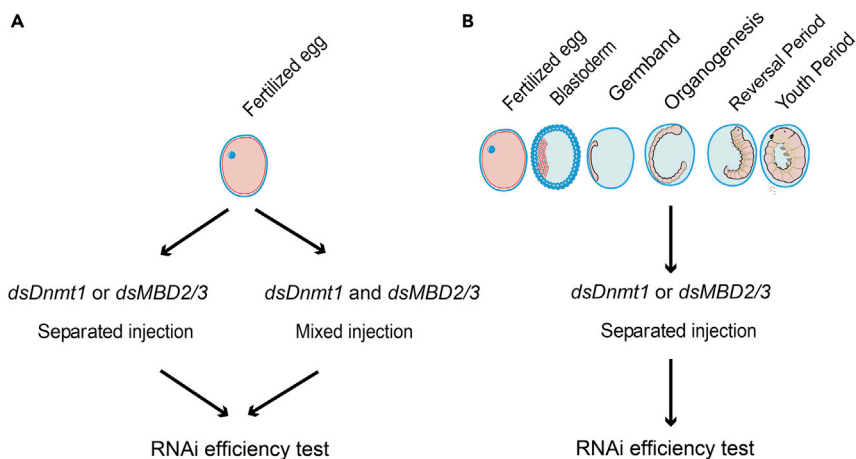


Figure 4. Model diagram of different injection methods of silkworm embryos

- dsRNA of one gene or multiple genes is injected at fertilized stage.
- dsRNA of *Dnmt1* or *MBD2/3* is injected at different stages of embryo development.

- a. After assembly, adjust the angle of the two needles and break the capillary end of the capillary glass needle using a tweezer (Anex, Niigata, Japan) under the microscope (SZX2, Olympus, Tokyo, Japan).
- b. Adjust and record the positions of the two needles to ensure that the capillary glass needle can enter the silkworm eggs and inject them from the holes made by the tungsten needles.
- c. Inject each silkworm egg with 30–50 ng dsRNA under the conditions of injection parameter: injection pressure (120), compensation pressure (50) (Figure 5).

Egg sealing and sterilization

⌚ Timing: 10–15 min

In this step, the injected embryos are sealed and sterilized to prevent death.

8. After microinjection, seal the injection hole with quick-drying glue (Kuosen chemical technology, Hangzhou, China) under the microscope to avoid embryonic death caused by loss of egg contents.

⚠ **CRITICAL:** The quick-drying glue only is needed to seal the injection hole, do not add too much to prevent the larvae from breaking the egg shell and dying.

9. Put the cotton soaked in 10% formaldehyde into a glass bottle, and put the sealed silkworm eggs into this bottle for sterilization with 10% formaldehyde steam for 5 min.
10. After sterilization, put the eggs in a sealed box, and move the insect box into an artificial climate chamber at 25°C under a photoperiod of 12 h light:12 h darkness.

⚠ **CRITICAL:** A little sterilized water can be added to the sealed box to ensure the humidity environment required for embryonic development.

11. On the fifth or sixth day of silkworm embryonic development, the formation of the silkworm embryo in the egg shell can be observed through an optical microscope.

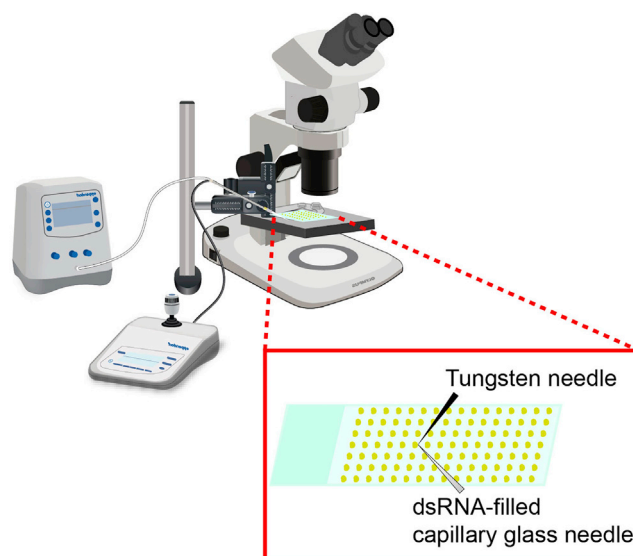


Figure 5. Schematic of silkworm fertilized egg microinjection

RNAi efficiency test

© Timing: 5 days

In this step, the RNAi efficiency of embryos at different developmental stages and different injection methods was detected by RT-qPCR.

12. Group one : inject dsRNAs of one and two genes into a fertilized egg (within three hours of fertilization), then collect samples (15–20 eggs per sample) at different time (24, 48, 72, 96 and 120 h) after injection (Figure 6), and detect RNAi efficiency by RT-qPCR.
13. Group two: inject dsRNAs into the *B. mori* embryos at different developmental stages (fertilized egg, blastoderm, germband, organogenesis, reversal period and youth period), then collect samples at 24 h after injection (Figure 7), detect RNAi efficiency by RT-qPCR.
14. The RNAi efficiency is based on three biological replicates each comprising three technical replicates.
 - a. RNA extraction and reverse transcription
 - i. Put 15–20 silkworm eggs into an EP tube, add 1 mL RNAex Pro Reagent (Accurate Biotechnology, Changsha, China), grind with a homogenizer on ice, and place the ground material on ice for 5 min.
 - ii. Add 200 μ L of chloroform: isoamyl alcohol = 24:1 (V/V), vigorously shake for 15 s, place on ice for 10 min.

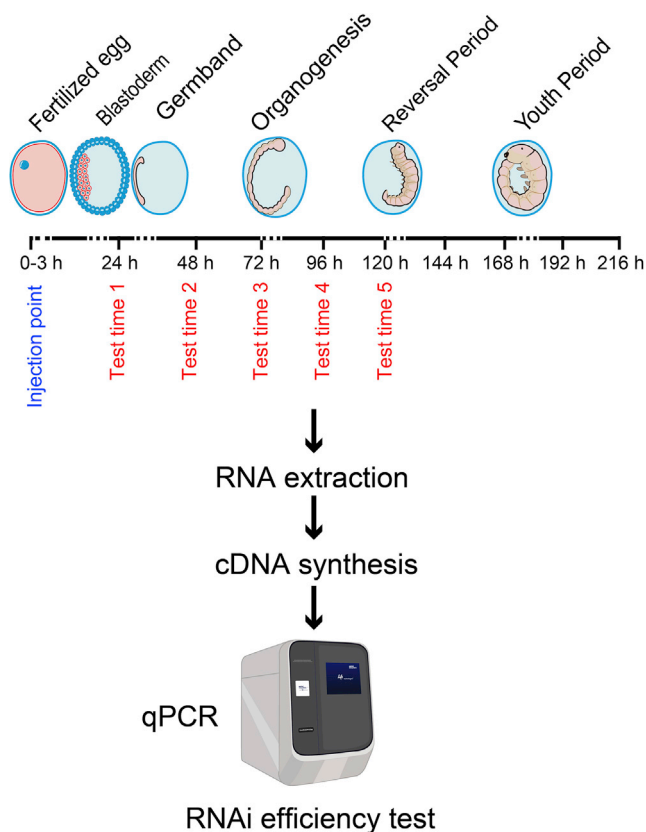


Figure 6. Model diagram of RNAi efficiency test at different times after fertilized eggs injected

dsRNAs were injected into fertilized eggs (within three hours of fertilization) and RNAi efficiency was tested at 24, 48, 72, 96 and 120 h after injection.

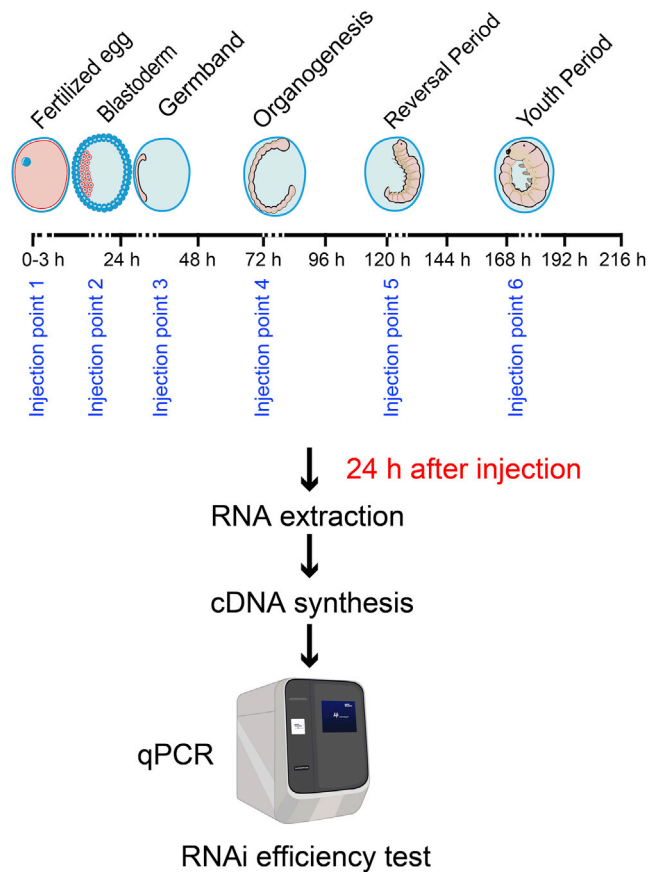


Figure 7. Model diagram of RNAi efficiency test of embryos dsRNA-injected at different developmental stages
dsRNAs were injected into the embryos at fertilized egg, blastoderm, germband, organogenesis, reversal period and youth period stage, respectively. RNAi efficiency was determined by RT-qPCR at 24 h after injection.

- iii. Centrifuge at 12,000 g for 4°C, 15 min, move the upper water phase to a new EP tube, add an equal volume of isopropanol, gently invert to mix, and place at -20°C for 0.5–1 h.
- iv. Collect RNA by centrifuge at 12,000 g for 4°C, 10 min. Discard the supernatant, add 1 mL of 75% ethanol to the pellet, briefly vortex and centrifuge at 12,000 g for 10 min to wash the RNA pellet. Repeat two more times.
- v. After drying, use 20 µL Nuclease-Free Water to dissolve RNA. Add 1 µL DNase I and 2.3 µL 10× DNase I buffer, incubate at 37°C for 30 min to remove DNA. Detect RNA purity and concentration with NANODROP 2000 spectrophotometer.
- vi. Prepare the RNA template solution according to the following table, and denature RNA at 65°C for 5 min.

Reagent	Amount
dNTP (10 mM)	5 µL
Oligo (dT) Primer	50 pmol
Template RNA	2 µg
RNase free water	Up to 10 µL

- vii. Prepare the reverse transcription reaction system according to the following table, and incubate the reaction at 42°C for 30–60 min. Inactivate the enzyme at 70°C for 15 min.

Reagent	Amount
RNA template solution	10 μ L
5 \times RTase M-MLV Buffer	4 μ L
RNase Inhibitor	20 U
M-MLV RTase (200 U/ μ L)	0.5 μ L
RNase free water	Up to 20 μ L

b. RT-qPCR

- i. Perform RT-qPCR using the HieffTM qPCR SYBR Green Master Mix Kit in QuantStudio™ 6 Flex Real-Time PCR System. The primers used for RT-qPCR are listed in following:

Primer	Sequence
<i>Dnmt1</i> primers	F: CTCTGCGAGCTTTGTTGGACATG R: CGCTGCCGCCAATATGATCAAC
<i>MBD2/3</i> primers	F: GAAACAAGCCATAAAAGACAA TCCA R: CTAGGCCACCGATA GAGCTTGT

- ii. Prepare the reaction solution according to the following table, and the PCR conditions are as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 31 s.

Reagent	Amount
Template	1 μ L
SYBR® Premix Ex Taq (2 \times)	10 μ L
Forward PCR Primer (10 μ M)	0.8 μ L
Reverse PCR Primer (10 μ M)	0.8 μ L
RNase free water	Up to 20 μ L

- iii. Normalize the relative mRNA level of gene expression to the expression level of a house-keeping gene encoding ribosomal protein 49 (*rp49*) (GenBank accession no. 778453), and analyze using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

EXPECTED OUTCOMES

The relative change of the mRNA level of *Dnmt1* or *MBD2/3* in the *dsRNA* and *dsGFP* group is measured. In the separated injected group, after 24–120 h of injection, the mRNA level of *Dnmt1* was decreased by 92.5%, 87.2%, 71%, 57% and 54.6%, respectively; and mRNA level of *MBD2/3* was decreased by 93.6%, 86.1%, 76.7%, 65.9% and 52.6%, respectively. In the mixed injected group, after 24–120 h of injection, the mRNA level of *Dnmt1* was decreased by 91.1%, 86.2%, 84.4%, 70.7% and 63.9%, respectively; and mRNA level of *MBD2/3* was decreased by 84.9%, 87.1%, 85.3%, 75% and 63%, respectively. These results confirm either *dsRNA* injection of one gene or *dsRNA* injection of multiple genes can significantly reduce the mRNA levels of target genes within 120 h (Figure 8).

When *dsRNA* was injected at different stages of embryo development, including fertilized egg, blastoderm, germband, organogenesis, reversal period and youth period, 24 h later, the mRNA level of *Dnmt1* was decreased by 91.3%, 84.6%, 84.3%, 67%, 48.9% and 12.1%, respectively; and the mRNA level of *MBD2/3* was decreased by 84.3%, 84%, 83.4%, 75.7%, 55% and 13.3%, respectively. These results showed that, *dsRNA* injection can significantly reduce the mRNA level of the target gene

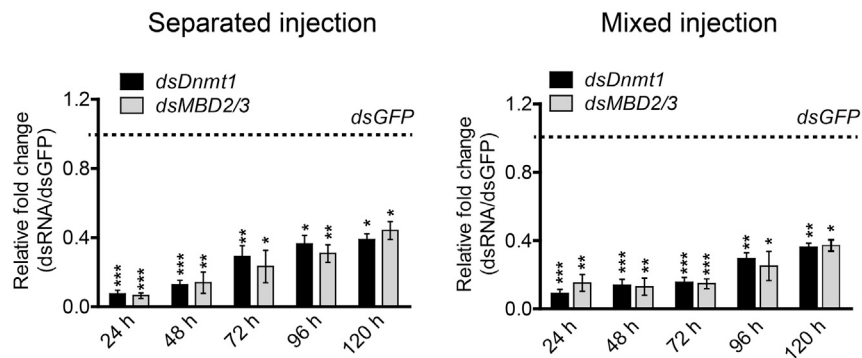


Figure 8. RT-qPCR determination of RNAi efficiency of the two injection methods within 120 h after injection at the fertilized egg stage

Significance of the differences was determined by t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

before reversal period stage. As the embryo matures, the RNAi sensitivity in silkworm is gradually decreased (Figure 9).

QUANTIFICATION AND STATISTICAL ANALYSIS

Perform statistical analyses using GraphPad Prism 6. Present data as the mean \pm SEM. Determine p-values using a two-tailed Student's t test. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

LIMITATIONS

Because it is necessary to perforate silkworm eggs with tungsten during the microinjection process, and then inject dsRNA with a capillary glass needle, it will inevitably cause damage to the silkworm embryos, leading to impaired development and even death of some silkworm embryos. Therefore, in our embryonic microinjection experiment, the embryonic hatching rate of the control group (*dsGFP*) is only about 35–45%.

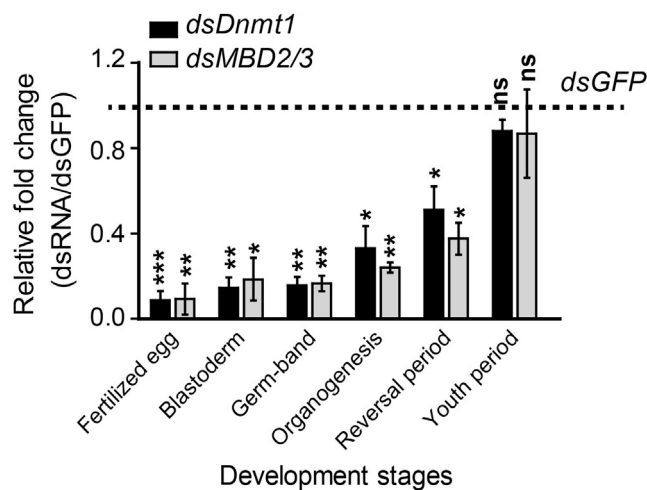


Figure 9. RT-qPCR determination of RNAi efficiency of the embryo dsRNA-injected at different developmental stages

dsDnmt1 and *dsMBD2/3* were separately injected into embryos at different developmental stages, and RT-PCR was performed 24 h after injection. Significance of the differences was determined by t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

TROUBLESHOOTING

Problem 1

dsRNA is degraded or the concentration is too low (Preparation of double-stranded RNAs step 7).

Potential solution

In order to prevent the degradation of dsRNA, the pipette tip, EP tube, PCR tube and water used in the dsRNA synthesis should be RNase-free. Experimenters wear mask and rubber gloves to prevent RNase contamination.

During dsRNA synthesis, several more reactions can be performed to ensure that the dsRNA concentration meets the experimental needs.

Problem 2

There are too many broken eggs when arranging eggs (step 3).

Potential solution

Before arranging eggs, we need to soften the egg shells in 20% NaOCl solution for 5–10 min. If the softening time is too long or the NaOCl concentration is too high, the egg shells will be too thin, resulting in damage when arranging the eggs. The NaOCl concentration should be appropriately reduced or the softening time should be reduced to within 3 min.

Problem 3

The capillary glass needle was blocked during injection (step 7).

Potential solution

When dsRNA is injected, the capillary glass needle needs to enter the egg, and the mucus in the egg, such as yolk protein, can easily block the capillary needle. We need to appropriately increase the compensation pressure parameter during injection to 70–90. If the blockage is severe, the capillary glass needle should be replaced.

Too high concentration of dsRNA solution will also cause the solution to be viscous, which is more likely to cause blockage of the capillary glass needle during injection. In our experiment, the dsRNA concentration is 5 $\mu\text{g}/\mu\text{L}$.

Problem 4

A large number of embryos died after dsRNA microinjection in the control group (mortality rate >80%) (steps 7 and 8).

Potential solution

This experiment requires a high level of microinjection technology, and the hole of the tungsten needle should not be too large, and the needle should not go too deep into the eggs to avoid a large number of embryonic deaths. The researcher needs to practice microinjection with H_2O or non-toxic dye first; the normal survival rate of silkworm eggs after injection is 35–45%. After microinjection, the injection hole needs to be sealed with quick-drying glue under the microscope within 10–15 min to avoid embryonic death caused by loss of egg contents.

Problem 5

The efficiency of RNAi is low (steps 12 and 13).

Potential solution

In our experiments, the 5 $\mu\text{g}/\mu\text{L}$ of dsRNA concentration was made, and each silkworm egg was injected with 30–50 ng of dsRNA. The amount of dsRNA injected per egg should not be less than 10 ng.

The dsRNA microinjection of silkworm embryos needs to be performed in the early embryos. In the late embryonic stage, due to the development and maturity of organs and tissues in the body, large number of nucleases may be synthesized and secreted, resulting in insensitivity to RNAi. It has been reported that large amounts of RNAi experiments are unsuccessful in lepidopteran larvae and adults (Terenius et al., 2011). The main reason is that there are large numbers of dsRNA-dependent ribonuclease in hemolymph of lepidopteran insects, which rapidly degrade dsRNA (Terenius et al., 2011). Therefore, it is speculated that the rapid degradation mechanism of hemolymph results in RNAi insensitivity of lepidopteran insects by injection (Garbutt et al., 2013; Wang et al., 2016).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sichun Zheng (sczheng@scnu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

No data or codes were generated in this study.

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AUTHOR CONTRIBUTIONS

G.X. conducted most of the experiments, participated in the data analyses, and drafted the manuscript. Y.T. and Y.P. conducted the microinjection of *B. mori* embryos. Z.S. conceived the design and oversaw the study, financially supported the research, and drafted and finalized the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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