bio-protocol

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RNA Interference Method for Gene Function Analysis in the Japanese Rhinoceros Beetle *Trypoxylus dichotomus*

Kazuki Sakura[1, #], Shinichi Morita[1, #] and Teruyuki Niimi[1, 2, *]

[1]Division of Evolutionary Developmental Biology, National Institute for Basic Biology, 38 Nishigonaka, Myodaiji, Okazaki, Japan
[2]Department of Basic Biology, School of Life Science, The Graduate University for Advanced Studies, SOKENDAI, 38 Nishigonaka, Myodaiji, Okazaki, Japan
[#]Contributed equally to this work
*For correspondence: niimi@nibb.ac.jp

Abstract

In the Japanese rhinoceros beetle *Trypoxylus dichotomus*, various candidate genes required for a specific phenotype of interest are listed by next-generation sequencing analysis. Their functions were investigated using RNA interference (RNAi) method, the only gene function analysis tool for *T. dichotomus* developed so far. The summarized procedure for the RNAi method used for *T. dichotomus* is to synthesize double-stranded RNA (dsRNA), and inject it in larvae or pupae of *T. dichotomus*. Although some dedicated materials or equipment are generally required to inject dsRNA in insects, the advantage of the protocol described here is that it is possible to inject dsRNA in *T. dichotomus* with one syringe.

Keywords: RNA interference, Japanese rhinoceros beetle, *Trypoxylus dichotomus*, Gene knock-down, Double-stranded RNA

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Background

The Japanese rhinoceros beetle *Trypoxylus dichotomus* is useful from different perspectives, such as in developmental biology, morphology, ethology, ecology, biomimetics, aerodynamics, and drug discovery. For instance, Siva-Jothy (1987) revealed the feeding time of *T. dichotomus* from ecological research, Miyanoshita *et al.* (1996) isolated a new antibacterial peptide from larvae of *T. dichotomus*, Hongo (2007) clarified the relationship between horn length and body size in *T. dichotomus*, Emlen *et al.* (2012) showed that signaling through the insulin receptor is involved in *T. dichotomus* horn development, Chen *et al.* (2017) clarified that the elytron plate of *T. dichotomus* has a mechanism of high energy absorption, and Ohde *et al.* (2018) identified several horn formation genes using RNA-sequencing analysis and RNAi techniques.

Recently, the genome of *T. dichotomus* was clarified (Ogata, 2021; Morita *et al.*, 2022), and various candidate genes required for a specific phenotype of interest were listed by RNA-sequencing analysis (Ohde *et al.*, 2018; Zinna *et al.*, 2018). Furthermore, the functions of the candidate genes were investigated using RNA interference (RNAi) method, the only gene function analysis tool for *T. dichotomus* developed so far (Emlen *et al.*, 2012; Ito *et al.*, 2013; Gotoh *et al.*, 2015; Adachi *et al.*, 2018, 2020; Ohde *et al.*, 2018; Morita *et al.*, 2019).

The RNAi method for small insects generally requires specialized setups for microinjection, such as those for preparing glass capillaries, loading double-stranded RNA (dsRNA) into microcapillaries, injecting dsRNA into insects using high pressure, and manipulating the needle for injection under a stereomicroscope (*e.g.*, Kim *et al.*, 2004; Niimi *et al.*, 2005; Masumoto *et al.*, 2009; Posnien *et al.*, 2009). The RNAi method in *T. dichotomus* does not require most of the above setups, it only requires a 1-mL syringe for dsRNA injection, since the beetle's larval body size is as large as a mouse. This study provides a detailed protocol for the RNAi method in the third instar larvae of *T. dichotomus* (Figure 1). This method can also be applied to the first and second instar larvae and pupae of *T. dichotomus*.

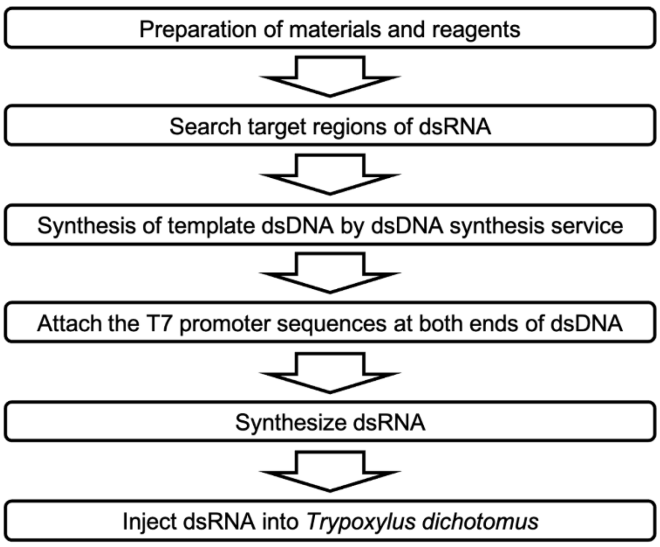

Figure 1. Overview of RNA interference method procedure in *Trypoxylus dichotomus*.

Materials and Reagents

1. Filter Pipette tips (RNase/DNase-free) (Labcon, catalog numbers: 1057-965-018-9 [P1000], 1059-965-008-9 [P200], 1055-965-018-9 [P40]; and QSP, catalog number: TF102-10-Q [P2])
2. Disposable needles No.30 (30-gauge) (Dentronics, No.30)
3. 0.2 mL Flat PCR Tube 8-Cap Strips (INA OPTIKA, catalog number: 3247-00)
4. 1.5 mL microtubes, flat-bottom, DNase/RNase-free (Watson, catalog number: 131-415C)

5. Plastic bottles (Hobby Club, blow container 750)
6. Lavender Nitrile Powder-free Exam Gloves (Kimberly-Clark, catalog number: 52818)
7. Styrofoam container
8. Aluminum foil
9. Third instar larvae of *Trypoxylus dichotomus* (Loiinne, third instar larva)
10. UltraPure™ DNase/RNase-Free Distilled Water (UPW) (Invitrogen, catalog number: 10977023)
11. Loading Dye (New England Biolabs, catalog number: B7024S)
12. 100 bp DNA Ladder (New England Biolabs, catalog number: N3231L)
13. *TaKaRa Ex Taq* (TaKaRa, catalog number: RR001B)
14. AmpliScribe™ T7-Flash™ Transcription Kit (Epicentre Technologies, catalog number: ASF3257)
15. Rnase AWAY (Thermo Fisher Scientific, catalog number: 7002)
16. Monarch PCR & DNA Cleanup Kit (5 μg) (BioLabs, catalog number: T1030)
17. Terumo Syringe 1 mL for Tuberculin Slip Tip White (Terumo, catalog number: SS-01P)
18. Ethanol (FUJIFILM Wako Chemicals, catalog number: 057-00456)
19. Phenol/Chloroform/Isoamyl alcohol (25:24:1) (PCI) (NIPPON GENE, catalog number: 311-90151)
20. Chloroform/Isoamyl alcohol (24:1) (CIA) (Sigma-Aldrich, catalog number: C0549)
21. 3 M Sodium Acetate (pH 5.2) (NIPPON GENE, catalog number: 316-90081)
22. Agarose S (NIPPON GENE, catalog number: 318-01195)
23. 10× TAE Buffer (NIPPON GENE, catalog number: 318-90301)
24. Ethidium Bromide Powder (SIGMA, catalog number: E7637-1G)
25. Humus (Dorcus Owner's shop, Osaka, Japan)
26. PCR Reaction mixture (see Recipes)
27. Solution for 1% agarose gel (see Recipes)

Equipment

1. Thermal cycler (Bio-Rad Laboratories, model: T100)
2. Pipetman (GILSON, catalog numbers: F123602 [P1000], F123601 [P200], F123600 [P20], and F144801 [P2])
3. Spectrophotometer (DeNovix, model: DS-11)
4. Electrophoresis chamber (TaKaRa, model: AD115)
5. Microwave oven (Panasonic, model: NE-MS261)
6. Gel imaging device (ATTO, model: WSE-6100)
7. Centrifuge (TOMY, model: MX-307)
8. Heat block (Major Science, model: MD-MINI)
9. Vortex mixer (LMS, model: VTX-3000L)

Procedure

A. Search target regions of dsRNA

1. Search target mRNA in RNA-seq database of *Trypoxylus dichotomus* (Ohde *et al.*, 2018; Zinna *et al.*, 2018).
2. Search for target regions of dsRNA.

Note: The lengths of target regions range from 150 bp to 600 bp. The target region should have low homology with any other T. dichotomus gene to avoid off-target effects. Therefore, it is desirable to not include any domains in the target design. Furthermore, the target region is checked for homology with other T. dichotomus genes, by performing a blastn search in the T. dichotomus database. dsRNA should be redesigned in other regions, if consecutive regions of 25-bp or more homological with other T. dichotomus genes are included.

3. Add the adapter sequence 5'-AACGAATTCGCCCTT-3' at the 5' end of the dsDNA, and the sequence 5'-AAGGGCGAATTCGCG-3' at the 3' end of dsDNA.

Note: The final sequence is as follows: 5'-AACGAATTCGCCCTT-[the sequence in the target region of dsRNA]-AAGGGCGAATTCGCG-3'. The adapter sequences are designed to be homologous with both ends of the pCR 4-TOPO vector (Invitrogen, catalog number: K457502).

4. Synthesize the above sequences using a dsDNA synthesis service (*e.g.*, IDT eBlocks).

B. Synthesize dsDNA

1. Add the T7 promoter sequences at both ends of dsDNA, by performing PCR using the following primers: 5'-taatacgactcactagggAACGAATTCGCCCTT-3' [melting temperature (Tm) = 74°C] and 5'-taatacgactcactagggCGCGAATTCGCCCTT-3' (Tm = 78°C).

Note: T7 promoter sequences are written in lowercase and underlined. The primers can also be used to attach T7 promoter sequences to inserts that are subcloned into the pCR 4-TOPO vector.

2. Perform PCR with a reaction mixture in which dsDNA (10–100 ng) synthesized in step A-4 is added as a template.

Note: The following steps constitute the PCR reaction: the initial denaturing step is 98°C for 30 s, followed by 30 cycles of denaturing at 98°C for 10 s, annealing at 55°C for 30 s, and an extension of for 60 s per 1 kb at 72°C, with an additional step of 72°C for 5 min. React about four 50 µL-reaction mixture tubes per target gene, to obtain more than 1 µg of dsDNA.

3. Collect PCR products from step B-2 into a 1.5-mL microtube (50 µL × 4 = total of 200 µL).
4. Add a sufficient amount of 1× TAE and 1 % agarose gel into the electrophoresis chamber.
5. Load a 100-bp DNA Ladder into the 1 % agarose gel well.
6. Load 5 µL of PCR product mixed (step B-3) with 1 µL of 6× DNA loading dye into a well.
7. Perform electrophoresis on full power (100 V) for 30 min.
8. Using a gel imaging device, check whether the PCR product size corresponds to the expected size.
9. Purify the PCR product from step B-3 with Monarch PCR & DNA Cleanup Kit (5 µg), according to the manufacturer's instructions.

Note: dsDNA is eluted with 100 µL of elution buffer.

10. Make dsDNA solution RNase-free.

Note: Lay aluminum foil on the laboratory table, and make the workplace and the experimental materials and equipment RNase-free with RNase AWAY. Wear rubber gloves.

a. Add 100 µL (equal to the volume of dsDNA solution) of Phenol/Chloroform/Isoamyl alcohol (PCI) to the result from step B-9 and vortex.
b. Centrifuge at 13,000 × *g* and 25°C for 15 min.
c. Transfer upper layer to new 1.5-mL RNase-free microtube.
d. Add 100 µL (equal to the volume of dsDNA solution) of chloroform, and vortex.
e. Centrifuge at 13,000 × *g* and 25°C for 5 min.
f. Transfer upper layer to new 1.5-mL RNase-free microtube.
g. Add 250 µL (2.5-fold the volume of dsDNA solution) of ethanol, and 10 µL (0.1-fold the volume of dsDNA solution) of 3 M Sodium Acetate (pH 5.2), and vortex.
h. Centrifuge at 20,400 × *g* and 4°C for 15 min.
i. Discard the supernatant.
j. Add 250 µL of 70% ethanol solution, made with UltraPure water (UPW), and vortex.
k. Centrifuge at 20,400 × *g* and 4°C for 5 min.
l. Discard the supernatant.
m. Open the lid of the tube, and dry for 5 min.
n. Add <7.3 µL of UPW.
o. Mix 1 µL of dsDNA solution from step B-10-n, and 9 µL of UPW, to make a 10-fold dilution.
p. Measure the concentration of the 10-fold diluted dsDNA solution from step B-10-o with the spectrophotometer.

C. Synthesize dsRNA

1. Synthesize dsRNA with 1 µg of dsDNA and AmpliScribe™ T7-Flash™ Transcription Kit according to the manufacturer's instructions.
2. Purify dsRNA

Note: Lay aluminum foil on the laboratory table, and make the workplace and the experimental materials and equipment RNase-free with RNase AWAY. Wear rubber gloves.

a. Add 80 µL of UPW to the result from step C-1, for a total volume of 100 µL.
b. Add 100 µL (equal to the volume of dsRNA solution) of PCI, and vortex for 30 s.
c. Centrifuge at 13,000 × *g* and 25°C for 15 min.
d. Transfer the upper layer to a new 1.5-mL RNase-free microtube.
e. Add 100 µL (equal to the volume of dsRNA solution) of chloroform, and vortex for 30 s.
f. Centrifuge at 13,000 × *g* and 25°C for 5 min.
g. Transfer the upper layer to a new 1.5-mL RNase-free microtube.
h. Add 250 µL (2.5-fold the volume of dsRNA solution) of ethanol, and 10 µL (0.1-fold the volume of dsRNA solution) of 3 M Sodium Acetate (pH 5.2), and vortex for 30 s.
i. Centrifuge at 20,400 × *g* and 4°C for 15 min.
j. Discard the supernatant.
k. Add 250 µL of 70% ethanol solution made with UPW, and vortex for 30 s.
l. Centrifuge at 20,400 × *g* and 4°C for 5 min.
m. Discard the supernatant.
n. Open the lid of the tube, and dry for <5 min.

Note: Do not dry RNA pellets for too long.

o. Add 20 µL of UPW.
p. Incubate the tube containing dsRNA solution from step C-2-o in a heat block at 65°C for 10 min.

q. Transfer the aluminum block of the heat block into a Styrofoam™ container, and cool it to room temperature, to anneal the dsRNA (Figure 2A).
r. Take an aliquot of the annealed dsRNA, and dilute it with UPW for quantification. We routinely dilute 1 µL of the dsRNA solution from step C-2-o with 9 µL of UPW.
s. Measure the concentration of 1 µL of 10-fold diluted dsRNA solution from step C-2-r with the spectrophotometer.
t. Add a sufficient amount of 1× TAE and 1% agarose gel into the electrophoresis chamber.
u. Load 100-bp DNA Ladder into the 1% agarose gel well.
v. Load 9 µL of 10-fold diluted dsRNA solution from step C-2-s mixed with 2 µL of 6× DNA loading dye into a well.
w. Perform electrophoresis on full power (100 V) for 30 min.
x. Check the concentration, and whether the dsRNA product size is the same as expected.
y. Add UPW to dsRNA solution.

Note: It is helpful to adjust the final concentration of the dsRNA solution to 5 µg/µL.

z. Store at −30°C until use.

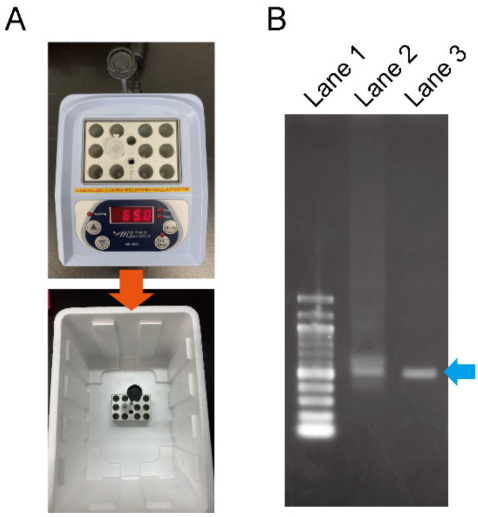

Figure 2. dsRNA annealing process.
A. The annealing process of dsRNA. B. Evaluation of dsRNA patterns via the annealing process. Lane 1; 100 bp DNA ladder. Lane 2; dsRNA before the annealing process looks smeared. Lane 3; dsRNA after the annealing process resembles a sharp band.

D. Inject dsRNA in *Trypoxylus dichotomus*

Note: EGFP is used as negative control.

1. Mix dsRNA (50–100 µg) and UPW.

Note: The final volume of dsRNA solution should be 50 µL.

2. Wear rubber gloves.

3. Softly hold the larva of *T. dichotomus* (Figure 3A).
4. Suck dsRNA aqueous solution using a 1-mL syringe with a 30-gauge needle.
5. Pierce the needle at the lateral body wall of the first thoracic segment (T1) of a larva, and inject the solution into the hemocoel, just beneath the epidermis (Figure 3B and C).

Note: It is possible for bubbles to be injected in T. dichotomus to a small extent.

6. Place the larva in a bottle filled with humus, and incubate it at room temperature (24–28°C) until the larva develops into the pupa or the adult.
7. Observe the phenotype in the pupa and/or the adult.

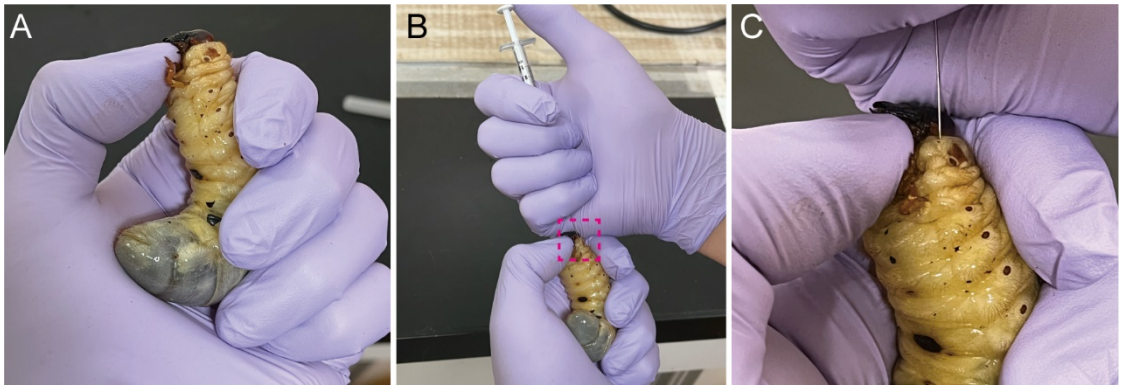

Figure 3. Injection of dsRNA into third instar larvae of *Trypoxylus dichotomus*.
A. How to hold a larva during the injection. To prevent a larva from moving its head during injection, place your thumbnail under the mandible and push the larval head upward. B. Injection of dsRNA into third instar larvae. C. A highly magnified view of the injection point in the first thoracic (T1) body segment. dsRNA is injected into the T1 of a larva. The needle should be inserted about 2 mm beneath the epidermis. The needle should be held in place for 10 sec after dsRNA injection, to prevent the backflow of dsRNA.

Recipes

1. PCR Reaction mixture

5 µL of 10× *Ex taq* Buffer
4 µL of dNTP Mixture (each 2.5 mM)
2 µL of Primer (10 mM) (1 µL of forward, and 1 µL of reverse)
0.5 µL of *Ex Taq*
38.5 µL of UPW (the amount of DNA template solution)

2. Solution for 1% agarose gel

150 mL of TAE (1×)
1.5 g Agarose S

Note: Dissolve the above solution in a microwave oven. Next, add 20 µL of 10 mg/mL ethidium bromide to the solution. Allow the solution to polymerize at room temperature.

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Competing interests

No conflict of interest is declared regarding this article.

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