BmICE-2 interference vector construction and its silencing effect in the *BmN-SWU1* cells

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ABSTRACT. Investigate the function of *BmICE-2* gene in silkworm cells by constructing a *BmICE-2* interference vector and analysing its interference effect. Online RNAi design software was used to predict the interference target sequence of Bmice-2. An optimized sequence was selected and inserted into the backbone of interference microRNA. The backbone of interference vector, microRNA-siBmICE-2, and a control vector, microRNA-siBmICE-2-control were designed. The synthesized gene segment was digested by Asc I and Sac II, and inserted into a PIZ/V5-dsRed vector. The interference vector PIZ/V5-BmICE-2-miRNA-dsRed and control vector PIZ/V5-BmICE-2-Control-miRNA-dsRed were designed and validated through digestion and sequencing. The interference and control vectors were then transfected into silkworm cells BmN-SWU1. qRT-PCR and Western blots were used to evaluate the effect of gene interference. Interference microRNA vector PIZ/V5-BmICE-2-miRNA-dsRed targeting *BmICE-2* was successfully constructed and transiently expressed in silkworm cells. The interference vector significantly inhibited the expression of *BmICE-2* at mRNA and protein levels. The constructed interfering vector significantly interfered with the expression of *BmICE-2* in silkworm cells BmNSWU1.

Key words: Silkworm; Bmice-2; RNA interference; RNAI vector

INTRODUCTION

RNA interference (RNAi) includes gene silencing on translational or transcriptional levels induced by a double-stranded RNA (dsRNA) that specifically and efficiently degrades the homologous mRNA of the target gene. It inhibits gene expression by inhibiting the transcription or translation of the specific gene (Fire 1998). When cells are treated with a double-stranded RNA homologous to the endogenous mRNA coding region, the targeted mRNA is degraded and the gene expression is silenced (Zamore 2000). Many studies show that dsRNA can work as an effective tool to interfere with specific gene expression in insects and to study gene function (Shabalina et al. 2008, Yamaguchi et al. 2011). Because of the high specificity, high efficiency, and high stability of RNAi and its ability to transfer across cells, it is widely applied to study the function of specific genes (Kim 2005, Winston et al.2002, Yamaguchi et al. 2011).

The silkworm is a holometabolous Lepidopteran insect that has significant economic importance (Chen et al. 2015, Xia et al. 2004). The silkworm *BmICE-2* gene is a pro-apoptotic gene recently identified from the silkworm cell apoptosis associated genes. It promotes apoptosis of the silkworm cell *BmN-SWU1* and BmE-SWU1 (Yi et al. 2014). In silkworm *BmN-SWU1* cells, endogenous *BmICE-2* accumulates in the nucleus in the form of a proenzyme of cysteinyl aspartate-specific proteinase (Caspase). In apoptotic cells, *BmICE-2* demonsrated the features of nuclear matrix translocation and mitochondrial repositioning (Yi 2014). The purpose of this study was to discover the molecular mechanism of *BmICE-2* in Caspase-dependent cell apoptosis in silkworm. We used RNAi techniques to design a RNA interference backbone, construct an interference vector, transfect silkworm *BmN-SWU1* cells, and detect the efficiency of interference using qRT-PCR.

MATERIALS AND METHODS

Plasmid and cells

PIZ/V5-dsRed plasmid was maintained by our laboratory; The silkworm embryo cell line: *BmN-SWU1* was cultured in a 27°C incubator using TC-100 insect cell culture medium containing fetal bovine serum (PAA), with the volume fraction of 10% (Pan et al.2007, Pan et al.2010).

Reagents and equipment

Liposomes were from Roche (USA); TC-100 insect cell culture medium was from Invitrogen in USA; 24-well circular cover clips were from Fisher (USA); fluorescent quantitative PCR kit (RR037A) and Asc I and Sac II enzymes were from Takara Engineering (Dalian, China); Rabbit *BmICE-2* protein polypeptide antibody (anti-BmICE-2, primary antibody) was from ZoonBio Biotechnology (Nanjing, China); Rabbit Tubulin antibody, goat anti-rabbit horseradish peroxidase secondary antibody, Western primary antibody dilution buffer (p0023a) and secondary antibody dilution buffer (P0023D), BeyoECL Star (super-sensitive ECL chemiluminescence kit) and PMSF (ST506) were from Beyotime Biotechnology (Hangzhou, China); the ultra-pure plasmid extraction kit (Qiagen, 12145), protein marker (DM201) and TransT 1 competent cells were from Transgene Biotech (China). All cell culture flasks, cell culture plates were from Corning (USA); fluorescence microscope (BX-51) was from Olympus (Japan); Vilber ultra high-sensitivity chemiluminescence imaging system (fusion FX6-XT) was used for immunoblotting membrane imaging analysis (Vilber Lourmat, France).

Design and synthesis of the microRNA interference backbone

The sequence of *BmICE-2* was obtained from GenBank (GenBank accession number: DQ 360829.1). siRNA target sequences were predicted using an online analysis tool (https://rnaidesigner. Invitrogen. COM rnaiexpress); non-specific binding siRNA sequences were excluded by comparing the sequences with the silkworm genome database. We selected the best interference sequence to silkworm cells, in which *BmICE-2* gene ORF coding region 212–230 bps (CGACAAGGAACGGTACATT) and 413–431 bps (CTGCCGACCAACCATACAA) were selected as targeting sequence (Yamaguchi et al. 2011). The reverse complementary sequences were AATGTACCGTTCCTTGTCG and TTGTATGGTTGGTCGGCAG, respectively. A siRNA interference fragment was designed based on data in reference (Zhang 2012), and was inserted into the bmo-mir 273a backbone sequence. A microRNA interference vector sequence was designed for microRNA-siBmICE-2. For the control vector, the target sequence encoding region 212–230 bps (CGACAAGGAACGGTACATT) and 413–431 bps (CTGCCGACCAACCATACAA) were randomly combined. We designed the sequence of microRNA-siBmICE-2-control for microRNA interference. Gene

synthesis was performed by Gen Script (Nanjing, Jiangsu, China). The synthetic gene sequence is shown in Figure 1.

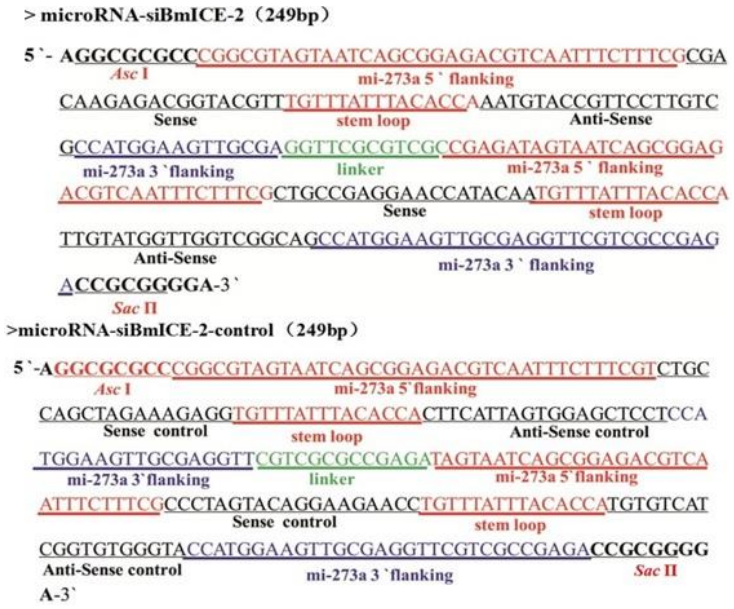

Figure 1. Interference vector sequence (microRNA-siBmICE-2) and the interference control vector sequence (microRNA-siBmICE-2-control).

Construction of miRNA interference vector

The synthesized plasmids microRNA-siBmICE-2, microRNA-siBmICE-2-control and PIZ/V5-dsRedicroRNA-si*bmice-2* were digested using *Asc*I and *Sac*II, followed by DNA extraction, overnight ligation using T4 DNA ligase, TransT1 competent cell transformation, and zeocin selection. Positive plasmid colonies of microRNA-siBmICE-2, microRNA-siBmICE-2-control and PIZ/V5-dsRedicroRNA-si*bmice-2* were validated by enzyme digestion using Asc I and Sac II. The undigested plasmid was used as the control. Sequencing was performed after enzyme digestion.

Interference plasmid transfecting silkworm cell

Silkworm *BmN-SWU1* cells were cultured to 90% confluence and transferred to a 24-well tissue culture slide with circular cover clip. A 800 ng amount of PIZ/V5-BmICE-2-miRNA-dsRed and PIZ/V5-BmICE-2-Control-miRNA-dsRed and 2 µL of liposome were incubated in 100 µL TC-100 medium for 30 minutes before transfection to *BmN-SWU1* cell. Cells were observed using a fluorescence microscope 36 h and 48 h after transfection (Yi et al. 2014).

qRT-PCR detection of *BmICE-2* interference

After transfection with PIZ/V5-BmICE-2-miRNA-dsRed and PIZ/V5-BmICE-2-Control-miRNA-dsRed ultra-pure plasmid, RNA from *BmN-SWU1* cells was collected at 48, 72 and 96 h and reverse-transcribed into cDNA. Silkworm *Actin4* was used as a reference gene in qRT-PCR with the primer of SW22934-F and SW22934-R. qRT-PCR primer for *BmICE-2* was QRT-PCR-ICE-2-F and QRT-PCR-ICE-2-R. The size of the amplicons was 180 bp (Table 1). For each gene, 3 biological replicates were performed. The PCR system was (15 µL): ddH2O 4.5 µL, SYBR 7.5 µL, prime F 1 µL, prime R 1 µL, cDNA 1 µL. The reaction procedure was 95°C, 30 s; 95°C, 5 s; 60°C, 30 s, 40 cycles. The specific protocol used was based on the reference (Yi et al. 2014).

Table 1. Main primers use in the experiment

Primer name	Primer sequence 5'→3'	Annealing temperature	Amplification size
BmiCE-2-F	TCTGTTGACGGTTATCTTTC	60 °c	180bp
BmiCE-2-R	TATTGTTGGTCTCCTGACAT		
SW22934-F	TTCGTACTGGCTCTTCTCGT	60 °c	180bp
SW22934-R	CAAAGTTGATAGCAATTCCCT		

Western blotting detecting the interference of *BmICE-2*

Healthy *BmN-SWU1* cells were plated in 12-well cell culture plates and transfected with PIZ/V5-BmICE-2-miRNA-dsRed and PIZ/V5-BmICE-2-Control-miRNA-dsRed, respectively. At 48, 72, 96, and 120 h after transfection, the supernatant was removed and protein samples were prepared according the procedure in reference (Yi et al. 2014). An equal amount of total cell proteins from interference group and control group at the different time points were analyzed using 12% SDS-PAGE gel electrophoresis. Proteins were transferred to the PVDF membrane using the semi-dry transferring method. The membrane was blocked in 50 g/L of skim milk powder solution for 2 h at room temperature. Anti-*BmICE-2* polypeptide antibody was diluted 1:1000 using Western antibody dilution solution containing 50 g/L of skim milk powder. Reference tubulin antibody was diluted 1:3000. The membrane was incubated overnight with antibody at 4°C. The membrane was then washed 5 times on a horizontal shaker with TBST, 5 mins per wash. Horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody was diluted 1:5000 and incubated at room temperature for 1 h. The membrane was then washed 3 times on a horizontal shaker with TBST, 10 mins per wash. Chemiluminescence development was performed according to the protocol of BeyoECL Star (Hypersensitive ECL chemiluminescent kit) (Beyotime Biotechnology, China). A chemiluminescence detector was used for film analysis.

Data analysis

The expression level of silkworm *BmICE-2* in *BmN-SWU1* cells was set as the control. The interference effects of the interfering vector PIZ/V5-BmICE-2-miRNA-dsRed and the control vector PIZ/V5-BmICE-2-Control-miRNA-dsRed in *BmN-SWU1* cells were analyzed using the $2^{-\Delta\Delta CT}$ method. The data was further analyzed and graphed using GraphPad 5.

RESULTS

Constructing the RNAi plasmid Targeting *BmICE*-2

The designed microRNA-si*BmICE-2* and microRNA-siBmICE-2-control sequences were synthesized by Gene script (Nanjing, China). Synthesized microRNA-siBmICE-2, microRNA-siBmICE-2-control and PIZ/V5-dsRed plasmids were digested using Asc I and Sac II, followed by DNA extraction, ligation, and TransT1 competent cell transformation. Zeocin resistant colonies on LB agar plates were selected for harvesting recombinant plasmids. Plasmids were validated through enzyme digestion using Asc I and Sac II. As shown in Figure 1, an expected 250 bp segment was observed. Sequencing data showed that the sequences of synthesized microRNA-si*BmICE-2* and microRNA-siBmICE-2-control successfully integrated into the PIZ/V5-dsRed eukaryotic expression vector. The sequencing result was identical to the designed sequence and no insertions, deletions or mutations were found.

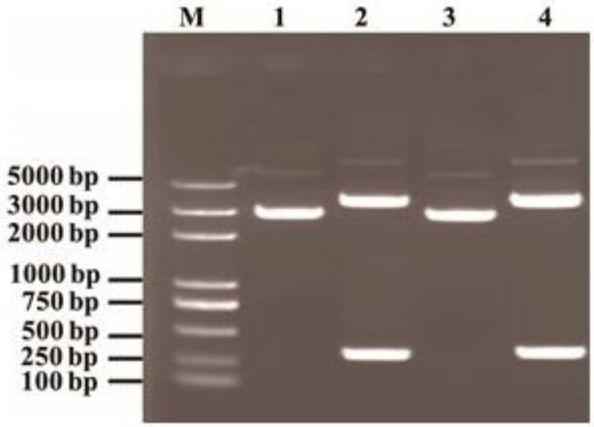

Figure 1. Identification of the PIZ/V5-BmICE-2-miRNA-dsRed and PIZ/V5-BmICE-2-Control-miRNA-dsRed recombinant plasmids by digestion with *Asc* I and *Sac* Π.

M. Marker DL 5 000; 1. RNAi vector of PIZ/V5-BmICE-2-miRNA-dsRed; 2. Production digested with *Asc* I and *Sac* Π enzyme of PIZ/V5-BmICE-2-miRNA-dsRed vector; 3. RNAi control vector of PIZ/V5-BmICE-2-Control-miRNA-dsRed; 4. Production digested with *Asc* I and *Sac* Π enzyme of PIZ/V5-BmICE-2-Control-miRNA-dsRed

Transient expression of the interference vector

Thirty-six h after *BmN-SWU1* cells were transfected with PIZ/V5-BmICE-2-miRNA-dsRed and PIZ/V5-BmICE-2-Control-miRNA-dsRed plasmids respectively, strong red fluorescence signals were observed in both groups. Red fluorescence signals indicated the existence of BmICE-2-miRNA-dsRed protein (Figures 2a and 2b) and BmICE-2-Control-miRNA-dsRed protein (Figures 2c and 2d). Cells transfected with PIZ/V5-BmICE-2-Control-miRNA-dsRed plasmid were taken as control.

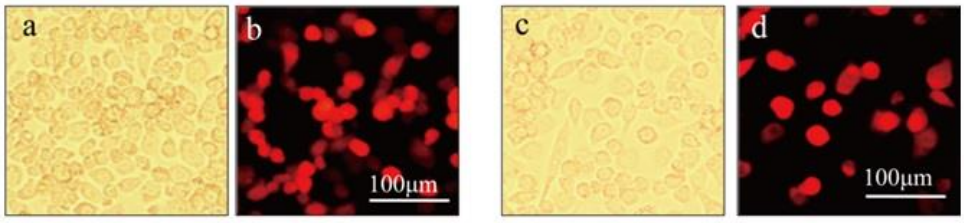

Figure 2. *BmN-SWU1* cells transfected with PIZ/V5-BmICE-2-miRNA-dsRed vector after 36 h.

Transfection with PIZ/V5-BmICE-2-miRNA-dsRed in *BmN-SWU1* cells (a and b); transfection with PIZ/V5-BmICE-2-control-miRNA-dsRed in *BmN-SWU1* cells (c and d); white photos a and c; fluorescence images b and d. Scale bars = 100 μm.

qRT-PCR Analyzing the Interference of *BmICE-2*

BmN-SWU1 cells were transfected with the constructed eukaryotic interference plasmids PIZ/V5-BmICE-2-miRNA-dsRed and PIZ/V5-BmICE-2-Control-miRNA-dsRed. After 24 h post-transfection, cells were cultured for another 24, 48 or 72 h in the zeocin-containing medium before total RNA was collected, respectively. RNA was reverse-transcribed into cDNA and tested for *BmICE-2* expression through qRT-PCR. qRT-PCR results showed that 72 h after the transient expression of PIZ/V5-BmICE-2-miRNA-dsRed interference plasmid, the expression of *BmICE-2* significantly decreased compared to control, $0.01<p<0.05$; 96 h after the transient expression, the significant p-value was 0.0011536 (Figure 3).

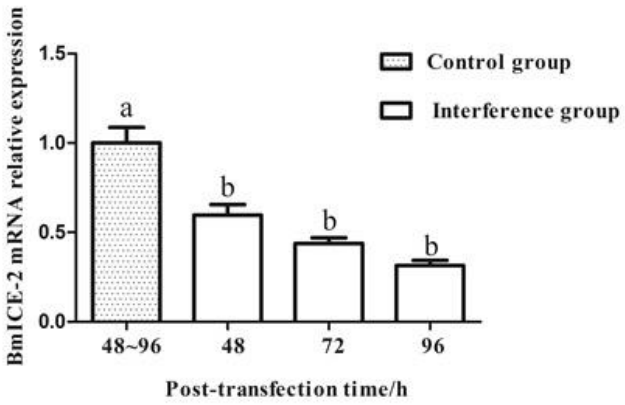

Figure 3. Silence efficiency detection of *BmICE-2* gene inducing by PIZ/V5-BmICE-2-miRNA-dsred in *BmN-SWU1* cells by qRT-PCR

Western blot analysis of *BmICE-2* interference

Silkworm cells *BmN-SWU1* were transfected with PIZ/V5-BmICE-2-miRNA-dsRed and PIZ/V5-Control-miRNA-dsRed respectively. Protein samples were prepared at 48, 72, 96, and 120 h after transfection for Western blotting. With increased interference time, cells transfected with PIZ/V5-BmICE-2-miRNA-dsRed exhibited down-regulated *BmICE-2* levels compared to the control (Figure 4).

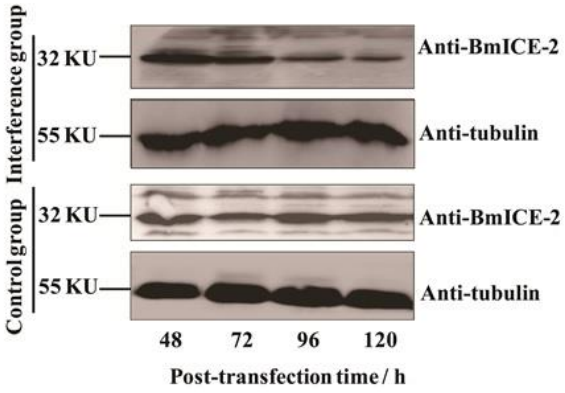

Figure 4. Silence efficiency detection of *BmICE-2* gene inducing by PIZ/V5-BmICE-2-miRNA-dsred in *BmN-SWU1* cells by Western blotting

DISCUSSION

RNAi is a sequence-specific and homology-dependent gene silencing phenomenon. It is ubiquitous in eukaryotes and functions to prevent nucleic acid intrusion and to regulate gene expression (Fire et al.1998, Shabalina et al. 2008, Zamore et al. 2000). In current RNAi interference technology, the sources of siRNA include chemical synthesis, *in vitro* transcription, endonuclease digestion, and *in vivo* expression using plasmids or viral vectors (Kamthan et al. 2015, Zhang et al. 2015, Zhao et al. 2015). MicroRNA (miRNA) is a class of RNA consisting of 18–25 non-coding nucleotides. Haley et al. (2008) showed that it was commonly involved in growth and development, apoptosis, tumorigenesis, anti-virus, and a number of other life processes. MicroRNA interfering vectors are widely used in genomic research, viral disease treatment, tumor treatment, autoimmune disease therapy, new drug development, and insect control (Andermatt et al. 2014, Haley et al. 2008, Haley et al. 2010, Naidu et al. 2014). Commercialized miRNA expression vectors have been developed for human and murine cells. There are also target gene silencing studies on the fruit fly (*Drosophila*) and silkworm, in which the interference vector with the backbone of miRNA was applied (Haley et al. 2008, Haley et al. 2010, Zhang et al.2012).

The miRNA expression vector in this study used the silkworm miRNA bmo-mir-273a as the basic backbone structure and PIZ/V5-BmICE-2-miRNA-dsRed as the interference vector. This expression vector effectively silenced the target gene *BmICE-2* in silkworm cells. Our data showed that a pair of oligonucleotide sequences targeting *BmICE-2* was correctly inserted into the bmo-mir-273a backbone sequence. The PIZ/V5-BmICE-2-miRNA-dsRed interfering vector could be transfected into silkworm *BmN-SWU1* cells. With low transfection efficiency, transfected cells could be selected by zeocin and cultured for analysis of *BmICE-2* silencing. Results of qRT-PCR and Western blotting showed that PIZ/V5-BmICE-2-miRNA-dsRed could significantly down-regulate the expression of *BmICE-2* at mRNA and protein levels. This suggests that the construction of gene silencing vector was stable and effective. This same vector could be used for other studies.

Yi et al. (2014) *showed that BmICE-2* was a pro-apoptosis caspase gene in the silkworm and played an important role in silkworm cell apoptosis.

CONCLUSION

Future studies might focus on the regulation of BmICE-2-dependent pathways and identification of the activation mechanism of *BmICE-2* in cells and its associated regulators. Our study provides information on the mechanism by which *BmICE-2* regulates silkworm cells apoptosis.

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