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*piggy*Bac transposon-derived targeting shRNA interference against the *Bombyx mori* nucleopolyhedrovirus (*Bm*NPV)

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Abstract The *Bombyx mori* nucleopolyhedrovirus (*Bm*NPV) is one of the most destructive diseases in silkworm, which has caused the main damage to sericulture industry. In this study, we developed a system of RNAi to prevent the *Bm*NPV infection using the *piggy*Bac transposon-derived targeting short hairpin RNA (shRNA) interference. The shRNAs targeting the genes of *i.e.*-1, *lef*-1, *lef*-2 and *lef*-3 of *Bm*NPV were designed and used to inhibit the intracellular replication or multiplication of *Bm*NPV in *Bm* cells. The highest activity was presented in the shRNA targeting the *i.e.*-1c of *Bm*NPV, of which the inhibition rate reached 94.5 % in vitro. Further a stable *Bm* cell line of *piggy*Bac transposon-derived targeting shRNA interference against *Bm*NPV was established, which has a highly efficacious suppression on virus proliferation. These results indicated that the recombinant shRNA expression system was a useful tool for resistance to *Bm*NPV in vitro. The approach by recombinant shRNAs opens a door of RNAi technology as a strategy that offering technically simpler, cheaper, and quicker gene knockdown for promising research and biotechnology application on silkworm lethal diseases.

Keywords Silkworm *Bombyx mori* L(Lepidoptera: Bombycidae) · Short hairpin RNA (shRNA) interference · *piggy*Bac transposon · *Bombyx mori* nucleopolyhedrovirus (*Bm*NPV)

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Introduction

Baculoviruses are divided into two genera based on occlusion body morphology, nucleopolyhedroviruses (NPVs) [1] and granulosis viruses (GVs) [2]. *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and *Bombyx mori* nucleopolyhedrovirus are the most characterized baculoviruses [3, 4].

*Bm*NPV has a circular, supercoiled DNA genome of 130 kbp [3]. Like most of the baculoviruses, *Bm*NPV is host-specific and highly infectious to *Bombyx mori* cultured cells and silkworm larvae whereas cannot replicate in the cells closely related lepidopteran *Spodoptera frugiperda* [5, 6]. *Bm*NPV is one of the most important viruses inducing the lethal disease in silkworm, and causing serious loss in sericulture industry [7].

Nine replication genes (*i.e.*-1, *i.e.*-2, *lef*-1, *lef*-2, *lef*-3, *helicase*, *DNApol*, *p*35 and *pe*38) of *Bm*NPV were identified within six regions previously shown to be essential for viral DNA replication in a transient assay [8, 9]. These genes encode IE-1 (an essential regulatory protein for viral gene expression and DNA replication) [10], LEF-1 (a DNA primase) [11], LEF-2 (interacts with LEF-1) [12], LEF-3 (a single-stranded DNA binding protein that interacts with DNA helicase) [13], DNA helicase [14] and DNA polymerase [15], respectively. Moreover, four of the six genes (*i.e.*-1, *lef*-1, *lef*-2, *lef*-3) are implicated as being necessary for DNA replication. Viral genes start transcribing in a temporal manner [16] with early genes (*i.e.*-1 and *i.e.*-2) [17–19]. Immediate early gene 1 (*i.e.*-1) encodes a protein of 67 kDa as a transcriptional activator [20, 21], which is one of the essential genes needed for viral replication [8, 9]. *i.e.*-1 has also been shown to activate promoters of early genes including *i.e.*-2, *39* K and *p*35 [22–24] and it is directly or indirectly involved in the expression of late genes [25]. Meanwhile, the deletion of *i.e.*-1

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induced inhibition of DNA replication [26]. It demonstrated that *i.e.*-1 directly involved in DNA replication and activated the expression of replication genes, especially the late expression factors (*lef*s) [27]. The *lef*s genes, *lef*-1, *lef*-2, were originally identified as required for late gene transcription [25, 28]. In addition, these two genes involved in DNA replication, and activated early genes expression [21, 29]. Late gene expression dependents on viral DNA replication and cannot be observed when DNA replication is inhibited [30–32]. A biochemical study showed a molecular mass (44.5 kDa) of infection-specific protein was found in nuclear extracts of *Ac*MNPV-infected *S. frugiperda* cells. This is consisted with that predicted for *lef*-3 [33].

All above genes are indispensable for replication or multiplication of *Bm*NPV in host both of cell line and larvae, thus may be considered as the appropriate target genes to inhibit infection of *Bm*NPV by RNA interference (RNAi). RNAi has been demonstrated in many insect species including *Bombyx mori* [7, 34]. More recently, it was reported that the introduction of double-stranded RNA (dsRNA) results in the repression of *Bm*NPV and *Ac*MNPV. The RNAi-mediate inhibition of *Bm*NPV proliferation by targeting *i.e.*-1 gene in an in vitro assay using dsRNA has been reported [6]. dsRNA targeting *ie*1, *lef*-1, *lef*-3, and *p*74 was partially effective to lower the *Bm*NPV proliferation [7, 35].

However, there were seldom reports targeting *lef*-2 and *lef*-3 of *Bm*NPV using short hairpin RNAs (shRNAs). Compared to dsRNA, shRNAs keep the interference stable and permanent. Once the shRNAs introduced into the host through vectors under U6 promoter, its function would be genetic. The IE1 promoter was derived from the *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV) immediate early gene and has been used to express several eukaryotic proteins [36]. U6 ubiquitous promoter is a polymerase III promoter, which was the most commonly used for shRNA expression, showed a comparably broad activity [37].

In this study, we developed a system of shRNAi for preventing the *Bm*NPV infection using the *piggy*Bac transposon-derived shRNAs targeting *i.e.*-1, *lef*-1, *lef*-2 and *lef*-3. The transformed cells were marked with the GFP under IE1 promoter. The inhibition efficacy was investigated through the analysis of shRNA-expression under *Bombyx mori* U6 (*Bm*U6) promoter [38]. Our results suggest that shRNAi technology might serve as an experimental basis and technical insight into the research on virus disease therapeutics.

Materials and methods

Construction of recombinant transformation plasmids

The enhanced green fluorescent protein (EGFP) gene under IE1 promoter was cloned in the *Bgl*II and *Hin*dIII sites of PXL-BACII (*Piggy*Bac transposition vector) to construct the PXL-BACII-GFP.

*Bm*U6 gene was amplified by PCR using silkworm genomic DNA as the template using specific primers (Forward primer: 5′-agatctAGGTTATGTATACACATTG-3′ and Reverse primer: 5′-ggatccACTTGTAGAGCACGATATT-3′). *Bgl*II and *Bam*HI restriction sites were introduced into the 5′ region, respectively. PXL-BACII-GFP then was digested with *Bgl*II and *Bam*HI, and the recovered DNA fragment was ligated with the 479 bp of the amplified *Bm*U6 PCR product, designed as PXL-BACII-GFP-*Bm*U6.

Design of shRNA and construction of recombinant plasmids

Targeting sequences in the coding region of the *i.e.*-1, *lef*-1, *lef*-2 and *lef*-3 of *Bm*NPV were selected and served as a basis for the design of the complementary shRNA template oligonucleotides (Table 1).

The shRNAs were synthesized, annealed under 95 °C/30 s, 72 °C/2 min, 37 °C/2 min, 25 °C/2 min, and replaced in the *Bam*HI and *Eco*RI sites of PXL-BACII-GFP-*Bm*U6. The resultant plasmid was designated PXL-BACII-GFP-*Bm*U6-shRNA. Finally the neomycin resistant gene (Neo^r) was inserted into the *Eco*RI–*Eco*RI sites of PXL-BACII-GFP-*Bm*U6-shRNA to construct transformable vector PXL-BACII-GFP-*Bm*U6-shRNA-Neo.

Viral challenge and investigation of interference against BmNPV

Transient transfection

*Bm*N cells were seeded onto 24-wells plates at a density of 2.0×10^6 cells per well in TC-100 medium (AppliChem GmBH, Darmstadt, Germany) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) at 27°C. After 24 h, the medium was removed and 0.8 μg of PXL-BACII-GFP-*Bm*U6-shRNAs were transfected using lipofectamine^{TM} 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. All transfections were conducted in triplicate.

Cloning of the transgenic BmN cell line

*Bm*N cells were co-transfected with the transformation plasmid PXL-BACII-GFP-*Bm*U6-shRNA-Neo and the transposase-producing helper plasmid (1:1). Green fluorescent protein expression was visible 24 h post-transfection under inverted fluorescence microscope.

A Neo^r was cloned into the above vector as a second selectable gene. Under the marker, the untransformed cells will be died, otherwise the transformed cells will keep alive

Table 1 Synthetic oligonucleotides for constructing shRNA expression plasmids

ORF	Name	Target sites (nts)	Targeting sequences (5′-3′)
i.e.-1	*i.e.*-1a	62–80	TCGACAACGGCTATTCAGA
	i.e.-1b	93–111	ACAACAGCCCAACGACTAT
	i.e.-1c[a]	139–159	GATGGAGCCGACACGGTAGTA
	i.e.-1d[a]	195–215	GGCAAGCGTCAATTCGTTAAC
	i.e.-1e	262–280	CTCGGAGAAGCAGTTAGTT
	i.e.-1f	349–367	GCGGAATCTTTTGAGCAGT
	i.e.-1 g	404–422	AACGGAAGCTGGACGAATA
	i.e.-1 h	99–117	GCCCAACGACTATTTGAAT
	i.e.-1i	600–618	GCCGTATTTGATGCGTTTC
	i.e.-1j	618–636	CGACGACAACGACTACAAT
lef-1	*lef*-1a	470–488	CCGGTATACATGACTCTTG
	lef-1b	104–122	CCATAACCAGGATAGCATT
	lef-1b2[a]	103–123	tCCATAACCAGGATAGCATTg
	lef-1c	109–127	CCAGGATAGCATTGCTTTA
	lef-1d	283–301	TGCATATGCGGAATGTACA
	lef-1e	341–359	GGCTGAATGCAATCACTAT
	lef-1e2[a]	341–361	GGCTGAATGCAATCACTATcc
	lef-1f	411–429	AGCGGACGTGATTTTAAAC
lef-2	*lef*-2a	163–181	CTGCGATTGTACATGCTGT
	lef-2b	411–429	TGCAAACGTGGTTTCGTGT
	lef-2c	497–515	GGGTGAAGTCATGCATCTT
	lef-2d	191–209	CGCCCACTATTAATGCAAT
	lef-2d2[a]	191–211	CGCCCACTATTAATGCAATta
	lef-2e	294–312	GCTGAACAGCAAGATCAAT
	lef-2e2[a]	292–312	atGCTGAACAGCAAGATCAAT
lef-3	*lef*-3a	120–138	CCAGCAGCATTGAGATTTG
	lef-3a2[a]	120–140	CCAGCAGCATTGAGATTTGat
	lef-3b	195–213	GACGTAGAAAACGGACTTG
	lef-3c	263–281	CGTTCAAATCGTCGCTCTT
	lef-3d	652–670	ACTTGAACCACGTCGGAAT
	lef-3e	943–961	TTGCGAATCGCCGTAGTAT
	lef-3e2[a]	943–963	TTGCGAATCGCCGTAGTATtc

[a] Targeting sequences of 21 bp

when adding the neomycin G418 into the medium. The medium was removed and cells were washed twice with PBS buffer, and added new medium with G418 (800 ug/ml). The medium was renewed every 3–5 days. The cells were cloned after selection of G418 for 15 days when most of the non-transformed cells died.

Viral challenge

24 h post-transfection with PXL-BACII-GFP-*Bm*U6-shRNAs or PXL-BACII-GFP-*Bm*U6-shRNA-Neo plasmids, the *Bm*N cells were infected with *Bm*NPV at a multiplicity of infection (MOI) of five. After incubation for 1 h at 27°C, the medium was removed and the fresh TC-100 medium supplemented 10 % FBS was added. Each viral challenge was carried out in triplicate.

BmNPV resistance in transformed BmN cell line by qPCR

*Bm*NPV specific primers for target genes were set as *Bm*NPV-F: 5′-TCGACAACGGCTATTCAGAG-3′; *Bm*NPV-R: 5′-CTGCAGTCTCGCTGTCAGAT-3′ according to *Bm*NPV sequence. The *Bm* actinA3 specific primers were used for housekeeping gene as actinA3-F: 5′-GCGCGGCTACTCGTTCACTACC-3′ and actinA3-R: 5′-GGATGTCCACGTCGCACTTCA-3′.

96 h post-challenged with *Bm*NPV, total RNA was isolated by using RNeasy Mini Kit (QIAGEN) from the *Bm*N cells. A total sample of (1–2 μg) RNA was used as template to synthesize cDNA using superscript II reverse transcriptase (Invitrogen).

qPCR was performed by using ABI7300 (Ambion, USA) and the fluorescence dye SYBR®Premix Ex Taq™ (TAKARA) on the resulting cDNA using pairs of primers mentioned above. The two-step amplification protocol consisted of a 30 s at 95 °C followed by target amplification via 40 cycles at 94 °C for 5 s, 60 °C for 31 s. After PCR, the absence of unwanted by-products was confirmed by automated melting curve analysis and agarose gel electrophoresis of the products. The transcript levels of the targeted fragments were normalized with *Bm* actinA3 transcript levels in the same samples. Relative qPCR was used to confirm the ectopic expression of *Bm*NPV transcripts. The relative levels of gene expression was analyzed according to the $2^{-\Delta Ct}$ method [39], where ΔCt is the Ct value for *Bm*NPV transcripts normalized to the *Bm*actinA3. All the experiments were performed in three independent biological replication and the reactions of each sample were carried out in triplicate.

Result

Construction of recombinant transformation plasmids

The investigation of shRNA interference against *Bm*NPV was conducted in two levels, transient shRNA expression for screening and selecting the better or best targeting sequence(s) and consistent expression for confirming its efficiency of interference in replication or multiplication of *Bm*NPV. Two recombinant plasmids were constructed (Fig. 1). The PXL-BACII-GFP-*Bm*U6-shRNA plasmid was used for screening and selecting the targeting sequence(s) through transient shRNA expression. The PXL-BACII-GFP-*Bm*U6-shRNA-Neo plasmid is for cloning of the transgenic *Bm*N cell line through neomycin

G418 selection, and for investigating the consistent efficiency of interference to *Bm*NPV.

Viral challenge and investigation of transient interference against BmNPV

*Bm*N cells were transfected with transient expression vector PXL-BACII-GFP-*Bm*U6-shRNA for 24 h. The green florescence of *Bm*N cells were observed under inverted fluorescence microscope as shown in Fig. 2. About 50–60 % of *Bm*N cells were screened of GFP expression when cells were transfected with vector PXL-BACII-GFP-*Bm*U6-shRNA.

24 h post-transfection with PXL-BACII-GFP-*Bm*U6-shRNA plasmids, the *Bm*N cells were infected with *Bm*NPV at a MOI of five. GFP-positive and GFP-negative *Bm*N cells were observed after infection with *Bm*NPV (Fig. 3). In Fig. 3a, the *Bm*N cell was transfected with PXL-BACII-GFP-*Bm*U6-shRNA plasmids which presented the green fluorescence under the inverted fluorescence microscope, and no virions were screened in this cell because of the targeting shRNA functions. Comparatively when the shRNA was not expressed in the *Bm*N cell, in which no GFP was screened, and the virions encapsulated within the polyhedral envelope were visible or released to environment by cell lysis and dissolution (Fig. 3b).

96 h after challenge with *Bm*NPV, the relative expression of virus was estimated by real-time quantity PCR (qPCR) analysis performed on the total RNA extracted from the cells (Fig. 4). Most transfected *Bm*N cells repressed the expression level of *Bm*NPV compared to control 96 h post-transfection. Although the shRNAs targeting *lef*-1a, *lef*-1b, *lef*-1c, *lef*-2d, *lef*-3b, *lef*-3d reduced the expression of *Bm*NPV, but their RNAi effects were relatively weaker (0.45–18.33 %). The shRNAs targeting *i.e.*-1c, *lef*-1b2, *lef*-3e, *lef*-3e2 presented relatively strong inhibition against *Bm*NPV infection. Among them, the highest suppression activity displayed in *i.e.*-1c sequence, its relative abundance was less than 5.0 % compared with the control (100 %). It means the inhibition rate of shRNA targeting *i.e.*-1c reached 95 %.

BmNPV resistance in the transgenic cell line

Transgenic *Bm*N cells carrying shRNA targeting the *i.e.*-1c of *Bm*NPV were tested for viral resistance with normal *Bm*N cells as a control. The expression level of *Bm*NPV implied the resistance of transgenic *Bm*N cell line to *Bm*NPV, which was analyzed by qPCR with *Bm*NPV specific primers according to *Bm*NPV sequence. As shown in Fig. 5, proliferation of virus went slowly in the first 24 h. The RNAi effects were appeared obviously 24 h post infection. The expression level of *Bm*NPV in the transgenic

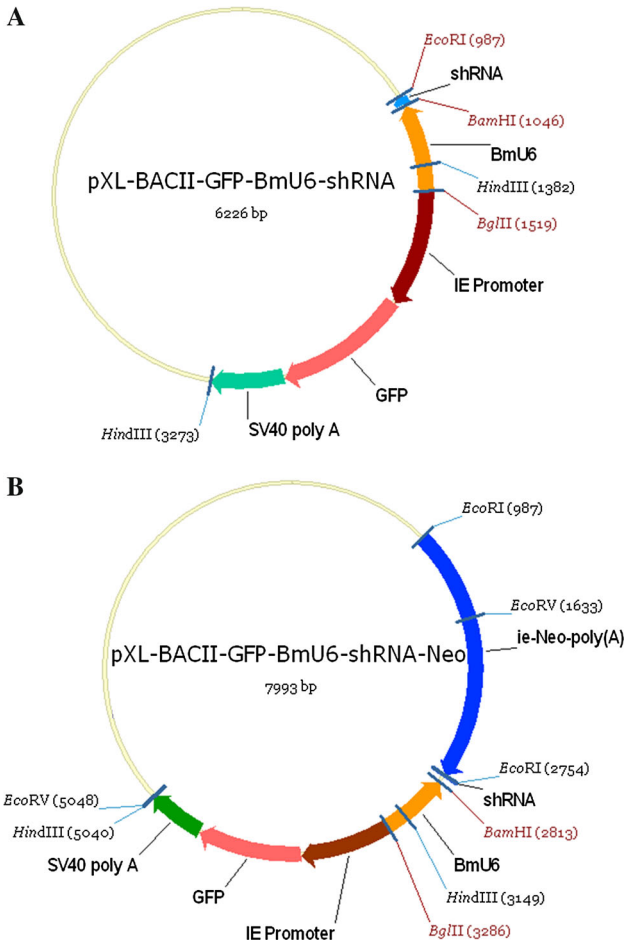

Fig. 1 Construction of recombinant transformation plasmids. **a** pXL-BACII-GFP-*Bm*U6-shRNA. The enhanced green fluorescent protein (EGFP) and *Bm*U6 genes were subcloned into pXL-BACII, and designated as pXL-BACII-GFP-*Bm*U6, and then the designed shRNA was subcloned in the *Bam*HI and *Eco*RI sites of pXL-BACII-GFP-*Bm*U6, and designated as pXL-BACII-GFP-*Bm*U6-shRNA. **b** pXL-BACII-GFP-*Bm*U6-shRNA-Neo. On above vector pXL-BACII-GFP-*Bm*U6-shRNA, the Neomycin resistant gene was inserted into the *Eco*RI–*Eco*RI sites

cells was significantly decreased with approximately 50 % less than the normal *Bm*N cells during 24–96 h post infection. Then the multiplication of *Bm*NPV kept smoothly until 96 h post infection not only in the control (56.23–64.03 %) but also the transgenic cell line (7.04–17.69 %). As the expression of *Bm*NPV increased to 80–90 % rapidly in normal *Bm*N cells from 96 h, the transgenic cells repressed *Bm*NPV approximate 50.06 % throughout after transfection. It displayed that the lysis of the infected normal *Bm*N cells whereas the transgenic cells did not.

Discussion

Currently, effective treatment for viral disease has been developed and put to practical use in silkworm, however,

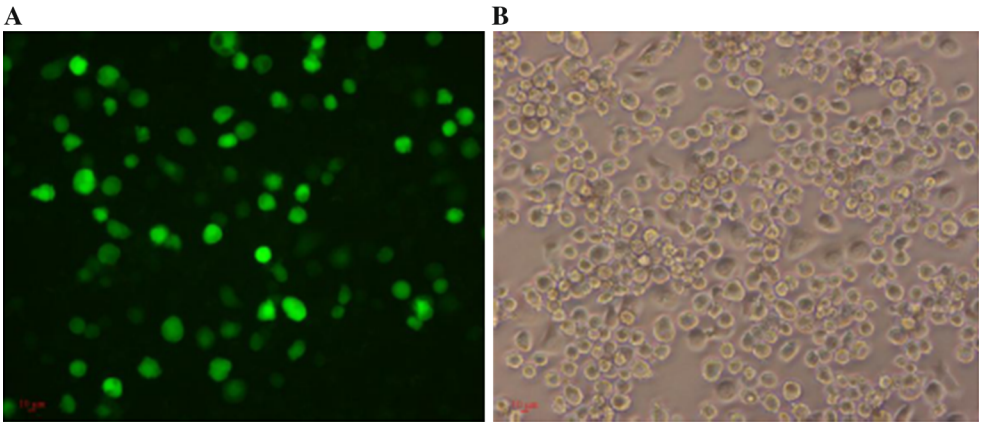

Fig. 2 Fluorescence observation of *Bm*N cells transfected with PXL-BACII-GFP-*Bm*U6-shRNAs. *Bm*N cells were transfected with transient expression vector PXL-BACII-GFP-*Bm*U6-shRNAs for 24 h. About 50–60 % of the *Bm*N cells were screened for the GFP expression when cells were transfected with vector PXL-BACII-GFP-*Bm*U6-shRNAs. **a** Transfected *Bm*N cells under inverted fluorescence microscope; **b** transfected *Bm*N cells under visible light

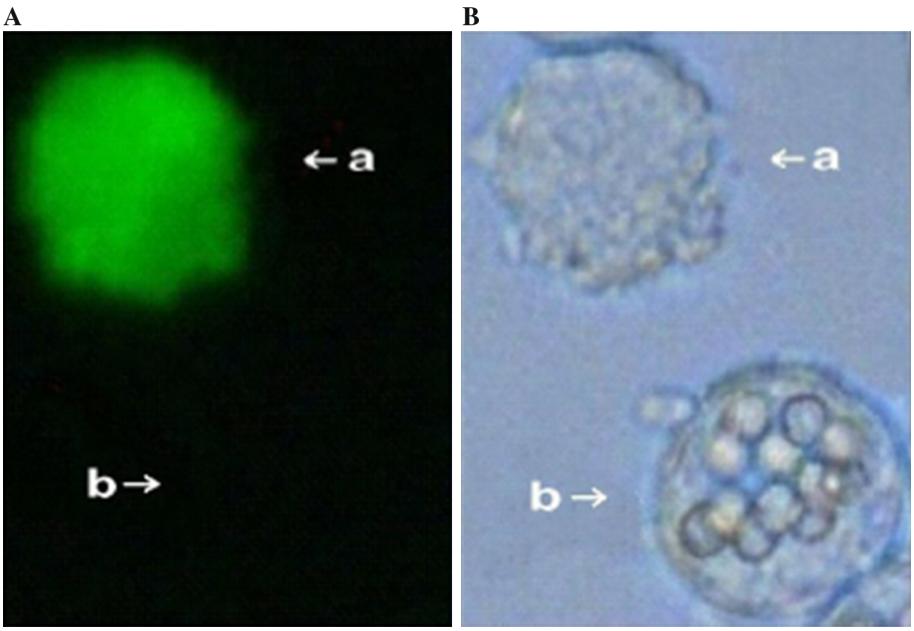

Fig. 3 Polyhedral envelope observed in GFP-negative *Bm*N cells. **a** *Bm*N cell was transfect with PXL-BACII-GFP-*Bm*U6-shRNAs plasmids which presented the *green* fluorescence under the inverted fluorescence microscope, and no virions were screened in this cell. **b** comparatively when the shRNA was not expressed in the *Bm*N cell, which no GFP was screened, the virions encapsulated within the polyhedral envelope were visible or released to the environment by cell lysis and dissolution. (Color figure online)

nucleopolyhedrosis disease caused by *Bm*NPV infection have not shown satisfactory results, and it is still harmful to the silkworm rearing and sericulture industry [7].

RNAi is a natural process by which dsRNA directs sequence-specific post-transcriptional gene silencing [40, 41]. Specific inhibition of endogenous or pathogen mRNA by RNAi can be triggered by introducing 21–23 nucleotide (nt) duplexes of RNA (siRNA) or by transcription of DNA precursor into the dsRNA homologous to target sequences [42, 43]. Therefore RNAi maybe provide a broad application in gene therapy for virus-related diseases.

In the past, researchers used to repress the viral DNA replication by dsRNA. However, the over-dose of dsRNA will lead to impaired of RNAi effectiveness. The high concentration not only raises the RNAi but also counteracts the effect of ADARs (RNA-dependent adenylate deaminase) [44, 45]. Excess dsRNA will cause the over-abundance of siRNA and disturb the combination of dsRNA and RNAi-specific enzymes, which will result in a toxic effect on cells. In this shRNA interference in transgenic silencing cell, the shRNA was cloned into *piggy*Bac transposon vector. It avoided the over-abundance of siRNA because its shRNA was transcripted in vivo accordingly.

Furthermore the chemical synthesized dsRNA usually be employed for transient gene silencing, which obviously is not appropriate for pathogens such as viruses. In order to solve these problems, we established a *piggy*Bac transposon-derived targeting shRNA system to keep long-term

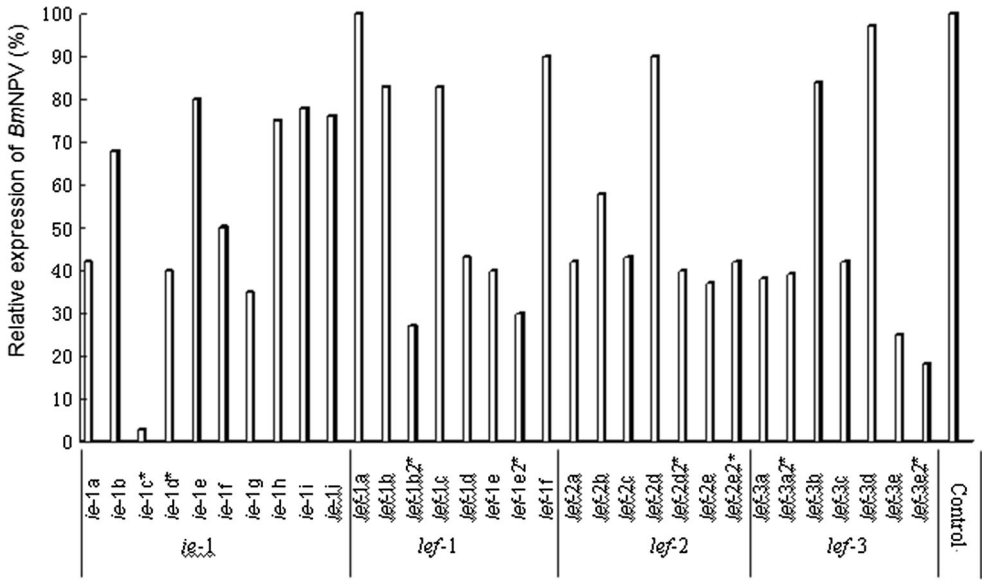

Fig. 4 Transient interference against *Bm*NPV after transfection of recombinant plasmid PXL-BACII-GFP-*Bm*U6-shRNAs. 96 h after challenge with *Bm*NPV, the relative expression of virus were investigated by qPCR analysis performed on the total RNA extract from the transfected *Bm*Ncells with *Bm*NPV specific primers according to *Bm*NPV sequence and *Bm*actinA3 specific primers as normalization. The relative expression of *Bm*NPV was used to estimate the shRNAs' interference efficiency for replication or multiplication of *Bm*NPV. All the experiments were performed in three independent biological replication and the reactions of each sample were carried out in triplicate

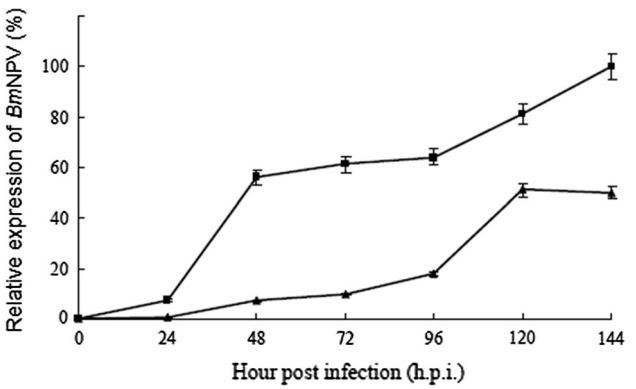

Fig. 5 Expression level of *Bm*NPV in transgenic *Bm*N cells post infection. The expression implied the *Bm*NPV resistance in transformed *Bm*N cell line, which was analyzed by qPCR. The curve indicates the relative expression level of *Bm*NPV in normal *Bm*N cells and transgenic cells infected with *Bm*NPV. *Filled square* normal *Bm*N cells (control); *filled triangle* transgenic cells. All the experiments were performed in three independent biological replication and the reactions of each sample were carried out in triplicate

gene silencing and stable concentration of siRNA. Although Kanginakudru et al. [6] and Isobe et al. [7] reported RNAi induces baculoviral resistance in transgenic silkworms, but this two were all depended on dsRNA interference.

We validated the best efficacy shRNA of *Bm*NPV resistance in both *Bm*N culture cells and silkworm larvae by targeting four of the essential genes of *Bm*NPV, *i.e.*-1, *lef*-1, *lef*-2 and *lef*-3. We found that most of these shRNAs can induce RNAi in silkworm cells, and can effectively inhibit the replication of *Bm*NPV. The results suggest the shRNA targeting *ie*1-c can inhibit the replication of *Bm*NPV the most effectively.

The feasibility of shRNA inhibiting *Bm*NPV replication may solve the practical problem in the silkworm industry. For this purpose, we designed a *piggy*Bac-based vector PXL-BACII-GFP-*Bm*U6-shRNA-Neo to trigger RNAi-mediated baculoviral repression in the transgenic *Bombyx mori* cell line. The inhibition of shRNA targeting *i.e.*-1c of *Bm*NPV in the transgenic cell line was tested. The approximate 50.06 % repression of *Bm*NPV replication was screened in the transgenic cells after PXL-BACII-GFP-*Bm*U6-shRNA-Neo transfection. The virus inhibition of transgenic *Bm*N cell line provided a basis for further construction of the antiviral silkworm.

In conclusion, the *piggy*Bac transposon-derived targeting shRNA interference is a promising way to inhibit the replication and multiplication of *Bm*NPV in cells and larvae. The highest activity was presented in the shRNA targeting *i.e.*-1c of *Bm*NPV, through/by which the inhibition rate reached 94.5 %. The established *Bm* cell line of *piggy*Bac transposon-derived targeting shRNA against *Bm*NPV presented efficacious suppression of virus proliferation. The shRNA antiviral model established in this study is useful in controlling silkworm diseases and applicable to the field of viral molecular biology.

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