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*These authors contributed equally to the work reported in this paper.

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Expression of anti-NbHK single-chain antibody in fusion with NSlmb enhances the resistance to *Nosema bombycis* in Sf9-III cells

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Abstract

Nosema bombycis is a destructive and specific intracellular parasite of silkworm, which is extremely harmful to the silkworm industry. *N. bombycis* is considered as a quarantine pathogen of sericulture because of its long incubation period and horizontal and vertical transmission. Herein, two single-chain antibodies targeting *N. bombycis* hexokinase (NbHK) were cloned and expressed in fusion with the N-terminal of Slmb (a *Drosophila melanogaster* FBP), which contains the F-box domain. Western blotting demonstrated that Sf9-III cells expressed NSlmb–scFv-7A and NSlmb–scFv-6H, which recognized native NbHK. Subsequently, the NbHK was degraded by host ubiquitination system. When challenged with *N. bombycis*, the transfected Sf9-III cells exhibited better resistance relative to the controls, demonstrating that NbHK is a prospective target for parasite controls and this approach represents a potential solution for constructing *N. bombycis*-resistant *Bombyx mori*.

Introduction

Microsporidia is a unique obligate intracellular pathogen. Microsporidia hosts are various and can infect invertebrates such as nematodes and insects, as well as vertebrates such as fish, mammals and even humans (Han and Weiss, 2017; Pan *et al.*, 2018). *Nosema bombycis*, the pathogen of *Bombyx mori*, is the first identified microsporidia and causes pébrine disease in silkworms. However, more than 100 years after *N. bombycis* was identified, sericulture production has been plagued by pébrine. Currently, the prevention and control of pébrine still adopt the conventional method of strict disinfection of parasite and there is an urgent need for novel approaches (Smyk *et al.*, 1952; Ishihara, 1969; Wang *et al.*, 2007).

Hexokinase is an essential enzyme in the energy metabolism pathway of eukaryotes. Hexokinase of microsporidia was first reported in *Nematocida parisii* (Cuomo *et al.*, 2012), and then researchers identified hexokinase in the secretory proteomics of *N. Parisii*, and hypothesized that it might be a secretory protein. Subsequently, it was found that the hexokinase of *Paranosema locustae* was secreted into the nucleus of host cells. In *Trachipleistophora hominis*, it was reported that hexokinase was localized on the surface of *T. hominis* and promoted the formation and aggregation of host ATP on its surface (Ferguson and Lucocq, 2019). These results suggest that the glycolytic pathway may not only act as an adenosine triphosphate (ATP) production pathway in microsporidia, but also play a vital role in host modulation processes. In our previous study, it was found that *N. bombycis* hexokinase (NbHK) was a secretory protein located in the cytoplasm and nucleus of host cells. In the intracellular proliferation stage, the down-regulation of NbHK expression inhibited the proliferation of *N. bombycis* (Huang *et al.*, 2018*b*), which means that NbHK participates in the energy metabolism of the host and becomes a potential target for the development of *N. bombycis*-resistant silkworm materials.

The single-chain antibody fragment (scFv) targeting pathogenically important proteins can inhibit pathogen infection to the host. The expression of anti-malarial parasite scFv in the salivary glands of *Anopheles mosquitoes* can effectively inhibit the growth of plasmodium in mosquitoes (Sumitani *et al.*, 2013), and the expression of a scFv against the plasmodium in *Metarhizium anisopliae* can both kill *A. mosquitoes* and block the transmission of malaria (Fang *et al.*, 2011). In addition, studies have shown that the scFv targeting *N. bombycis* spore-wall protein can significantly inhibit the infection and proliferation of *N. bombycis* in silkworms (Huang *et al.*, 2018*a*).

Ubiquitin pathway is a key component of selective protein degradation in eukaryotes and it participates in modulating the cell cycle, growth, apoptosis, metastasis, differentiation, gene

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expression, transcriptional modulation, signal transduction, injury repair, inflammation and immunity, and almost all life activities (Nandi *et al.*, 2006; Grumati and Dikic, 2018). The result of protein ubiquitination is that the labeled protein is broken down by proteases into smaller polypeptides, amino acids, and ubiquitin that can be reused (Swatek and Komander, 2016). E3 ubiquitin ligase complex binds to the substrate during ubiquitination of eukaryotes, which is composed of polypeptides – Skp1, Cullin1/3, and Roc1/Rbx1 – and a fourth variable adapter, the F-box protein. The F-box protein determines the specificity of the substrate (Dui *et al.*, 2012). Some research findings illustrate that, for many proteins, the first ubiquitin moiety is fused linearly to the α-NH_2 group of the N-terminal residue (Ciechanover and Ben-Saadon, 2004; Ciechanover, 2005). Slmb is an F-box protein of *Drosophila*, which performs various functions in development and cell physiology, and its F-box domain is located at the N-terminal of the protein. The fusion of N-terminal F-box domain of Slmb with single-chain antibody fragment can specifically degrade the target protein (Caussinus *et al.*, 2011).

In this study, we cloned two single-chain antibodies (scFv-7A and scFv-6H of NbHK), which were subsequently used to fuse with the N-terminal of Slmb (a *Drosophila melanogaster* FBP) containing an F-box domain (hereafter called NSlmb–scFv) for expression in insect cells. Further studies showed that the expression of NSlmb–scFv significantly inhibited the growth of *N. bombycis* in insect cells.

Materials and methods

Preparation of N. bombycis *and cell cultivation*

We acquired *N. bombycis* CQ1 mature spores (CVCC no. 102059) from the China Veterinary Culture Collection Center. The mature spores were fed on the fourth-instar silkworm for expanded culture, and the spores were separated and purified by percoll density gradient centrifugation (40 min, 21,000 ***g***) at pupal stage for further use (He *et al.*, 2020). Sf9-III cells were cultured in Sf-900™ III SFM (Thermo Fisher Scientific, Santa Clara, CA, USA) at 28°C.

Monoclonal antibody preparation

In our previous studies, the constructed plasmid (pET-28-NbHK) expressing recombinant NbHK (rNbHK) proteins was transformed into *Escherichia coli* Rosetta that were then 0.5 mM isopropyl-β-D-thiogalactopyranoside in Lysogeny broth (LB) medium was employed to trigger recombinant protein expression (4 h) at 37°C. Nickel chelating affinity chromatography (Roche, Basel, Switzerland) was employed to purify the rNbHK protein. The Laboratory Animals Ethics Review Committee of Southwest University (Chongqing, China) approved the mice experiments (AERCSWU2017-7). Female BALB/c mice (6 to 8-weeks-old) were subcutaneously administered with purified rNbHK (100 µg per mouse) mixed with Freund's complete/incomplete adjuvant (1:1; Sigma, St. Louis, MO, USA) four times. The interval of injection was 7 days. The produced monoclonal antibodies (mAbs) were harvested and assessed as documented previously (Huang *et al.*, 2018*a*). The mouse monoclonal antibody isotyping kit (Roche, Switzerland) was employed to assay the subtypes of mAb immunoglobulin G (IgG) as described by the manufacturer (Zheng *et al.*, 2021).

scFv sequence modifications

On the basis of the sequence assessment results, we designed primers for overlapping polymerase chain reaction (PCR). The 7A cloning of heavy-chain variable region was done with primer H7A-F: 5′-CTTCCGGAATTCSARGTNMAGCTGSAGSAGTCWGG-3′ and primer scVH7A-R: 5′-GGAAGATCTAGGGGCCAGTGGATAGACTGATGG-3′, whereas cloning of the light-chain variable region was performed with primer scVL7A-F: 5′-GAYATTGTGMTSACMCARWCTMCA-3′ and primer scVL7A-R: 5′-GGATACAGTTGGTGCAGCATC-3′. Primer H7A-F: 5′-ATGGACATTGTGCTCACCC-3′ and primer scVL7A-R1: 5′-GGAAGATCTCTTGACCAG-3′ were employed to connect the two variable regions via overlapping PCR, and with an insertion of a short polypeptide linker $(G4S)_3$ between the two variable regions at this step. Similarly, cloning of the 6H heavy-chain variable region was done with primer H6H-F: 5-CTTCCGGAATTCSARGTNMAGCTGSAGSAGTCWGG-3 and primer scVH6H-R: 5′-GGAAGATCTCTTGACCAGGCATCCTAGAGTCA-3′, whereas cloning of the light-chain variable region was performed with primer scVL6H-F: 5′-GAYATTGTGMTSACMCARWCTMCA-3′ and primer scVL6H-R: 5′-GGATACAGTTGGTGCAGCATC-3′. Connection of these two variable regions was accomplished via overlapping PCR with primer H6H-F: 5′-ATGGACATTGTGCTCACCC-3′ and primer scVL6H-R1: 5′-GGAAGATCTAGGGGCCAG-3′. All primers in this study were synthesized by Shanghai Sangon Co, Ltd.

The cloned single-chain antibody sequences named scFv-7A and scFv-6H were individually inserted to the expression vector phIA-[IE2-NSlmb-V5-6 × His-PA], which was constructed in our laboratory based on pSL1180 plasmid for subsequent expression in Sf9 cells (fig. 1a).

Protein preparation

The plasmid constructs encoding NSlmb–scFv-7A and NSlmb–scFv-6H single-chain antibodies were individually transfected into Sf9 cells and at 48 h post transfection, glass-bead break methods were employed to prepare total proteins of the transfected cells as documented previously (Li *et al.*, 2012). Briefly, RIPA lysis buffer (Beyotime, Shanghai, China) enriched with a protease inhibitor (phenylmethylsulfonyl fluoride) was employed to lyse the mixture of the harvested cells and 0.4 g glass beads (212–300 µm), and then a Bioprep-24 homogenizer (ALLSHENG, Hangzhou, China) was employed to crush the lysate for 5 min at 4°C. The treated samples were spun at 12,000 ***g*** for 5 min and the supernatant was aliquoted to a fresh tube. These samples were used to detect the expression of NSlmb–scFv.

In order to detect the specificity of NSlmb–scFv, the total protein of the Sf9 cell transfected with the recombinant plasmid was extracted using phosphate-buffered saline (PBS), so as to ensure the activity of the expressed NSlmb–scFvs.

The transfected cells were infected by *N. bombycis* mature spores (spore:cell = 10:1) at 48 h post transfection and cultured for additional 96 h. The total proteins from infected cells as well as untreated Sf9 control cells were prepared with the same method as descripted for the transfected cells. In order to detect the degradation of NbHK, the total protein of 96 h after mature spore infection was extracted. The transfected NSlmb–scFv-7A and NSlmb–scFv-6H recombinant plasmid Sf9 cells were used as the experimental group, and the transfected blank plasmid Sf9 cells were used as the control group.

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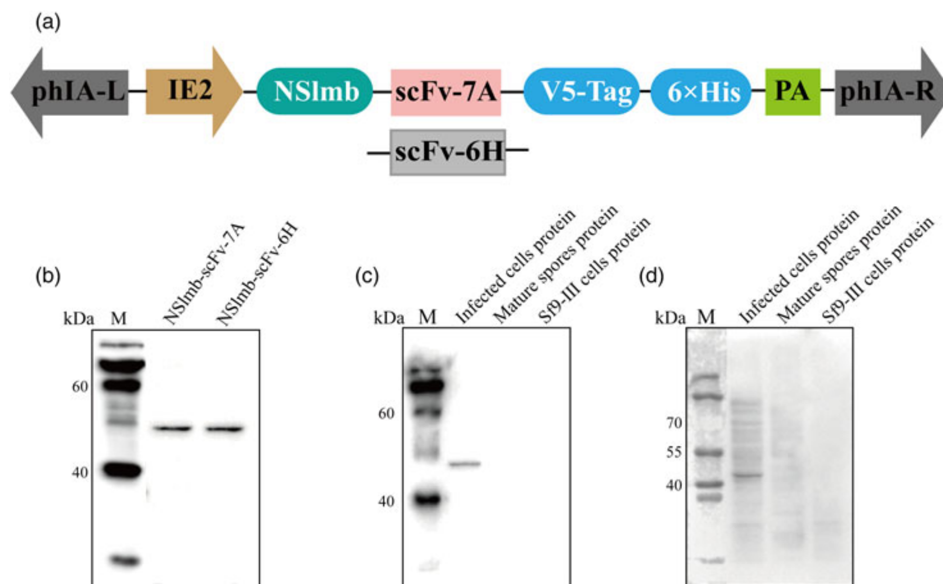

Figure 1. Expression of fusion protein and analysis of NSlmb–scFv specificity. (a) Schematic diagram of the expression plasmids (NSlmb–scFv-7A or NSlmb–scFv-6H). (b) Detection of NSlmb–scFv expression in Sf9 cells. Total proteins from scFv-transfected Sf9-III cells were extracted and analyzed by western blotting with the V5 mAb being used as the primary antibody. (c) Verification of NSlmb–scFv-7A specificity. (d) Verification of NSlmb–scFv-6H specificity. Proteins extracted from the infected Sf9-III cells, mature spores, and healthy Sf9-III cells were subjected to western blot using NSlmb–scFv against NbHK. M: protein marker (Transgene, Shanghai, China).

Western blot analysis

Total proteins prepared from the Sf9 cells were fractionated on an SDS-PAGE gel. After that, the proteins were blotted onto poly (vinylidene fluoride) membranes (Roche, Switzerland). Subsequently, 5% dry milk in TBST (150 mM NaCl, 20 mM Tris-HCl, 0.05% Tween-20) was employed to block the membranes. Next, the membranes were inoculated with the anti-V5 mouse antibody (Sigma, Saint Louis, USA; 1:3000) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Bio-Rad, Richmond, California, USA; 1:6000) sequentially with washing in between. Afterward, ECL Plus Western Blotting Detection Reagents (Bio-Rad, Richmond, California, USA) were employed to detect the bound antibodies. Specificity of NSlmb–scFv was assessed by the same western blot protocol except the NSlmb–scFv-7A or NSlmb–scFv-6H antibody, which was isolated from the transfected Sf9 cells, was used as the primary antibody.

To test the specificity of NSlmb–scFv, total cell proteins extracted with PBS, including the active NSlmb–scFv-7A or NSlmb–scFv-6H antibodies, were incubated with total spore proteins as primary antibodies. Then the V5 antibody and HRP-conjugated goat anti-mouse IgG were used to detect its mutual recognition with native NbHK protein.

In order to evaluate whether the expression of NSlmb–scFv antibody can effectively lead to the degradation of NbHK, we used the polyclonal antibodies against NbHK to detect its expression, and the β-tubulin of Sf9 cells as internal reference (Huang *et al.*, 2018*b*).

Real-time quantitative PCR (RT-qPCR)

A DNA extraction kit (Omega, Norcross, GA, USA) was employed to isolate gDNAs of the control Sf9 cells or the cells transfected with single-chain antibodies or the cells transfected and then infected by mature spores. *Nbβ-tubulin gene* copy number was employed as an internal reference for the count of the *N. bombycis*. The gDNAs were analyzed by qPCR. The sequences of the primers were: *Nbβ-tubulin*-qF 5′-AGAACCAGGAACAA TGGACG-3′ and *Nbβ-tubulin*-qR 5′-AGCCCAATTATTACCA GCACC-3′ and the real-time PCR reagent from Novoprotein Scientific Inc., Shanghai, China. The standard template was documented in previous investigation (Huang *et al.*, 2018*a*). The standard curve covered six orders of magnitude (1.3×10^2–10^7). The data were presented after normalization.

Results

Development of mAbs against NbHK

Purified rNbHK served as antigens to immunize BALB/c mice and screen hybridomas. We harvested splenocytes from the immunized BALB/c mice and polyethylene glycol 1500 was employed to fuse them with SP2/0 cells. The 7A subtypes were assessed using 7A hybridoma serum-free medium and the test strip confirmed that the subtype of 7A was IgG2b-κ. In the same method, 6H hybridoma serum-free medium was employed to detect the subtype of 6H and its subtype was confirmed as IgG2a-κ.

Construction of scFv and NSlmb–scFv-7A and NSlmb–scFv-6H plasmids

Degenerate primers selected according to 7A subtypes were employed to amplify the scFv-7A light-chain along with heavy-chain variable regions using 7A hybridoma cDNA as the template (fig. 2a, b). After that, we inserted these two amplicons into pMD19-T vector and subsequently sequenced using M13-F/R primers. The sequencing analysis confirmed that the incorporated variable region of 7A was cloned into the vector. Finally, on the basis of the sequencing data, we designed the primers of the subsequent overlap PCR, which was employed to connect the light-chain to the heavy-chain with $(G4S)_3$ linkers (fig. 3a). The same steps were used to get scFv-6H (figs 2c, d and 3b).

Expression and specificity of NSlmb–scFv

The NSlmb–scFv recombinant plasmids were transfected into Sf9 cells. Western blot analysis showed that the fusion proteins NSlmb–scFv-7A and NSlmb–scFv-6H were successfully expressed

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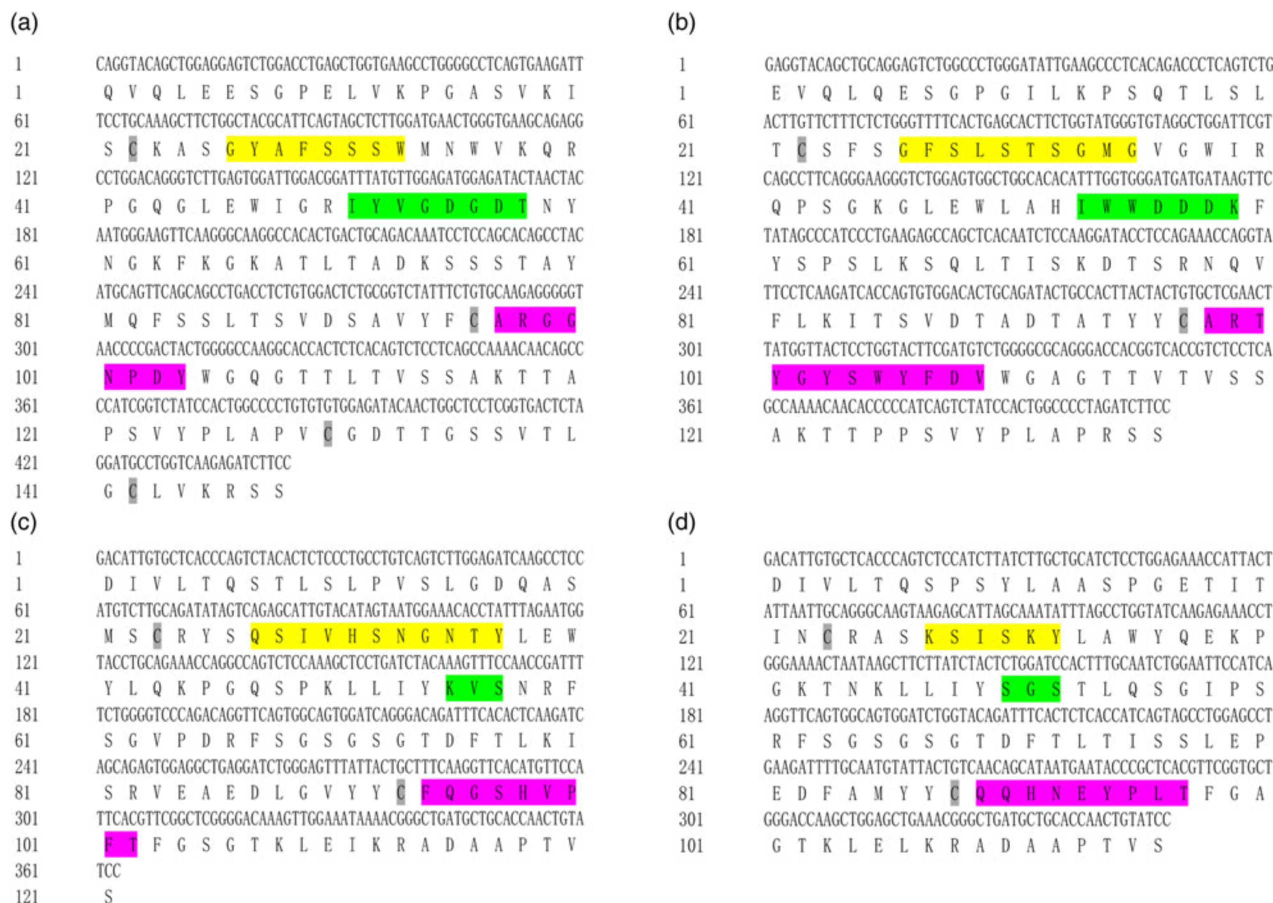

Figure 2. Clone of scFv-7A and scFv-6H heavy-chain, light-chain variable regions. (a) scFv-7A light-chain variable region sequence. (b) scFv-7A heavy-chain variable region sequence. (c) scFv-6H light-chain variable region sequence. (d) scFv-6H heavy-chain variable region sequence. Gray, cysteine; yellow, complementarity-determining region (CDR)1; green, CDR2; pink, CDR3.

based on the examination by western blot using V5 mAb as primary antibody (fig. 1b).

NbHK protein is secreted into host cells to hijack energy from host and typically cannot be detected in mature spore cell lysates (Huang *et al*., 2018*b*). In this part, western blotting data exhibited a unique positive band of NSlmb–scFv-7A and NSlmb–scFv-6H in the infected cell proteins. This band was approximately 50 kDa, which is equal to the molecular weight of the native form of NbHK (fig. 1c, d). The results indicated that the NSlmb–scFv-7A and NSlmb–scFv-6H could recognize native NbHK in infected cells protein.

Expression of NSlmb–scFv inhibited N. bombycis*' proliferation*

To access the functionality of expressed NSlmb–scFv proteins in Sf9 cells, the transfected cells were infected by mature spores. At 96 h post inoculation the cell lysates were prepared. First, we evaluated whether the expression of NSlmb–scFv antibodies can effectively result in NbHK degradation. For this purpose, the cell lysates prepared from the infected cells as well as the control cells were subjected to western blot using the polyclonal antibodies of NbHK as primary antibody. The result demonstrated that the protein expression level of NbHK was remarkably lowered in contrast to the control group, indicating that NbHK was partially degraded in the host (fig. 4a).

To examine whether the expression of NSlmb–scFv antibodies can repress the proliferation of *N. bombycis* in insect cells, we quantified parasite loads based on the relative copy numbers of *Nbβ-tubulin* genes by real-time PCR (Huang *et al*., 2018*a*). As shown in fig. 4b, the parasite loads were significantly lowered in the cells transfected with NSlmb–scFv-7A or NSlmb–scFv-6H at 96 h post infection (fig. 4b), suggesting that NSlmb–scFvs can effectively repress the proliferation of *N. bombycis* in host cells.

Discussion

Energy metabolism is the most basic feature of the living body. By absorbing nutrients from the outside world, the organism carries out a series of complex decomposition and transformation in the body, releasing the energy contained in the nutrients to maintain life activities (Li *et al*., 2012; He *et al*., 2020). As the eukaryotes with the smallest genome, microsporidia have lost several metabolic pathways, like tricarboxylic acid cycle, oxidative phosphorylation, and fatty acid β-oxidation, so does *N. bombycis* (Katinka *et al*., 2001; Pan *et al*., 2013). Metabolism in the intracellular stages exhibit well adaption of microsporidia to parasitism. According to genome research, microsporidia have lost many genes compared with fungal relatives. So it has to adopt sophisticated strategies to survive in the host with the least payment. They

(a)

```
1    ATGGACATTGTGCTCACCCAGTCTACACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCC
1     M  D  I  V  L  T  Q  S  T  L  S  L  P  V  S  L  G  D  Q  A
61   TCCATGTCTTGCAGATATAGTCAGAGCATTGTACATAGTAATGGAAACACCTATTTAGAA
21    S  M  S  C  R  Y  S  Q  S  I  V  H  S  N  G  N  T  Y  L  E
121  TGGTACCTGCAGAAACCAGGCCAGTCTCCAAAGCTCCTGATCTACAAAGTTTCCAACCGA
41    W  Y  L  Q  K  P  G  Q  S  P  K  L  L  I  Y  K  V  S  N  R
181  TTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAG
61    F  S  G  V  P  D  R  F  S  G  S  G  S  G  T  D  F  T  L  K
241  ATCAGCAGAGTGGAGGCTGAGGATCTGGGAGTTTATTACTGCTTTCAAGGTTCACATGTT
81    I  S  R  V  E  A  E  D  L  G  V  Y  Y  C  F  Q  G  S  H  V
301  CCATTCACGTTCGGCTCGGGGACAAAGTTGGAAATAAAACGGGCTGATGCTGCACCAACT
101   P  F  T  F  G  S  G  T  K  L  E  I  K  R  A  D  A  A  P  T
361  GTATCCGGTGGTGGTGGAGCGGTGGTGGCGGCTCTGGCGGCGGCGGATCACAGGTACAG
121   V  S  G  G  G  G  S  G  G  G  G  S  G  G  G  G  S  Q  V  Q
421  CTGGAGGAGTCTGGACCTGAGCTGGTGAAGCCTGGGGCCTCAGTGAAGATTTCCTGCAAA
141   L  E  E  S  G  P  E  L  V  K  P  G  A  S  V  K  I  S  C  K
481  GCTTCTGGCTACGCATTCAGTAGCTCTTGGATGAACTGGGTGAAGCAGAGGCCTGGACAG
161   A  S  G  Y  A  F  S  S  S  W  M  N  W  V  K  Q  R  P  G  Q
541  GGTCTTGAGTGGATTGGACGGATTTATGTTGGAGATGGAGATACTAACTACAATGGGAAG
181   G  L  E  W  I  G  R  I  Y  V  G  D  G  D  T  N  Y  N  G  K
601  TTCAAGGGCAAGGCCACACTGACTGCAGACAAATCCTCCAGCACAGCCTACATGCAGTTC
201   F  K  G  K  A  T  L  T  A  D  K  S  S  S  T  A  Y  M  Q  F
661  AGCAGCCTGACCTCTGTGGACTCTGCGGTCTATTTCTGTGCAAGAGGGGGTAACCCCGAC
221   S  S  L  T  S  V  D  S  A  V  Y  F  C  A  R  G  G  N  P  D
721  TACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCAGCCAAAACAACAGCCCCATCGGTC
241   Y  W  G  Q  G  T  T  L  T  V  S  S  A  K  T  T  A  P  S  V
781  TATCCACTGGCCCCTGTGTGTGGAGATACAACTGGCTCCTCGGTGACTCTAGGATGCCTG
261   Y  P  L  A  P  V  C  G  D  T  T  G  S  S  V  T  L  G  C  L
841  GTCAAGAGATCTTCC
281   V  K  R  S  S
```

(b)

```
1    ATGGACATTGTGCTCACCCAGTCTCCATCTTATCTTGCTGCATCTCCTGGAGAAACCATT
1     M  D  I  V  L  T  Q  S  P  S  Y  L  A  A  S  P  G  E  T  I
61   ACTATTAATTGCAGGGCAAGTAAGAGCATTAGCAAATATTTAGCCTGGTATCAAGAGAAA
21    T  I  N  C  R  A  S  K  S  I  S  K  Y  L  A  W  Y  Q  E  K
121  CCTGGGAAAACTAATAAGCTTCTTATCTACTCTGGATCCACTTTGCAATCTGGAATTCCA
41    P  G  K  T  N  K  L  L  I  Y  S  G  S  T  L  Q  S  G  I  P
181  TCAAGGTTCAGTGGCAGTGGATCTGGTACAGATTTCACTCTCACCATCAGTAGCCTGGAG
61    S  R  F  S  G  S  G  S  G  T  D  F  T  L  T  I  S  S  L  E
241  CCTGAAGATTTTGCAATGTATTACTGTCAACAGCATAATGAATACCCGCTCACGTTCGGT
81    P  E  D  F  A  M  Y  Y  C  Q  Q  H  N  E  Y  P  L  T  F  G
301  GCTGGGACCAAGCTGGAGCTGAAACGGGCTGATGCTGCACCAACTGTATCCGGTGGTGGT
101   A  G  T  K  L  E  L  K  R  A  D  A  A  P  T  V  S  G  G  G
361  GGGAGCGGTGGTGGCGGCTCTGGCGGCGGCGGATCAGAGGTACAGCTGCAGGAGTCTGGC
121   G  S  G  G  G  G  S  G  G  G  G  S  E  V  Q  L  Q  E  S  G
421  CCTGGGATATTGAAGCCCTCACAGACCCTCAGTCTGACTTGTTCTTTCTCTGGGTTTTCA
141   P  G  I  L  K  P  S  Q  T  L  S  L  T  C  S  F  S  G  F  S
481  CTGAGCACTTCTGGTATGGGTGTAGGCTGGATTCGTCAGCCTTCAGGGAAGGGTCTGGAG
161   L  S  T  S  G  M  G  V  G  W  I  R  Q  P  S  G  K  G  L  E
541  TGGCTGGCACACATTTGGTGGGATGATGATAAGTTCTATAGCCCATCCCTGAAGAGCCAG
181   W  L  A  H  I  W  W  D  D  D  K  F  Y  S  P  S  L  K  S  Q
601  CTCACAATCTCCAAGGATACCTCCAGAAACCAGGTATTCCTCAAGATCACCAGTGTGGAC
201   L  T  I  S  K  D  T  S  R  N  Q  V  F  L  K  I  T  S  V  D
661  ACTGCAGATACTGCCACTTACTACTGTGCTCGAACTTATGGTTACTCCTGGTACTTCGAT
221   T  A  D  T  A  T  Y  Y  C  A  R  T  Y  G  Y  S  W  Y  F  D
721  GTCTGGGGCGCAGGGACCACGGTCACCGTCTCCTCAGCCAAAACAACACCCCCATCAGTC
241   V  W  G  A  G  T  T  V  T  V  S  S  A  K  T  T  P  P  S  V
781  TATCCACTGGCCCCTAGATCTTCC
261   Y  P  L  A  P  R  S  S
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Figure 3. Construction of scFv-7A and scFv-6H. (a) Analysis of constructed scFv-7A sequence. (b) Analysis of constructed scFv-6H sequence. Yellow, light-chain variable region sequence; gray, $(G4S)_3$ linker; green, heavy-chain variable region sequence.

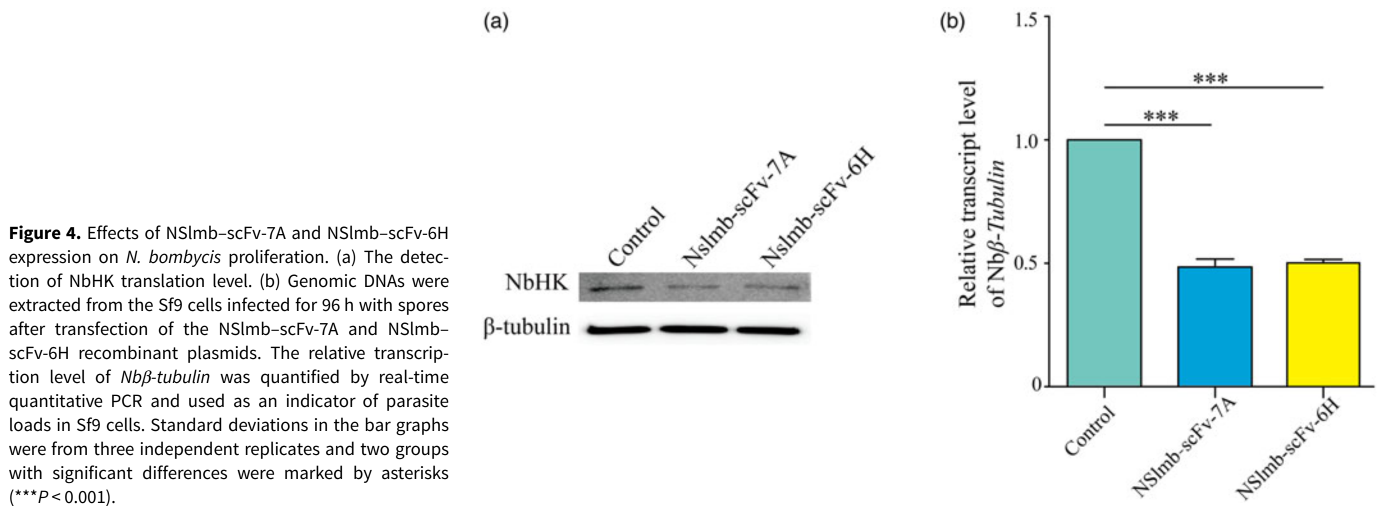

Figure 4. Effects of NSlmb–scFv-7A and NSlmb–scFv-6H expression on *N. bombycis* proliferation. (a) The detection of NbHK translation level. (b) Genomic DNAs were extracted from the Sf9 cells infected for 96 h with spores after transfection of the NSlmb–scFv-7A and NSlmb–scFv-6H recombinant plasmids. The relative transcription level of *Nbβ-tubulin* was quantified by real-time quantitative PCR and used as an indicator of parasite loads in Sf9 cells. Standard deviations in the bar graphs were from three independent replicates and two groups with significant differences were marked by asterisks (***$P < 0.001$).

can directly bind with host mitochondria, cluster ATP-delivering mitochondrial porin voltage-dependent anion channel (VDAC) and steal energy using plasma membrane-located nucleotide transporters (Hacker *et al.*, 2014; Heinz *et al.*, 2014; Dean *et al.*, 2016; Han *et al.*, 2019). Molecules were secreted to regulate the host metabolism resulting in more rewards. Hexokinase of *N. bombycis* can be secreted to the cytoplasm and nucleus of host cell during intracellular stages (Huang *et al.*, 2018*b*). Microsporidia do not have typical mitochondria, but instead only have a highly reduced mitochondrial structure, called mitosome (Shiflett and Johnson, 2010), suggesting that microsporidia may not have a complete oxidative phosphorylation pathway. The

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microsporidia genome is so simplified that it lacks many of the genes necessary for energy metabolism (Weidner *et al*., 1999; Williams *et al*., 2002; Embley and Martin, 2006). However, it is interesting that hexokinase is retained in all the species of microsporidia investigated, indicating its importance in the evolutionary process (Nakjang *et al*., 2013).

Expression of single-chain antibodies is a promising approach of breeding *N. bombycis*-resistant silkworms, because single-chain antibodies can directly neutralize proteins of intracellular pathogens. In Anopheles, transposon-mediated transformation was employed in generating m2A10, m1C3, and m4B7 single-chain antibodies, and the transgenic mosquitoes expressing the scFv gene had remarkably lower infection levels of *Plasmodium falciparum* (Isaacs *et al*., 2011, 2012). Based on successful attempts in *Plasmodium*, our lab also tried to use these methods to antagonize *N. bombycis*. We generated mAb scFv-G4 targeting SWP12, the first identified Bin/Amphiphysin/Rvs domain-containing protein which may function in membrane structure formation (Chen *et al*., 2013; Huang *et al*., 2018*a*). *In vitro* assay suggested that, the transgenic Sf9-III cell line expressing scFv-G4 showed better resistance relative to the controls when infected by *N. bombycis* (Chen *et al*., 2013). As single-chain antibodies directly affect protein function of *N. bombycis*, this strategy will play important role in breeding of resistant strains.

Ubiquitin degradation mechanism in eukaryotes can degrade some waste proteins and foreign proteins *in vivo* (Ciechanover and Ben-Saadon, 2004; Nandi *et al*., 2006), but this degradation mechanism does not have high specificity. Recent studies have found that the nanobody in fusion with the F-box domain of E3 ligase in the ubiquitination degradation system can lead to the degradation of the recognized target protein (Zhang *et al*., 2003; Wang *et al*., 2017). Herein, two single-chain antibodies targeting NbHK were developed and were later used to fuse with NSlmb to direct specific degradation of NbHK proteins in insect cells. When the insect cells expressing such antibodies were infected by *N. bombycis*, the secreted NbHK proteins derived from the parasites were partially degraded by the expressed fusion antibody and therefore hinder parasite to hijack energy from the host cells, resulting in proliferation inhibition of *N. bombycis*.

Conclusions

In the present study, we constructed an NbHK single-chain antibody-guided ubiquitination system, which can specifically target NbHK protein produced by *N. bombycis* during infection and degrade it. It was also confirmed that this method could significantly inhibit the proliferation of *N. bombycis* in the host. Our research represents a novel strategy of creating *N. bombycis* resistant silkworm strains when combined with additional genetic manipulation techniques.

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