Antiviral Research 98 (2013) 171–173

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Short Communication

A transgenic animal with antiviral properties that might inhibit multiple stages of infection

Liang Jiang [a,1], Ping Zhao [a,1], Tingcai Cheng [a], Qiang Sun [a,b], Zhengwen Peng [a], Yinghui Dang [a], Xiangwei Wu [a], Genhong Wang [a], Shengkai Jin [a], Ping Lin [a], Qingyou Xia [a,b,*]

[a] State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing 400715, PR China
[b] College of Biotechnology, Southwest University, Chongqing 400715, PR China

ARTICLE INFO

Article history:
Received 6 November 2012
Revised 15 February 2013
Accepted 22 February 2013
Available online 4 March 2013

Keywords:
Antivirus
BmNPV
RNAi
Overexpression
Silkworm
Transgenic

ABSTRACT

Bombyx mori nucleopolyhedrovirus (BmNPV) is the primary pathogen of silkworms, causing severe economic losses in sericulture. To create antiviral silkworm strains, we constructed a transgenic vector in which the dsRNA for five tandem BmNPV genes was controlled by the BmNPV hr3 enhancer and IE1 promoter. The antivirus gene *Bmlipase-1* was driven by *B. mori* midgut-specific promoter P2. Transgenic strains (SW-H) were generated via embryo microinjection using the practical silkworm strain SW. After infection with a high dose of BmNPV, the survival rates of SW-H and non-transgenic SW were 64% and 13%, respectively. SW-H could be the first transgenic animal that is highly antiviral and that might inhibit the virus at multiple stages of infection.

Virus infection is a severe threat to plants, animals, and humans, so antiviral research occurs worldwide. We use *Bombyx mori*, a common lepidopteran model (Xia et al., 2009, 2004), for antiviral studies. Silkworm is an important economic insect used in the silk industry. In 2010, the Chinese industrial output of cocoons was 19.11 billion Yuan and the silk output was 195.92 billion Yuan. However, silkworm diseases can cause serious losses of almost 20% of the cocoons each year.

B. mori nucleopolyhedrovirus (BmNPV) is the primary pathogen of silkworms. BmNPV is enveloped with a circular double-strand DNA genome (Kondo and Maeda, 1991). For many years, silkworm breeders have attempted to generate antiviral strains. Traditional breeding methods have several limitations however, so far, no success case has been reported. Transgenic technology could help to generate antiviral species. Previous reports demonstrated that overexpression of antiviral genes (Jiang et al., 2012a,b) or silence of viral genes (Jiang et al., 2012c; Kanginakudru et al., 2007) by transgenic technology can enhance the resistance of silkworm to viruses. In the study presented here, we optimized and integrated the two methods that overexpression of antiviral gene and RNAi of viral genes, and created a new transgenic silkworm strain (SW-H1) that is strongly anti-BmNPV.

Endogenous Bmlipase-1 is a midgut-specific protein with antiviral function in the gut juice of silkworm larvae (Ponnuvel et al., 2003). Overexpression of *Bmlipase-1* under the control of BmNPV IE1 promoter (IE1P) results in activity in almost all tissues that can enhance the viral resistance of silkworms (Jiang et al., 2012b). As an improvement, we cloned the midgut-specific P2 promoter (GenBank accession number KC573068) with high activity in the silkworm (Jiang et al., 2013) to drive *Bmlipase-1* in pb-HPFL (Fig. 1). Silencing the BmNPV gene, which is essential for the virus, inhibited viral proliferation. IE1P combined with BmNPV hr3 enhancer was an ideal promoter for RNAi (Jiang et al., 2012c). Previous studies suggested that targeting multiple pathogen genes by RNAi is a valid strategy to improve therapeutic effects (Ramirez-Carvajal and Long, 2012; Shah and Schaffer, 2011). In the study presented here, we generated a synthetic DNA sequence named FS (Sup. 1) that contained fragments of *i.e.-1*, *gp64*, *lef-1*, *lef-2*, and *dnapol*, which are BmNPV essential genes (Gomi et al., 1999; Schultz and Friesen, 2009; Zhou and Blissard, 2008). The dsRNA of the five tandem genes was controlled by the hr3 enhancer and IE1P in pb-HPFL (Fig. 1).

SW is a practical diapause silkworm strain. The generation of nondiapause embryos, microinjection, and screening of transgenic silkworms were performed as previously described (Jiang et al., 2012a,b,c) to generate 387 injected embryos and 226 hatched

* Corresponding author at: State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing 400715, PR China. Tel.: +86 23 68251996; fax: +86 23 68251128.
E-mail address: xiaqy@swu.edu.cn (Q. Xia).
[1] These two authors contribute equally to this work.

0166-3542/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.antiviral.2013.02.015

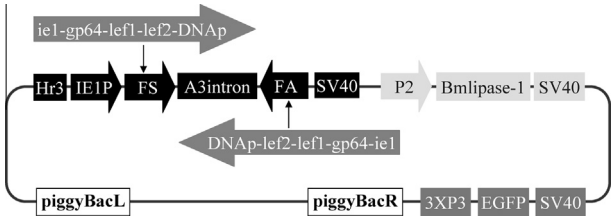

Fig. 1. Schematic diagram of the transgenic vector. 3×P3-EGFP-SV40 is the report marker of transgenic basic vector *PiggyBac* [3×p3 EGFP afm]; piggyBacL and piggyBacR, left and right terminal inverted repeats. Hr3, BmNPV enhancer; IE1P and P2, BmNPV IE1 promoter and a *B. mori* midgut-specific promoter; FS, synthetic DNA sequence with fragments of five essential BmNPV genes: *ie-1*, *gp64*, *lef-1*, *lef-2*, and *dnapol*; FA, reverse complementary sequence of FS; A3intron, spacer (Jiang et al., 2012c); Bmlipase-1, coding sequences of *Bmlipase-1*; SV40, polyadenylation signal.

larvae. The method resulted in 51 G1 broods and 21 EGFP-positive broods. We randomly selected three transgenic lines (named SW-H1, SW-H2, and SW-H3) for investigation. Each transgenic line contained only one insertion and the insertion site was located in an intergenic region (data not shown).

Resistance was investigated by oral inoculation of wild BmNPV with 5×10^5 occlusion bodies (OB) per third instar larva (Jiang et al., 2012a,b,c). Each transgenic line and nontransgenic SW had three infected replicates with three noninfected replicates as controls. Each replicate included 60 larvae. Cumulative survival rates were recorded daily until 10 days post infection (dpi) (Jiang et al., 2012a,c). Survival rates were 64% for SW-H1, 61% for SW-H2, 60% for SW-H3, and 13% for SW. Compared to SW, the resistance of SW-H was significantly increased and the survival rate of SW-H1 was increased by 51%. All untreated silkworms SW(C) survived (Fig. 3). Repeat experiments showed similar results (data not shown). Results of qPCR showed that, at 48 h post infection (hpi), the level of accumulated BmNPV DNA (Jiang et al., 2012a,b,c) in SW-H was less than 20% of the level of SW (Fig. 2C). These results indicated that SW-H significantly suppressed BmNPV proliferation.

Bmlipase-1 inhibits BmNPV at the initial stage of viral infection (Ponnuvel et al., 2003). Increased expression in the midgut could enhance the resistance of transgenic silkworms (Jiang et al., 2012b). The mRNA levels of *Bmlipase-1* in third instar larvae and the fifth instar silkworm midgut of SW-H were about 40% and 90% higher than SW, respectively (Fig. 2A). These results suggested that SW-H might suppress BmNPV at the initial stage of infection. Knockdown of a BmNPV gene suppresses the virus at the proliferation stage (Jiang et al., 2012c; Kanginakudru et al., 2007). Recently, Subbaiah et al. (2013) generated an improved transgenic silkworm by silencing multiple BmNPV genes, which conferred greater protection than targeting a viral gene (Subbaiah et al., 2013). To analyze data at 48 hpi, the expression of FS in SW-H1 was set to 1 for standardization. By this method, the mRNA of *ie-1* in SW-H was about 17% and *gp64* was about 25% compared to SW (Fig. 2B). This suggested that SW-H inhibited viruses at the multiplication stage by silencing BmNPV genes. The results showed that SW-H might inhibit BmNPV at multiple stages of viral infection. This strategy could be a valuable reference for studies with other animals. To the best of our knowledge, SW-H is the first transgenic animal with strong antiviral properties that might suppress virus at multiple infection stages. The economic traits of SW-H were not changed (Fig. S1) although viral resistance was significantly enhanced (Fig. 3). Currently, SW-H is being tested for safety.

In conclusion, we created a transgenic silkworm strain with strong antiviral characteristics that could be used in sericulture to prevent economic loss. This transgenic silkworm might suppress

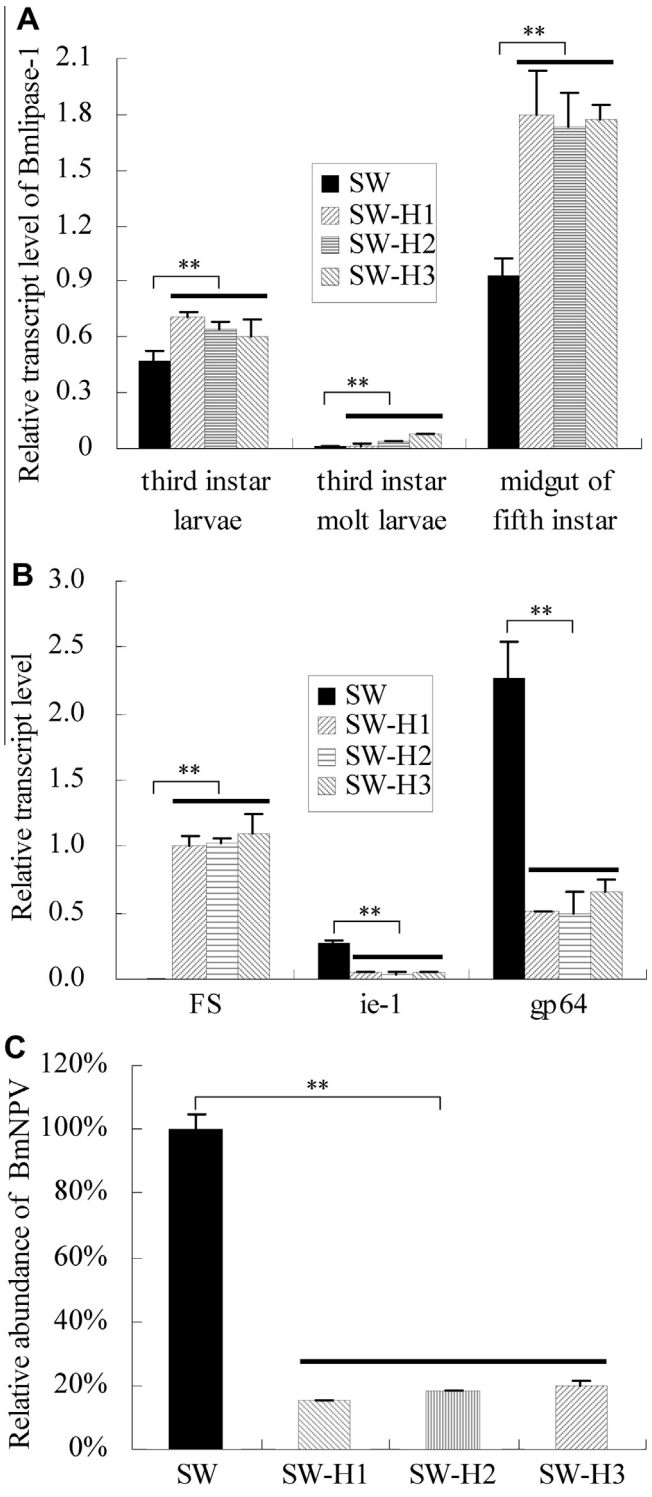

Fig. 2. qPCR analysis of transgenic silkworms. (A) Expression of *Bmlipase-1* in larvae and midguts. RNA of newly exuviated third instar larvae, third instar molt larvae, and midgut of fifth instar larvae of SW, SW-H1, SW-H2 and SW-H3, extracted and digested with RNase-free DNase I. RNA samples were reverse-transcribed to cDNA for analysis of *Bmlipase-1* by qPCR using primers Bmlip-1QRT (Sup. Table 1). (B) Viral gene mRNA. Total RNA was extracted from SW, SW-H1, SW-H2 and SW-H3 at 48 hpi. RNA samples were obtained from 10 treated larvae. FS, *ie-1*, and *gp64* were analyzed by qPCR with primers FS-QRT, ie1-QRT, and gp64-QRT (Sup. Table 1). Average FS level in SW-H1 was 1 for normalization. (C) Analysis of viral DNA content. Total DNA was extracted from 10 treated larvae at 48 hpi. Accumulated BmNPV DNA content was analyzed by qPCR using primers GP41 and BmGAPDH (Jiang et al., 2012a,b). SW average was 100% for standardization of transgenic lines. Bars are standard deviations. Significant differences of Student's *t*-test: $^{**}P < 0.01$.

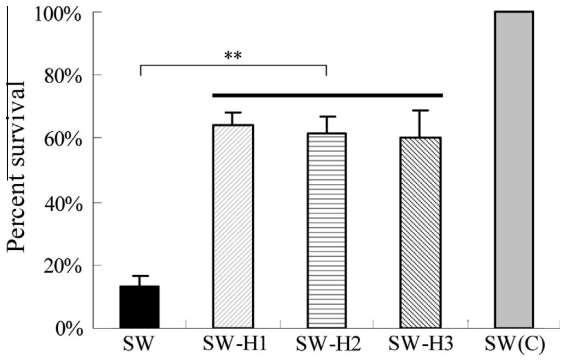

Fig. 3. Survival curve of transgenic silkworms. Four silkworm strains, SW, SW-H1, SW-H2, and SW-H3, were orally inoculated with BmNPV with 5×10^5 OB per third instar larva. Each larva ingested the same OBs dose. Accumulative survival rate to 10 dpi is shown from an average of three infection replicates. SW(C), noninfected control. Bars are standard deviations. Significant differences of Student's *t*-test: $^{**}P < 0.01$.

virus at multiple stages of viral infection, and could pave the way for antiviral studies in the other animals.

Acknowledgments

This work was funded by the National Basic Research Program of China (No. 2012CB114600), the National Hi-Tech Research and Development Program of China (No. 2011AA100306, No. 2013AA102507), the Foundation for Sci & Tech Research Project of Chongqing (CSTC2009AA1024), the National Natural Science Foundation of China (No. 30901054), the Production, Education & Research of Guangdong and Ministry of Education (No. 2010A090200079).

We thank Dr. Qiong Yang for gifting the BmNPV virus and Dr. Huawei He for critical reading of the manuscript. Thank the editor and reviewers for their critical review, valuable suggestions and highly favorable comments on our manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.02.015.

References

Gomi, S., Majima, K., Maeda, S., 1999. Sequence analysis of the genome of *Bombyx mori* nucleopolyhedrovirus. J. Gen. Virol. 80, 1323–1337.

Jiang, L., Cheng, T.C., Zhao, P., et al., 2012a. Resistance to BmNPV via overexpression of an exogenous gene controlled by an inducible promoter and enhancer in transgenic silkworm, *Bombyx mori*. Plos One 7.

Jiang, L., Wang, G.H., Cheng, T.C., et al., 2012b. Resistance to *Bombyx mori* nucleopolyhedrovirus via overexpression of an endogenous antiviral gene in transgenic silkworms. Arch. Virol. 157, 1323–1328.

Jiang, L., Zhao, P., Wang, G., et al., 2012c. Comparison of factors that may affect the inhibitory efficacy of transgenic RNAi targeting of baculoviral genes in silkworm, *Bombyx mori*. Antiviral Res. 97, 255–263.

Jiang, L., Cheng, T.C., Dang, Y.H., et al., 2013. Identification of a midgut-specific promoter in the silkworm *Bombyx mori*. Biochem Bioph Res Co. in press.

Kanginakudru, S., Royer, C., Edupalli, S.V., et al., 2007. Targeting *ie-1* gene by RNAi induces baculoviral resistance in lepidopteran cell lines and in transgenic silkworms. Insect Mol. Biol. 16, 635–644.

Kondo, A., Maeda, S., 1991. Host range expansion by recombination of the baculoviruses *Bombyx-Mori* nuclear polyhedrosis-Virus and *Autographa-Californica* nuclear polyhedrosis-virus. J. Virol. 65, 3625–3632.

Ponnuvel, K.M., Nakazawa, H., Furukawa, S., et al., 2003. A lipase isolated from the silkworm *Bombyx mori* shows antiviral activity against nucleopolyhedrovirus. J. Virol. 77, 10725–10729.

Ramirez-Carvajal, L., Long, C.R., 2012. Down-regulation of viral replication by lentiviral-mediated expression of short-hairpin RNAs against vesicular stomatitis virus ribonuclear complex genes. Antiviral Res. 95, 150–158.

Schultz, K.L.W., Friesen, P.D., 2009. Baculovirus DNA replication-specific expression factors trigger apoptosis and shutoff of host protein synthesis during infection. J. Virol. 83, 11123–11132.

Shah, P.S., Schaffer, D.V., 2011. Antiviral RNAi: translating science towards therapeutic success. Pharm. Res. 28, 2966–2982.

Subbaiah, E.V., Royer, C., Kanginakudru, S., et al., 2013. Engineering silkworms for resistance to baculovirus through multigene RNA interference. Genetics 193, 63–75.

Xia, Q.Y., Guo, Y.R., Zhang, Z., et al., 2009. Complete resequencing of 40 genomes reveals domestication events and genes in silkworm (*Bombyx*). Science 326, 433–436.

Xia, Q.Y., Zhou, Z.Y., Lu, C., et al., 2004. A draft sequence for the genome of the domesticated silkworm (*Bombyx mori*). Science 306, 1937–1940.

Zhou, J., Blissard, G.W., 2008. Identification of a GP64 subdomain involved in receptor binding by budded virions of the baculovirus *Autographica californica* multicapsid nucleopolyhedrovirus. J. Virol. 82, 4449–4460.