IJIE Int. J. Indust. Entomol. 37(2) 49-54 (2018)
ISSN 1598-3579, http://dx.doi.org/10.7852/ijie.2018.37.2.49

ISSN 1598-3579 (Print)
ISSN 2586-4785 (Online)

The innate immune response transcription factor *Bombyx mori Relish1* induces high-level antimicrobial peptides in silkworm

Seong-Wan Kim[1], Seong-Ryul Kim[1], Tae-Won Goo[2], and Kwang-Ho Choi[1,*]

[1]*Department of Agricultural Biology, National Institute of Agricultural Science, RDA, Jeonju 55365, Republic of Korea*
[2]*Department of Biochemistry, School of Medicine, Dongguk University, Gyeongju, 38066, Republic of Korea*

Abstract

To artificially enhance antimicrobial peptide expression in *Bombyx mori*, we constructed genetically engineered silkworms overexpressing Rel family transcription factor. The truncated *BmRelish1* (*BmRelish1t*) gene contained a Rel homolog domain (RHD), nuclear localization signal (NLS), acidic and hydrophobic amino acid (AHAA)-rich region, and death domain (DD), but no ankyrin-repeat (ANK) domain. The *BmRelish1t* gene was controlled by *B. mori* cytoplasmic actin 3 promoter in the PiggyBac transposon vector. Chromosome analysis of G1 generations of a transgenic silkworm with *EGFP* expression confirmed stable insertion of *BmRelish1t*. *BmRelish1t* gene overexpression in transgenic silkworms resulted in higher mRNA expression levels of *B. mori* antimicrobial peptides such as lebocin(~20.5-fold), moricin(~8.7-fold), and nuecin(~17.4-fold) than those in normal silkworms.

Int. J. Indust. Entomol. 37(2), 49-54 (2018)

Received : 20 Nov. 2018
Accepted : 4 Dec. 2018

Keywords:
Bombyx mori,
Relish,
Transgenic silkworm,
Antimicrobial peptide,
Silkworm

Introduction

The antibacterial immune response requires the Drosophila Rel protein, Relish (Dushay *et al.*, 1996). Relish is a homolog of the mammalian p105 precursor of the NF-κB p50 protein (Furukawa *et al.*, 2009). It consists of both an N-terminal Rel homology domain (RHD) and a C-terminal IκB-like ankyrin-repeat domain (ANK) that is believed to inhibit its own nuclear translocation (Goo *et al.*, 2008). The Relish protein is activated by infection with gram-negative bacteria and leads to activation of the IMD pathway (Furukawa *et al.*, 2009). Subsequently, the N-terminal fragment, including the RHD of Relish, translocates into the nucleus and activates antimicrobial peptide genes (Goo *et al.*, 2008). Recent studies have identified recognition molecules for invading microorganisms, such as gram-negative bacteria recognition protein (GNBP) and peptidoglycan recognition proteins (PGRP) (Tanaka and Yamakawa, 2011). PGRP-LC and PGRP-LE are involved in both recognition of gram-negative bacteria and activation of the IMD pathway (Tanaka and Yamakawa, 2011). *BmRelish1* and 2 genes that are conserved the Rel homology domain were cloned from the silkworm *Bombyx mori* (Hara and Yamakawa, 1995). *BmRelish1* contains a Rel homology domain (RHD), nuclear localization signal (NLS), acidic and hydrophobic amino acid (AHAA) - rich regions, ankyrin repeats (ANK), and death domain (DD). Transfection experiments using *D. melanogaster* mbn-2 cells showed that *BmRelish1* do not activate promoters of any *B. mori* antimicrobial peptide genes, whereas *BmRelish1* lacking ANK strongly activates promoters of these genes (Hara *and Yamakawa*, 1995).

***Corresponding author.**
Kwang-Ho Choi
National Institute of Agricultural Science, RDA, Jeonju 55365, Republic of Korea
Tel: +82-63-238-2113 / FAX: +82-63-238-3832
E-mail: ckh@rda.go.kr

Recently, a germline transformation method using the PiggyBac transposon was successfully developed in the silkworm *B. mori* (Hedengren *et al.*, 1999). Silkworm transgenesis is now a routine procedure leading to satisfactory yield of transformed animals and to reliable expression of transgenes in multiple successive generations (Hedengren *et al.*, 1999; Hoffmann, 2003; Ip *et al.*, 1993; Kaneko *et al.*, 2007). As a result, *B. mori* has received considerable attention as one of the most attractive host systems for the mass production of recombinant proteins (Kaneko *et al.*, 2008). In this study, in order to develop silkworm as an alternative to antibiotics for livestock diets, we constructed transgenic silkworms that overexpressed the innate immune response transcription factor *BmRelish1t* gene under BmA3 promoter using a PiggyBac-derived vector. Injection mixtures containing *BmRelish1t* vectors were microinjected into 1800 eggs of bivoltine silkworms and screened. Using the transgenic silkworms, we investigated mRNA expression levels of antimicrobial peptide genes.

Materials and Methods

Silkworm strains

The *B. mori* bivoltine strain "Baegokjam" (Jam123 × Jam124) was obtained from the National Academy of Agricultural Science (Jeonju, Korea). The silkworms were reared at 25°C and fed mulberry leaves and an artificial diet. DNA-injected eggs were maintained at 25°C in moist petri dishes. The hatched larvae were fed an artificial diet and reared in groups under standard conditions.

Transformation vector construction

Total RNA was prepared from fifth-instar silkworms using TRI Reagent (Molecular Research center, USA), according to the manufacturer's protocol. The quality and quantity of each sample were analyzed by agarose gel electrophoresis and spectrophotometry. Poly A^+ RNA was prepared from total RNA using a Micro-FastTrack 2.0 mRNA isolation kit (Invitrogen, USA), according to the manufacturer's protocol. cDNA synthesis was performed using a Poly A^+ RNA and Marathon cDNA Amplification kit (Clontech, USA). The *BmRelish1t* cDNA was generated by PCR amplification with specific primers (forward primer, 5'-AAAATGTCTACAACTGCCAGTG-3' and reverse primer 5'-TTATAATACATAAGTCAATGGAT-3') into which the *Nhe I* and *Afl II* sites were introduced. PCR products were subcloned into the pGEM-T easy vector (Promega, USA) and sequenced. *B. mori* cytoplasmic actin A3 gene promoter fragments and SV40 polyA signal fragments were inserted between the *Asc I* and *Nhe I* sites and *Afl II* and *Fse I* sites of pBac3xP3EGFP, respectively (Kim *et al.*, 2011), resulting in transfer vector PG-3xP3EGFP-BmA3. *BmRelish1t* was inserted into the PG-3xP3EGFP-BmA3 vector sites digested with *Nhe I* and *Afl II*. Transfer vectors PG-3xP3EGFP-BmA3BmRelish1t was generated. The transfer vector pBac3xP3EGFP (Kobayashi *et al.*, 2011) and the helper vector pHA3PIG (Hedengren *et al.*, 1999) were provided by Dr M. Jindra (Academy of Sciences of the Czech Republic, Czech Republic). Each of the vectors (PG-3xP3EGFP-BmA3BmRelish1t and pHA3PIG) was purified with an EndoFree Plasmid Maxi Kit (QIAGEN Gmbh, Germany) and used to generate transgenic silkworms.

Transgenesis and screening of silkworms

In order to generate eggs, male and female moths were allowed to mate for at least 4h at 25°C. The mating moths were stored overnight at 4°C. The female moths were placed on a plastic sheet and left in dark boxes for 1 h. The eggs they laid were immersed in HCl (specific gravity 1.0955, 25°C) for 30 min at 25°C, rinsed with distilled water, and finally dried. The transfer vectors (PG-3xP3EGFP-BmA3BmRelish1t) and the helper vector (pHA3PIG) were dissolved in 5mM KCl and 0.5mM phosphate buffer (pH7.0) at a concentration of 0.2 μg/μL and mixed at a ratio of 1:1. Approximately 5-10 nL of this mixture were injected using an IM-300 microinjector (Narishige Scientific Instrument Lab., Japan) into pre-blastoderm embryos at 2-8 h after oviposition (Hedengren *et al.*, 1999). Injected embryos were allowed to develop at 25°C in moist chambers. G1 embryo and larvae were screened under a fluorescence stereomicroscope equipped with a GFP filter (Leica, Switzerland).

Relative quantification analysis of mRNA by real-time PCR

Total RNA was extracted from fifth-instar, five-day-old larvae of normal silkworms and G1 transgenic silkworms with *BmRelish1t* genes. Total RNA was prepared using TRI Reagent according to the manufacturer's protocol (Molecular Research Center, USA). Total RNA (2 μg) was amplified by a three-step protocol using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems,

Table 1. Identification of the genomic insertion of PG-3xP3EGFP-BmA3BmRelish1t vector into the G1 transgenic silkworm genome by inverse PCR. Insertion length means the length of genomic DNA between the *Sau3AI* site and the 3′ insert boundaries of the vector. TTAA-duplicated sequences appearing at 3′ insert boundaries are underlined.

5' Genomic sequences			3' Genomic sequences
PG-3xP3EGFP-BmA3BmRelish1t	ATACAAGAATTGCTCGTTTAA TCAGATTATATATATATTAAT CGTTGGAGCCGTTTCCGAGAT CCTACTAAATTTTATGAAAAT GCCCCCGAAATCCCCTGAATA GATTTATC	-piggyBac-	TTAAAGGTATAAGATTAAATA TAGTCAGTCT

Table 2. Primers used in the real-time PCR. Primer design was based on the GenBank database.

No.	Primer name	Sequence	GeneBank Accession no.
1	Cecropin D2-F	5'- TTAAAGAGCTTGAAGGTGTG -3'	NM_001043459
	Cecropin D2-R	5'- CTTCCTTTGATTCAGAACTGT -3'	
2	Enbocin-F	5'- ATTTGGAACAGCATTTCCCC -3'	U30289
	Enbocin-R	5'- AAGCAATTTGTGAACGCGAG -3'	
3	Gloverin1-F	5'- ACGGCACCAGAGTATTAG -3'	AB289654
	Gloverin1-R	5'- GCCTGTTCAAGTCAATAGC -3'	
4	Lebocin-F	5'- TAGTATTCAGTTCAGTTCTGGT -3'	S79612
	Lebocin-R	5'- TATTATCGGACGCTGTGTT -3'	
5	Moricin-F	5'- TGGCAATGTCTCTGGTGTCA -3'	NM_001043364
	Moricin-R	5'- TGGCGATATTGATGGCTCTT -3'	
6	Nuecin-F	5'- GTAGCGTTGTTGTTGTTGT -3'	AF005384
	Nuecin-R	5'- GTTCCATCCGAGTTCACT -3'	
7	RP49-F	5'- TCGAAGAGACACCGTGAGCGATCT -3'	NM_001098282
	RP49-R	5'- CGGTTCCAACAAGAAGACCCGTCA -3'	
8	Transferrin-F	5'- ATAGAATGGAGTGTCTGAATTAC -3'	DQ375762
	Transferrin-R	5'- AACGACGAAGTCCTGATT -3'	

USA). The cDNA mix was diluted to 1:5 and stored at -20°C for subsequent RT-PCR. Real-time PCR amplification was performed using the gene-specific primers listed in Table 2 and normalized to a *B. mori* ribosomal protein 49 (RP49) gene. The real-time PCR assay was carried out in an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, USA). The amplifications were performed in a 25 μl reaction volume containing 12.5 μl of 2 × SYBR green master mix (TaKaRa, JAPAN). The thermal profile for RT-PCR was 95°C for 15 s followed by 40 cycles at 95°C for 15 s and 46°C for 32 s and a final step at 95°C for 15 s, 46°C for 32 min, and 95°C for 15 s. Each sample was run in triplicate and data analysis was performed with SDS software V2.0 (Applied Biosystems, USA). This experiments were repeated three times.

Statistical analysis

All data are presented as the mean ± S.D. from three or more independent experiments, unless otherwise stated. Different treatments were compared with Student's *t*-test, one-way ANOVA with Dunnett's multiple comparisons test, or chi-square tests using SPSS software (version 18.0; SPSS Inc., Chicago, IL). Differences with a *p*-value less than 0.05 were considered to be statistically significant.

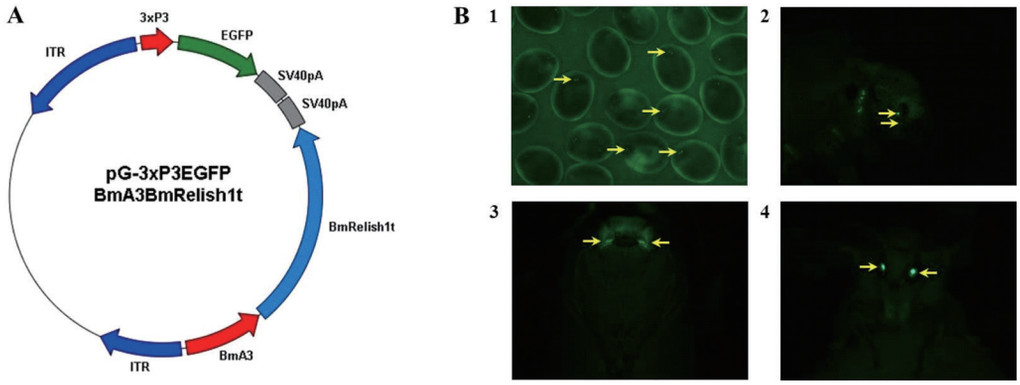

Fig. 1. Construction of the PG-3xP3EGFP-BmA3BmRelish1t vector and fluorescent image of transgenic silkworms. (A) Schematic of the PG-3xP3EGFP-BmA3BmRelish1t vector. The *BmRelish1t* gene was inserted into the PiggyBac plasmid between the BmA3 promoter and SV40 poly A. The EGFP gene was used as a transformation marker. (B1) G1 broods with EGFP-positive embryos at the seventh day of embryonic development were viewed under fluorescence. (B2)The G1 third-instar larva was viewed under fluorescence. Arrows in panels 1, 2, point to the ocelli and the abdominal nervous system, respectively. (B3,4) Pupae and adults were also viewed under fluorescence. Arrows in panels 3, 4 point to the compound eyes.

Results

Synthesis of BmRelish genes from *B. mori*

A cDNA sequence of BmRelish1t gene was identified in the GeneBank, (database accession numbers NM_001102465.1) and synthesized by RT-PCR. The truncated BmRelish1 (BmRelish1t) gene has a 1,686 bp cDNA contained in an open reading frame encoding 561 amino acid residues, and consists of an N-terminal Rel homology domain (RHD), nuclear localization signal (NLS), and acidic and hydrophobic amino acid (AHAA)-rich regions but no ankyrin repeats (ANK) or death domain (DD).

Generation of transgenic silkworms

To express the BmRelish1t gene under the control of *B. mori* cytoplasmic actin 3 promoter (BmA3), we constructed PG-3xP3EGFP-BmA3BmRelish1t plasmid DNA vector (Fig. 1A). Each of these vectors was mixed with the helper plasmid pHA3PIG and microinjected into 1800 eggs (pre-blastoderm stage) of Baegokjam bivoltine silkworms. Hatched larvae (G0) were allowed to develop into moths. Moths were mated within the same family or backcrossed with the wild-type moths. During this process, the 3xP3-EGFP system allowed us to screen a large number of G1 broods and to identify transgenic silkworms, because EGFP fluorescent signals were easily detected in the stemmata and nervous system at early embryonic and larval stages (Lemaitre and Hoffmann, 2007). The resulting G1 broods were screened for EGFP fluorescence. EGFP fluorescence became visible in the ocelli and peripheral nervous system on the seventh day of embryonic development (Fig. 1B1). Fluorescence in the ocelli was observed throughout the larval stages (Fig. 1B2). Fluorescence was also observed in the compound eyes of pupa and moth (Fig. 1B3, 4). The rates of successful transgenesis for G1 broods were 24.5%.

Expression levels of BmRelish1t mRNA transgenic silkworms

BmRelish1t mRNA level in a fifth-instar larva of G1 transgenic silkworm was analyzed by real-time RT-PCR. The mRNA abundance was calculated as the ribosomal protein RP49 mRNA. BmRelish1t mRNA was detectable in transgenic silkworm and normal silkworm. But BmRelish1t mRNA was more abundant (5-fold) than normal silkworm (Fig. 2A).

Expression levels of antimicrobial peptide genes in transgenic silkworms

Expression of seven antimicrobial peptide genes was analyzed in transgenic silkworms with over-expressed BmRelish1t gene. Transcript expression levels of antimicrobial peptides such as cecropin D2, enbocin, gloverin1, lebocin, moricin, nuecin, and transferrin were compared to those of normal silkworms and analyzed by real-time PCR. As shown in Figure 2, overexpression of the BmRelish1t gene in

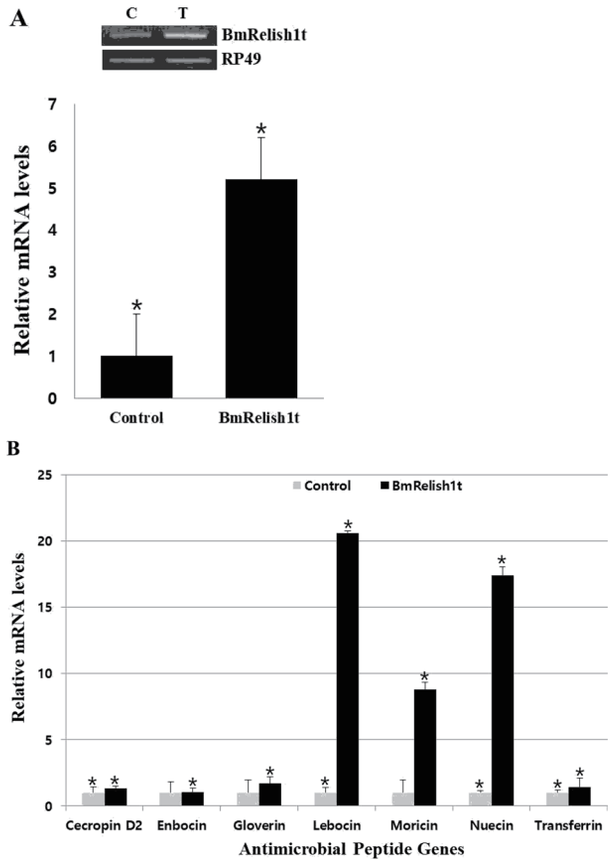

Fig. 2. Comparison of mRNA levels of the *BmRelish1t*-inducible antimicrobial peptide genes in transgenic silkworm larvae. (A) Comparison of mRNA levels of the BmRelish1t genes in transgenic silkworm. mRNA quantification was normalized to ribosomal protein RP49 mRNA. Results of triplicate experiments are shown with their standard deviations. (B) mRNA quantification was normalized to ribosomal protein RP49 mRNA. Results of triplicate experiments are shown with their standard deviations. Antimicrobial peptide genes are CecropinD2 (NM_001043459), Enbocin (U30289), Gloverin1 (AB289654), Lebocin (S79612), Moricin (NM_001043364), Nucin (AF005384), and Transferrin (DQ375762). Control: a fifth-instar larva of silkworm; BmRelish1t, a fifth-instar larva of G1 *BmRelish1t*-induced transgenic silkworm. Relative expression levels are presented as the mean ± SD (n = 3); *$p < 0.05$ versus control.

transgenic silkworm resulted in higher mRNA expression levels of *B. mori* antimicrobial peptides such as lebocin(~20.5-fold), moricin(~8.7-fold) and nuecin(~17.4-fold) than in normal silkworms (Fig. 2B).

Discussion

Many countries have banned or rigorously limited antibiotic use in the animal industry. Considering the movement toward banning the use of antibiotics, the animal industry cannot help but become interested in alternatives to antibiotics for growth promotion and maintenance of health under commercial conditions. Recently, antimicrobial peptides (AMPs) are receiving attention as an alternative to conventional antibiotics (Steward, 1987). Antimicrobial peptides (AMPs) are important components of the innate immune systems in all living organisms (Stoven *et al.*, 2000). Produced by a wide variety of organisms as part of a non-specific immune response, these peptides are involved in the directed destruction of various microorganisms. In *B. mori*, six different families of antimicrobial peptides have been identified: cecropin (Sugiyama *et al.*, 1995), attacin (Tamura *et al.*, 2000), lebocin (Tanaka *et al.*, 2008), moricin (Tanaka *et al.*, 2007), goverin (Tanaka *et al.*, 2007), and defensin (Tanaka *et al.*, 2009). The induction mechanism of these genes is divided into two distinct pathways: the Toll and IMD pathways (Thomas *et al.*, 2002). Silkworms are considered a good source of high-quality protein and lipids because of their high protein (55.6%) and lipid content (32.2%) (Thomas *et al.,* 2002).In this study, we constructed transgenic silkworms expressing immune-inducible antibacterial peptides using truncated *BmRelish1* (*BmRelish1t*) lacking the ankyrin repeats domain (ANK). These genes were induced to express antimicrobial peptide genes by infection of gram-positive and gram-negative bacteria (Hedengren *et al.,* 1999). However, the activation of these genes by the Toll pathway is lower than the activation by the IMD pathway (Wurm, 2003). Transfection experiments using *D. melanogaster* mbn-2 cells showed that *BmRelish1* do not activate promoters of any *B. mori* antimicrobial peptide genes, whereas BmRelish1 lacking the ANK strongly activates promoters of these genes (Hedengren *et al.,* 1999). Thus, we removed the ANK from the *BmRelish1* gene and generated germline transformation using the PiggyBac-derived vector in *B. mori.* The G1 broods were screened by EGFP fluorescence (Fig. 1B). The rate of successful transgenesis for G1 broods of *BmRelish1t* was 24.5%. To confirm whether *BmRelish1t* gene was correctly inserted into the genome of the transgenic silkworms, inverse-PCR experiments were performed using genomic DNA from G1 moths (Table 1). Chromosome analysis of the G1 generations confirmed the stable insertion of *BmRelish1t* into the genome. In addition, in order to analyze the effect of *BmRelish1t* overexpression on the transcription of *B. mori* antimicrobial peptide genes in transgenic silkworms, we carried out relative quantification analysis of mRNA by real-time relative PCR. The mRNA expression levels of lebocin(~20.5-fold), nuecin(~17.4-fold) and moricin(~8.7-fold) were higher than in normal silkworms (Fig. 2B). Our results suggest that the antibacterial peptide genes were highly expressed in the *BmRelish1t* gene-overexpressed

transgenic silkworm. These results suggest that the expression of antimicrobial peptide genes in transgenic silkworms is upregulated by the *BmRelish1t* gene, and that transgenic silkworms overexpressing *BmRelish1t* may be useful as a feed additive in livestock diets to reduce the need for antibiotics.

Acknowledgements

This work was supported by Agenda Program (PJ012709) and Cooperative Research Program for Agriculture Science & Technology Development (PJ012089) from Rural Development Administration, Republic of Korea.

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