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Production of the BmCecB1 antimicrobial peptide in transgenic silkworm

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Abstract

This peptide has antibacterial activity against several Gram-positive and Gram-negative bacteria. *Bombyx mori* cecropinB1(BmCecB1) is antimicrobial peptides from *Bombyx mori* and belongs to cecropin family. Antimicrobial peptides are important components of the innate immune systems in all living organism. To produce the BmCecB1 antimicrobial peptide, we constructed transgenic silkworm that expressed BmCecB1 gene under the control BmA3 promoter using *piggyBac* vector. The use of the 3xP3-driven EGFP cDNA as a marker allowed us to rapidly distinguish transgenic silkworm. Mixtures of the donor vector and helper vector were micro-injected into 600 eggs of bivoltin silkworms, Baegokjam. In total, 49 larvae (G0) were hatched and allowed to develop into moths. The resulting G1 generation consisted of 22 broods, and we selected 2 broods containing at least 1 EGFP-positive embryo. The rate of successful transgenesis for the G1 broods was 9%. We identified 9 EGFP-positive G1 moths and these were backcrossed with wild-type moths. With the aim of identifying a BmCecB1 as antimicrobial peptide, we investigated the Radical diffusion Assay (RDA) and then demonstrated that BmCecB1 possesses high antibacterial activities against Gram-negative bacteria.

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Introduction

Insects have powerful innate immunity against microorganisms. Their immune system comprises both humoral and cellular reactions. The humoral reactions involve soluble proteins in the hemolymph, such as phenoloxidase, antimicrobial proteins (AMPs), lysozymes, and lectins; whereas hemocytes mediate cellular reactions such as phagocytosis, encapsulation, and nodule formation (Tanaka *et al*., 2011). Among these, cecropins are antimicrobial peptides and small proteins of about 31 - 37 amino acid residues mainly active against Gram-negative bacteria (Mourgues *et al*., 1998). Cecropin, originally isolated from the giant silkmoth, *Hyalophora cecropia*, consists of about 40 amino acid residues and is heat-stable. Three subtypes of cecropin (A, B and D) have been reported from *B. mori* (Morishima *et al*., 1990). Structure of cecropin consists of mainly alpha helixes, determined by solution NMR. Protein molecular weight = 4203.4g/M. At low peptide to lipid ratios ion channels are formed, at high peptide to lipid ratios pores are formed (Christensen *et al*., 1988).

Recently, a germline transformation method using the

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PiggyBac transposon was successfully developed in the silkworm *B. mori* (Tamura *et al*., 2000). Silkworm transgenesis is now a routine procedure leading to satisfactory yield of transformed animals and to reliable expression of transgenes in multiple successive generations (Tamura *et al*., 2000; Berghammer *et al*., 1999; Hoffmann *et al*., 2003). As a result, *B. mori* has received considerable attention as one of the most attractive host systems for the mass production of recombinant proteins (Wurm *et al*., 2003). In this study, in order to develop silkworm as an alternative to antibiotics for livestock diets, we constructed transgenic silkworms that overexpressed the BmCecB1 under BmA3 promoter using a PiggyBac-derived vector. Injection mixtures containing BmCecB1 vector was microinjected into 600 eggs of bivoltine silkworms and screened. Using the transgenic silkworms, we investigated the antibacterial activity against various pathogenic bacteria.

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.

Plasmid DNA Construction

We constructed the transition vector pG-3xP3-EGFP-BmA3-bPDIsp-BmCecB1. The BmA3 promoter was generated by PCR amplification with specific primers (forward primer, 5’-GGCGCGCCGCGCGTTACCATATATGGTG -3 ’ and reverse primer 5’- GCTAGCCTTGAATTAGTCTGCAAGAAA -3’). The PCR product was cloned into the pGEM-T-easy vector (Promega, Madison, Wi, USA) and named pGEM-BmA3. BmA3 promoter was excised from pGEM-BmA3 with AscI and *Nhe*I and cloned into a p3×P3 promoter fragment from pBac3×P3EGFP to generate pG-3xP3-EGFP-BmA3. The bPDIsp-BmCecB1 cDNA was generated by PCR amplification with specific primers (forward primer, 5’-GCTAGCATGCGTGTTTTAATTTTCACG-3’ and reverse primer 5’- CTTGGTTCGGCTAAAGCTATACTTAAG-3’) into which the *Nhe I* and *Afl II* sites were introduced. PCR products were subcloned into the pGEM-T easy vector (Promega) and sequenced. bPDIsp-BmCecB1 was inserted into the pG-3xP3-EGFP-BmA3 vector sites digested with *Nhe I* and *Afl II*, respectively. Transfer vectors pG-3xP3-EGFP-BmA3-bPDIsp-BmCecB1 was generated.

Transgenesis and screening of silkworms

For egg preparation, male and female moths were allowed to mate for at least 4 h 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 laid eggs were immersed in HCl (specific gravity 1.0955, 25°C) for 30 min at 25°C, rinsed with distilled water, and finally dried. The transition vector pG-3xP3-EGFP-BmA3-bPDIsp-BmCecB1 and the helper vector pHA3PIG were dissolved in 5 mM KCl and 0.5 mM phosphate buffer (pH 7.0) 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 IM300 microinjector (Narishige Scientific Instrument Lab., Japan) into pre-blastoderm embryos at 2–8 h after oviposition (Tamura et al. 2007). 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, Wetzlar, Germany).

Inverse PCR

Genomic DNA was purified using a QIAamp DNA Mini Kit (QIAGEN Gmbh, Germany) from G1 moths. Purified genomic DNA was digested with *Sau*3AI (NEB, Hitchin, Hertfordshire, UK) and circularized by overnight ligation at 16°C with T4 DNA ligase (Promega, USA). PCR was performed on the ligated DNA using primer sequences between the restriction site and the end sequence of the transition vector. For the 5’ junction, the primer pair was 5’-ATCAGTGACACTTACCGCATTGACA-3’ and 5’-TG ACGAGCTTGTTGGTGAGGATTCT-3’. For the 3’ junction, the primer pair was 5’-TACGCATGATTATCTTTAACGTA-3’ and 5’-GGGGTCCGTCAAAACAAAACATC-3’ (Tamura et al. 2000). PCR products were cloned into the pGEM-T-easy vector (Promega, USA) and sequenced. Sequencing data were analyzed

using the DNASTAR LaserGene program (DNASTAR Inc. Madison, WI, USA).

Analysis of antimicrobial activity

Transgenic silkworms were tested for antibacterial activity with an inhibition zone assay. In brief, 0.5 g of tissue from fifth-instar larvae of *B. mori* was added to a mixture of 0.3% acetic acid solution and 100 mL NaCl solution. This solution was stirred for 1 h and centrifuged at 10,000 rpm for 30 min. The upper phase was transferred to a new tube and heat-treated at 80°C for 30 min, then centrifuged at 10,000 rpm for 30 min. After removing the supernatant, the sample was concentrated by freeze-drying. The freeze-dried sample was dissolved in the 0.01% acetic acid solution. Bacterial strains *Escherichia coli, Salmonella Enteritidis, Salmonella Gallinarum, Salmonella Typhimurium, and Salmonella Pullorum* were grown overnight at 37°C and shaken at 200 rpm in Tryptic Soy Broth (TSB, Difco, USA). The culture was then washed twice with autoclaved 10 mM sodium phosphate buffer (pH 7.4), and re-suspended in fresh TSB to a final concentration of 2 x 10^4 CFU/mL. The *E. coli, S. Enteritidis, S. Gallinarum, S. Typhimurium and S. Pullorum* were washed once with 10 mM Tris (pH 7.4). Next, 4 x 10^6 bacterial colony-forming units (CFU) were added to 10 mL of the underlay agarose gel (1% (v/v) TSB, 1% (w/v) LE agarose (Lonza, USA), 1% (v/v) 0.1M Citrate buffer, and 1% (v/v) 0.1M Sodium phosphate). The underlay was poured into an 85 mm petri dish. After agarose solidification, 4 mm-diameter wells were punched and 10 µL of test sample was added to each well. Plates were incubated at 37ºC for 3 h to allow diffusion of the peptides. The underlay gel was then covered with 10 mL of molten overlay (6% (w/v) TSB, 1% (w/v) LE agarose, in dH_2O). Antimicrobial activity was visible as a zone of clearing around each well after 18-24 h of incubation at 37°C.

Results

Generation of transgenic silkworm

To express the BmCecB1 gene under the control of *B. mori* cytoplasmic actin 3 promoter (BmA3), we constructed pG-3xP3-EGFP-BmA3-bPDIsp-BmCecB1 plasmid DNA vectors (Fig. 1). This vector was mixed with the helper plasmid pHA3PIG and microinjected into 600 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. 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. 2A). Fluorescence in the ocelli was observed throughout the larval stages (Fig. 2B). Fluorescence was also observed in the compound eyes of the pupa and moth (Fig. 2C, 2D). The rates of successful transgenesis for G1 broods was 9% (Table 1).

Chromosome analysis

We isolated one of the G1 broods showing the highest level of fluorescence. To analyze the insertion sites in this transgenic silkworm, inverse-PCR experiments were performed using genomic DNA from the G1 moths. As shown in Table 2, we identified one genomic junction sequence. This sequence revealed the characteristic TTAA duplication at the PiggyBac junction with chromosomal DNA. We searched using KAIKOBLAST (http://sgp.dna.affrc.go.jp) and matched this sequence with contiguous sequences in the database. Thus, inverse PCR analysis of the G1 generations confirmed the stable insertion of BmCecB1 into the genome.

Antimicrobial Activity

The antimicrobial activities of of BmCecB1 was examined against various poultry pathogens such as *E.coli, S. Enteritidis, S. Gallinarum, S. Typhimurium, and S. Pullorum.* We performed an inhibition zone assay and compared the transgenic silkworms and normal silkworms. BmCecB1 antimicrobial peptide showed the strongest activity against *S. Enteritidis* and *S. Pullorum*. They showed moderate activity against *E. coli*, and weak activity against *S. Gallinarum* and *S. Typhimurium*. These differences may result from differences in the membrane potential of each bacterium (Fig. 3).

Discussion

Globally, antibiotics have been used in the animal industry for growth promotion and the prevention and treatment of disease for more than fifty years (Looft *et al*., 2012). Despite the many advantages of antibiotics, their repeated use in livestock feed has caused many problems, such as the emergence of antibiotic-resistant bacteria, antibiotic residues in edible animal products, and disturbance to normal intestinal microflora (Barton *et al*., 2000). As a result, 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 (Baltzer *et al*., 2011). Antimicrobial peptides (AMPs) are important components of the innate immune systems in all living organisms (Andreu *et al*., 1998). 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, attacin, lebocin, moricin, goverin, and defensin (Steiner *et al*., 1981; Sugiyama *et al*., 1995; Tanaka *et al*., 2008; Hara *et al*., 1995; Kaneko *et al*., 2007; Kaneko *et al*., 2008). The induction mechanism of these genes is divided into two distinct pathways: the Toll and IMD pathways (Lemaitre *et al*., 2007). Silkworms are considered a good source of high-quality protein and lipids because of their high protein (55.6%) and lipid content (32.2%) (Tomotake *et al*., 2010). In this study, we constructed transgenic silkworms expressing antibacterial peptide using BmCecB1 genes. This gene was induced to express antimicrobial peptide genes by infection of gram-negative bacteria. The G1 broods were screened by EGFP fluorescence (Fig. 2). The rate of successful transgenesis for G1 broods of BmCecB1 was 9% (Table 1). To confirm whether BmCecB1 gene was correctly inserted into the genome of the transgenic silkworms, inverse-PCR experiments were performed using genomic DNA from G1 moths. Chromosome analysis of the G1 generations confirmed the stable insertion of BmCecB1 into the genome. In the inhibition zone assay, BmCecB1 antimicrobial peptide showed antimicrobial activity against various poultry pathogens such as *Escherichia coli, Salmonella Enteritidis, Salmonella Gallinarum, Salmonella Typhimurium,* and *Salmonella Pullorum* (Fig. 3). These results suggest that the BmCecB1 antimicrobial peptide in transgenic silkworms is overexpressed, and that transgenic silkworms overexpressing BmCecB1 peptide may be useful as a feed additive in livestock diets to reduce the need for antibiotics.

Conclusion

Our results confirmed that for achieving best antibacterial peptide productivity of BmCecB1 in Silkworm. To produce the BmCecB1 antimicrobial peptide, we constructed transgenic silkworm and selected 2 broods containing at least 1 EGFP-positive embryo. With the aim of identifying a BmCecB1 as antimicrobial peptide, we investigated the radical diffusion assay (RDA) and showed antimicrobial activity against Gram-negative bacteria. These results suggest that BmCecB1 peptide may be useful as a feed additive in livestock diets.

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References

Andreu D and Rivas L (1998) Animal antimicrobial peptides: an overview. Biopolymers 47(6), 415-433.

Baltzer SA and Brown MH (2011) Antimicrobial peptides: promising alternatives to conventional antibiotics. Journal of molecular microbiology and biotechnology 20(4), 228-235 .

Barton MD (2000) Antibiotic use in animal feed and its impact on human healt. Nutrition research reviews 13(2), 279-299.

Berghammer AJ, Klingler M and Wimmer EA (1999) A universal marker for transgenic insects. Nature 402(6760), 370-371.

Christensen B, Fink J, Merrifield RB and Mauzerall D (1988) Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes. Proceedings of the National Academy of Sciences of the United States of America 85(14), 5072-5076.

Hara S and Yamakawa M (1995) Moricin, a novel type of antibacterial

peptide isolated from the silkworm, Bombyx mori. J Biol Chem 270(50), 29923-29927.

Hoffmann JA (2003) The immune response of Drosophila. Nature 426(6962), 33-38.

Kaneko Y, Furukawa S, Tanaka H and Yamakawa M (2007) Expression of antimicrobial peptide genes encoding Enbocin and Gloverin isoforms in the silkworm, Bombyx mori. Biosci Biotechnol Biochem 71(9), 2233-2241.

Kaneko Y, Tanaka H, Ishibashi J, Iwasaki T and Yamakawa M (2008) Gene expression of a novel defensin antimicrobial peptide in the silkworm, Bombyx mori. Biosci Biotechnol Biochem 72(9), 2353-2361.

Lemaitre B and Hoffmann J (2007) The host defense of Drosophila melanogaster. Annu Rev Immunol 25, 697-743.

Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD, Sul WJ, Stedtfeld T M, Chai B, Cole JR, Hashsham SA, Tiedje JM and Stanton TB (2012) In-feed antibiotic effects on the swine intestinal microbiome. Proceedings of the National Academy of Sciences of the United States of America 109(5), 1691-1696.

Morishima I, Suginaka S, Ueno T and Hirano H (1990) Isolation and structure of cecropins, inducible antibacterial peptides, from the silkworm, Bombyx mori. Comp Biochem Physiol B 95(3), 551-554.

Mourgues F, Brisset MN and Chevreau E (1998) Strategies to improve plant resistance to bacterial diseases through genetic engineering. Trends Biotechnol 16(5), 203-210.

Steiner H, Hultmark D, Engstrom A, Bennich H and Boman HG (1981) Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature 292(5820), 246-248.

Sugiyama M, Kuniyoshi H, Kotani E, Taniai K, Kadono-Okuda K, Kato Y, Yamamoto M, Shimabukuro M, Chowdhury S and Xu J (1995) Characterization of a Bombyx mori cDNA encoding a novel member of the attacin family of insect antibacterial proteins. Insect biochemistry and molecular biology 25(3), 385-392.

Tamura T, Thibert C, Royer C, Kanda T, Abraham E, Kamba M, Komoto N, Thomas JL, Mauchamp B, Chavancy G, Shirk P, Fraser M, Prudhomme JC and Couble P (2000) Germline transformation of the silkworm Bombyx mori L. using a piggyBac transposon-derived vector. Nature biotechnology 18(1), 81-84.

Tanaka H, Ishibashi J, Fujita K, Nakajima Y, Sagisaka A, Tomimoto K, Suzuki N, Yoshiyama M, Kaneko Y, Iwasaki T, Sunagawa T, Yamaji K, Asaoka A, Mita K and Yamakawa M (2008) A genome-wide analysis of genes and gene families involved in innate immunity of Bombyx mori. Insect biochemistry and molecular biology 38(12), 1087-1110 .

Tanaka H and Yamakawa M (2011) Regulation of the innate immune responses in the silkworm, Bombyx mori. Isj-Invert Surviv J 8(1), 59-69.

Tomotake H, Katagiri M and Yamato M (2010) Silkworm pupae (Bombyx mori) are new sources of high quality protein and lipid. Journal of nutritional science and vitaminology 56(6), 446-448.

Wurm FM (2003) Human therapeutic proteins from silkworms. Nature biotechnology 21(1), 34-35.