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SHORT COMMUNICATION

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Production of antibacterial *Bombyx mori* cecropin A in mealworm-pathogenic *Beauveria bassiana* ERL1170

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Abstract Efforts are underway to produce antimicrobial peptides in yellow mealworms (*Tenebrio molitor*), which can be developed as more effective and safer animal feed additives. In this work, we expressed *Bombyx mori* (*Bm*) cecropin-A in mealworms by the infection of transformed entomopathogenic *Beauveria bassiana* ERL1170. The active domain of *Bm cecropin A* gene was tagged with a signal sequence of *B. bassiana* for extracellular secretion, and the fragment was inserted into ERL1170 by the restriction enzyme-mediated integration method. Transformant D-6 showed antibacterial activity against *Bacillus subtilis* and *Listeria monocytogenes*. Against *T. molitor* larvae, D-6 had similar mortality to wild-type, and D6-infected mealworm suspension showed strong antibacterial activity against the two bacteria, but not in the wild-type-infected mealworms, thereby increasing the value of mealworms as animal feed additives.

Keywords *Tenebrio molitor* · Animal feed · Cecropin A · *Beauveria bassiana* · Antibacterial activity

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Introduction

Entomopathogenic fungi can be considered as practical platforms for the expression of functional proteins in insects, and the infected mealworm complex has a potential to be used as animal feed additives [13]. Functional proteins can be produced in insects by the infection of genetically engineered-entomopathogenic fungi, and ultimately the mycotized insects can be used as raw materials for feed additives. Yellow mealworm, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) has been added to animal feeds, because it has large amount of nutrition [16]. Mealworms in their larval and pupal stages are rich in protein and are easy to rear [7]. Proteins from insects are clean and safe to animals and insects have little chance of being infected by harmful microorganisms or of acquiring diseases [4, 5].

One of the functional proteins, cecropin is a family of potent antibacterial peptides involved in the immune hemolymph of the *Cecropia* moth [17]. Cecropin is synthesized in fat bodies as a preproprotein consisting of 31–39 amino acid residues and adopt an α-helical structure upon interaction with bacterial membranes, resulting in the formation of ion channels [3]. At low concentrations (0.1–5 μM), cecropin exhibits lytic antibacterial activity against several bacteria, but not against eukaryotic cells [10, 15].

Cecropin has been widely used in many applications [1, 8, 14] but expression of this protein in entomopathogenic fungal expression platforms has not yet been fully exploited. A mealworm-fungus complex containing *B. mori* cecropin A could possibly control bacterial infection in animals. In this work, the antibacterial gene was integrated into an entomopathogenic *Beauveria bassiana* ERL1170 isolate by a restriction enzyme-mediated integration method to investigate the antibacterial activity of a transformed fungus and the infected mealworm complex.

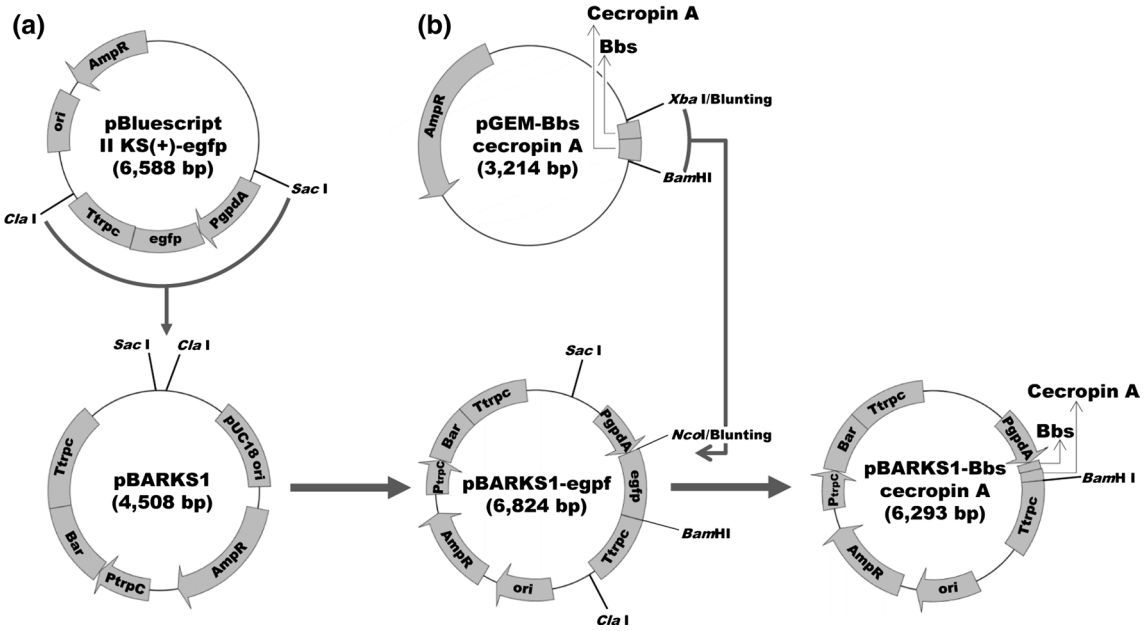

Fig. 1 Construction of the fungal transformation vector, pBARKS1-Bbs-cecropin A containing *B. bassiana* signal (Bbs) and *B. mori* (*Bm*) cecropin A. **a** Integration of the egfp expression cassette to pBARKS1; and **b** exchange of egfp with Bbs-Bm cecropin A fragment

Materials and methods

Microbial strains and culture media

Beauveria bassiana ERL1170 (ARSEF2060 in USDA-ARS in Itheca) [11] were maintained on fourth-strength Sabouraud dextrose agar (SDA/4) in darkness at 25 °C for colonial growth. Conidia were harvested and suspended in 0.03 % (v/v) siloxane solution (Silwet L-77®, Loveland, CO, USA) as a wetting agent. *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, USA) was used for DNA manipulation, and cultured on Luria–Bertani (LB) medium containing 50 mg/mL ampicillin.

Vector construction

A fungal transformation vector, pBARKS1 (Fungal Genetics Stock Center, USA) was used as a plasmid for vector construction. pBARKS1 (4,508-bp) has a phosphinothricin (PPT)-resistant gene, which is expressed under the control of *trpC* promoter (Fig. 1). First, the 2,393-bp egfp-expression cassette including *gpdA* promoter (P*gpdA*) and *trpC* terminator (T*trpC*) was digested from the pBluescript II KS(+)-egfp [12] using *Cla*I and *Sac*I (Fig. 1a). The egfp-expression cassette was ligated to pBARKS1, which was previously digested using the same endonucleases. The generated plasmid was designated as pBARKS1-egfp (6,824-bp). Using a HT-oligo™ synthesizer from BIONEER (http://www.bioneer.co.kr), *cecropin A* gene (GenBank: D17394) from *B. mori* was synthesized. In the gene synthesis, the 5′-end of cecropin A active domain was tagged to the 3′-end of 84-bp *B. bassiana* signal (*Bbs*) fragment of chitinase (GenBank: AY145440) (Fig. 1b). The *Bbs-cecropin A* fragment was flanked with *Xba*I at the 5′-end and *Bam*HI at the 3′-end for ligation and was ligated to pGEM-T vector, ultimately producing pGEM-Bbs-cecropin A (3,214-bp). pGEM-Bbs-cecropin A was digested with *Xba*I/blunting (Klenow pol.) and *Bam*HI, and the *Bbs-cecropin A* (198-bp) was isolated using a QIAquick Gel Extraction Kit (Qiagen, CA, USA). pBARKS1-egfp was digested with *Nco*I/blunting (Klenow pol.) and *Bam*HI, and the egfp-removed fragment (6,107-bp) was isolated using the same extraction kit. The two isolated fragments were ligated and finally pBARKS1-Bbs-cecropin A (6,293-bp) was generated. All plasmids were confirmed by sequencing.

Fungal transformation

pBARKS1-Bbs-cecropin A was linearized by *Hin*dIII and integrated into the genomic DNA of ERL1170 by the restriction enzyme-mediated integration method based on protoplasts [6]. Transformants were collected and sequentially grown on 600 μg/mL PPT+ Czapek's solution agar. Putative transformants were sub-cultured twice on SDA/4 at 25 °C. Genomic DNAs were extracted from 5-day old mycelial masses using the quick fungal genomic DNA extraction method [2], and the *bar* and *Bbs-cecropin A* were detected by PCRs with primers Bar-F and Bar-R and primers Bbs-cecropin A-F and Bbs-cecropin A-R (Table 1).

Table 1 Primers used in this study

Primer name	Sequence	Size of amplified fragment
Bb 18S rRNA-F	5′-TTA CGT CCC TGC CCT TTG TA-3′	166 bp
Bb 18S rRNA-R	5′-CCA ACG GAG ACC TTG TTA CG-3′	
Bar-F	5′-AGT CGA CCG TGT ACG TCT CC-3′	241 bp
Bar-R	5′-GAA GTC CAG CTG CCA GAA AC-3′	
Bbs-cecropin A-F	5′-CTC GCG CTC CTT CCA TT-3′	156 bp
Bbs-cecropin A-R	5′-TCG CTT GCC CTA TGA CG-3′	

Verification of expression

Transcription of *Bbs-cecropin A* in the putative transformants was examined by the extraction of RNAs from 5-day-old fungal mycelial masses using the TRIZOL (Invitrogen, CA, USA) method and reverse transcription PCR (RT-PCR) with the primers Bbs-cecropin A-F and Bbs-cecropin A-R. As controls, RT-PCRs with the *B. bassiana* 18S rRNA-specific primers (Table 1) were conducted. For western blotting, transformants and wild-type were cultured in fourth strength Sabouraud dextrose broth (SDB/4) at 25 °C and 150 rpm of shaking for 5 days. Cultured broth was filtered using sterile 3 M filter papers and syringe filters (0.25 μm), and the filtrate was concentrated by ultrafiltration using Amicon tubes (Millipores, MA, USA). The concentrates were subjected to 12 % SDS–polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. A polyclonal antibody against cecropin (Novus Biologicals, CO, USA) was used to detect the expression of cecropin A which was secreted from the mycelia of the transformants. The PVDF membrane was incubated with a 1,000-fold dilution of the polyclonal antibody and a 2,000-fold dilution of goat anti-rabbit IgG horseradish peroxidase (HRP) as the second antibody. Visualization was conducted under a chemiluminescence image analysis system (Uvitec, Cambridge, UK) using the luminol reagent, SC-2048 (Santa Cruz Biotechnology Co., USA).

Antibacterial activity assay

Antibacterial activities of putative transformants were assayed against two bacteria on LB medium. The two bacteria, *Bacillus subtilis* (KACC15591) and *Listeria monocytogenes* (KACC10550) from the Korean Agricultural Culture Collection (http://www.genebank.go.kr) were cultured on LB medium at 27 °C for 3 days. A 50-μl aliquot of bacterial suspension (OD_{600}: ~1.0) was spread on LB medium in a 60-mm diameter Petri dish, and the agar block (5 mm diameter) of a 7-day-old putative transformant was placed on the LB medium with three replicates. Wild-type served as a control. All dishes were held at room temperature (~25 °C), and antibacterial activities of transformants were examined daily by measuring the diameter of clear zone of the agar blocks (when no clear zone was observed, it was recorded as zero).

Fungal inoculation to mealworms

To inoculate putative transformants to mealworm larvae, 3rd instar larvae were placed in a 60-mm diameter Petri dish (10 larvae/dish) containing 2 g of wheat bran as diet, and they were placed at 4 °C for 20 min to reduce their mobility. For inoculation, a 10-mL aliquot of conidial suspension (1×10^7 conidia/mL) was applied to ten larvae in a dish using a micro sprayer, and the dish was covered with a lid to maintain high humidity conditions. It was replicated three times (three Petri dishes/treatment). Wild-type served as a control. Untreated mealworms served as another control. All dishes were held at 25 °C and at 16:8 (L/D) regimes in an incubator. Mortality was examined daily by counting the number of dead larvae, and the mycelial outgrowth was observed. In 7 days, dead mealworms were collected and freeze-dried to make mealworm powder, and it was subjected to an antibacterial activity assay with some modification to inhibit the fungal growth from the powder. Fluazinam (CAS NO.: 79622-59-6) as an antimycotics was added to phosphate buffered saline (PBS) at 0.25 g/L, and the freeze-dried mealworm powder (0.01 g) was suspended in the 1 mL PBS solution to make 1 % (w/v) suspension. In a preliminary test, fluazinam did not show any clear zone against the bacteria. After the spreading of the bacterial suspension on LB medium, the freeze-dried mealworm suspension was dropped on a sterile paper disk (5 μL/6-mm diameter paper disk).

Results and discussion

Characterization of cecropin A-producing transformant

After the restriction enzyme-mediated fungal transformation, putative transformants (30–50 colonies/dish) were observed on the regeneration medium including phosphinothricin (PPT). A transformant with strong antibacterial activity was selected, and it was designated as D-6. The

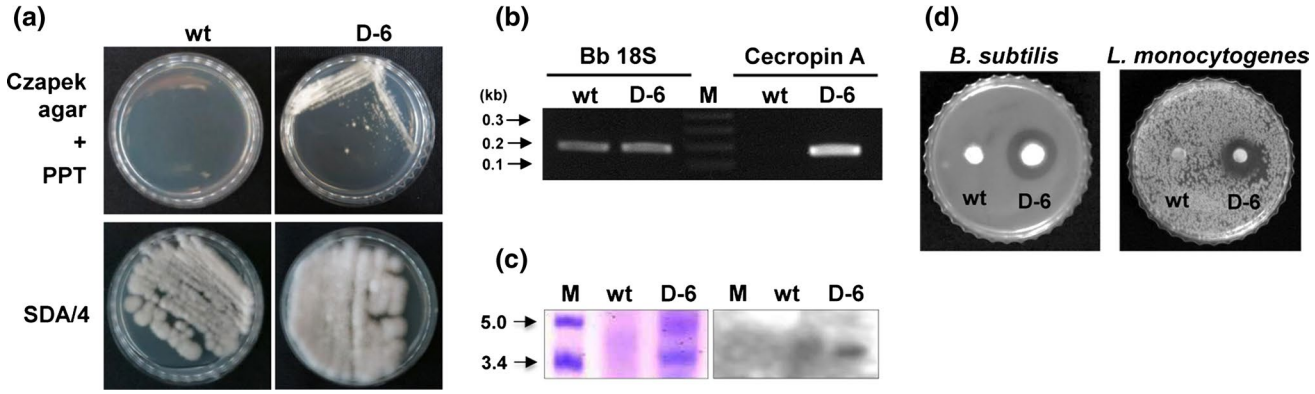

Fig. 2 Characterization of *B. mori* cecropin A-producing transformant D-6. **a** Mycelial growth of wild-type and transformant D-6 on Czapek's solution agar containing phosphinothricin (PPT) and SDA/4 (without PPT); **b** Reverse transcription (RT) PCR of wild type and transformant D-6 (*B. bassiana* 18S rRNA was used as a control); **c** SDS-PAGE and western blot of wild-type and D-6 supernatants; and **d** Antibacterial activity of wild-type and transformant D-6 against *B. subtilis* and *L. monogenesis* on LB agar in three days

transformant had a stable growth on the Czapek's solution agar containing PPT during sub-cultures, whereas wild type did not grow on the selection medium (Fig. 2a). On the SDA/4 medium, D-6 showed a slightly different morphology (more mycelial mass was observed) compared to the wild-type (Fig. 2a). From the sequencing of the ITS region of transformant D-6 with primers ITS1-F and ITS4-R, it was identical to the sequence of wild-type (data not shown). The slightly different morphology of D-6 [Less number of conidia were produced in D-6 (c. 5×10^7 conidia/cm^2) compared to wild-type (c. 2×10^8 conidia/cm^2)] might be resulted from the possible integration of *cecropin A* gene into conidiogenesis-related genes in wild-type genomic DNA, but it needs to be further investigated in a gene characterization study.

Transcription of *Bbs-cecropin A* gene was confirmed by RT-PCR analysis (Fig. 2b) and the expression of cecropin A was confirmed by western blot analysis (Fig. 2c). RT-PCR of transformant D-6 with the primers Bbs-cecropin A-F and Bbs-cecropin A-R showed 156-bp target fragment, but not in wild-type. As an internal control, 18S rRNA-specific RT-PCR product (166-bp) was observed in both wild-type and transformant D-6. The western blot analysis of D-6 supernatant detected ~3.8 kDa protein, but not in the wild-type supernatant, which confirmed that *Bm cecropin A* gene was transcribed and successfully expressed in the fungus. In this work, the production efficiency of cecropin A was investigated by measuring the clear zone of the transformant D-6 in the antibacterial activity assay, however, it needs to be investigated more accurately.

The transformant D-6 showed strong antibacterial activity against *B. subtilis* and *L. monocytogenes* (Fig. 2d), whereas wild-type did not show any significant activity. Compared to the wild-type with no clear zone, transformant D-6 showed 19.3 ± 0.6 mm of diameter (*B. subtilis*) and 13.1 ± 0.5 mm of diameter (*L. monocytogenes*) of clear zones in 3 days after inoculation. The expressed *B. mori* cecropin A could be mainly involved in the antibacterial activity. If the transformant D-6 was cultured earlier than the bacterial inoculation, it might show much higher antibacterial activity. Control of bacterial growth can be significantly related to the time when the transformant is treated. Additionally, the speed of fungal growth is possibly involved in the antibacterial activity.

Antibacterial activity of the infected mealworm powder

After the infection, transformant D-6 showed similar mortality to wild-type, and mycelial outgrowth (mycosis) was observed in D-6 and wild type 7 days post-infection (Fig. 3a). Any significant difference of mortality between wild-type and D-6 was not observed. In 5 days, >90 % of populations were dead in the fungal treatments (D-6 and wild type), but the untreated control showed <10 % mortality. The median lethal time (LT_{50}) of transformant D-6 was 3.2 ± 0.4 days, and it was similar to the LT_{50} of wild-type (3.7 ± 0.5 days). The fungal transformation possibly did not disrupt the virulence factors of *B. bassiana*, although it needs to be further investigated by the identification of the flanking region of the fungal genomic DNA using TAIL-PCR or iPCR method. The mortality might be accelerated by increasing the dose of inoculum or raising the relative humidity at fungal infection stage. The stage of *T. molitor* larvae when they were infected by the fungus might be another critical factor of mortality.

The D-6-infected mealworm suspension showed strong antibacterial activity against the two bacteria on LB medium in 3 days, but not in the wild type-infected mealworms (Fig. 3b). The *B. mori* cecropin A was possibly accumulated in *T. molitor* larvae, and the infected

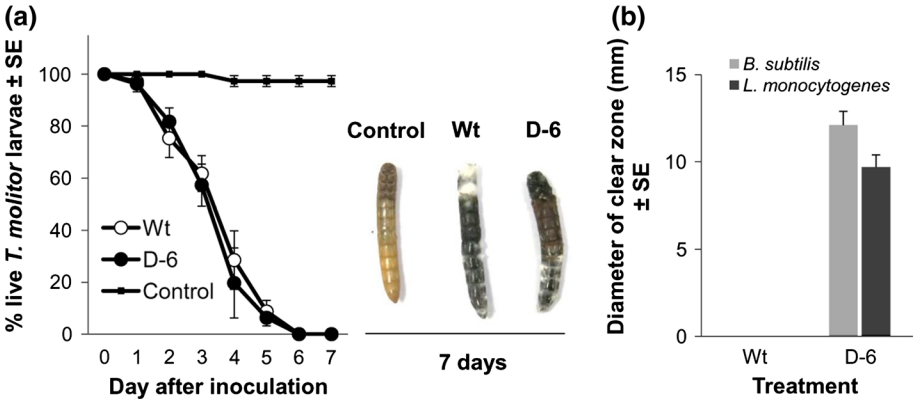

Fig. 3 Antibacterial activity of the transformant D-6-infected mealworm (*T. molitor*) powder. **a** Percentage (%) of live *T. molitor* larvae after the treatment of wild-type and D-6 conidial suspensions (1×10^7 conidia/mL). In 7 days, mycelial outgrowth was observed in both of wild-type and D-6 treatments; and **b** Antibacterial activity of the infected mealworm powder against *B. subtilis* and *L. monogenesis* on LB agar 3 days after treatment. Infected mealworms were freeze-dried and suspended in phosphate buffer saline at 1 % (w/v), which contained fluazinam as an antimycotics to inhibit fungal growth, and the infected mealworm suspension was dropped on a paper disk (5 μL/6-mm diameter disk)

mealworms could be used as an antibacterial material in animal feeds. However, one concern about the utility of the system is the reproducibility of the process (concentration of active cecropin A in the final product in different batches). It can be overcome when the transformant stably keeps the gene over the generations and inoculation factors such as larval stage and quantity of conidia are consistent in batches. Another concern is the safety of the final product containing *B. bassiana* proteins and the antibiotic resistance gene. *Beauveria bassiana* has been reported as safe to human and mammalian [13] and Bm cecropin A has been reported long times ago and now used as additives for foods [14]. However, we need to investigate the safety of the mealworm-*B. bassiana* complex in the following studies. The secreted protein in *T. molitor* larvae will be characterized in the next works, followed by the investigation of feasibility in industrialization.

Some other functional genes can be expressed in this mealworm-based fungal expression platform, such as those encoding edible vaccines, digestive enzymes or other functional peptides. The final mealworm-fungal complex can be used as an animal feed additive and its application possibly expands to human health care products. Integration of AMPs, edible vaccines and useful amino acids to *Cordyceps* species could significantly improve their biological activities [9].

This work suggests that *B. mori* cecropin A was successfully expressed in yellow mealworms by the infection of transformed *B. bassiana* ERL1170 containing the cecropin A-expression cassette. The transformant-infected mealworms showed strong antibacterial activity against *B. subtilis* and *L. monocytogenes*, thereby increasing the value of mealworms as animal feed additives.

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