# Suppression of a catalase gene (*catE7*) expression in an entomopathogenic fungus, *Beauveria bassiana,* by RNA interference

Chalermkiart Youngthong[1*], Duriya Chantasingh[2,] and KusolPootanakit[1]

[1]*Institute of Molecular Biosciences, Mahidol University, Salaya Campus, Phuttamonthon Distric, NakhonPathom 73170, Thailand*
[2]*Enzyme Technology Laboratory, Bioresources Technology Unit, National Center for Genetic Engineering and Biotechnology, 113 Thailand Science Park, Phahonyothin Rd., KhlongLuang, PathumThani 12120, Thailand*
**e-mail:arraideewa@gmail.com*

## Abstract

*Beauveria bassiana* is an entomopathogenic fungus that kills a broad range of insects. Recently, we found that after 2 hours of *Spodoptera exigua* larvae infection, a catalase enzyme (CatE7) was found to be up-regulated and that overexpression of this catalase in transgenic *B. bassiana* enhanced its toxicity. In this study, we further investigated the function of this catalase in relation to the pathogenesis of *B. bassiana via* RNA interference approach. Plasmid for knockdown of *catE7* gene was constructed and was transferred into *B. bassiana* by *Agrobacterium tumefaciens*-mediated transformation. Five transformed colonies were randomly selected for PCR amplification of silencing cassette. The result showed that three out of five fungal clones (#2, #3 and, #5) harbored intact silencing cassette. Catalase activity assay showed that the transgenics had lower catalase activity (24.70, 39.42 and, 14.99 U/mg protein) than the wild type (49.47 U/mg protein). Semi-quantitative RT-PCR of *catE7* transcript also showed that transcription level of *catE7* in the transgenic was decreased to the maximum of 94% (Bb::pPZP-SicatE7#5) when compared to the wild type. These results depicted the success of gene silencing in *B. bassiana* BCC2659.

**Keywords**: entomopathogenic fungus, catalase, RNAi, semi-quantitative RT-PCR

## Introduction

Nowadays, accumulating chemical insecticide increases environmental pollution and even more troubling is the difficulty in eliminating it. Furthermore, chemical insecticide also affects non-target organisms and gives rise to insecticide-resistance pests (Sharmaa et al., 2011). The biological control agents are alternative ways to solve these problems. These agents are organisms which are the pest natural enemies and have less environmental impacts (Zaim and Guillet, 2002, Crowder et al., 2010). However, biopesticides are less effective, in that, they require longer time to kill than chemical pesticides (Thomas and Read, 2007). To date, there are many organisms that can be used as biological control agents: viruses (Progar et al., 2010), bacteria (Federici, 2007, Romeis et al., 2006), nematodes (Malan et al., 2011) and fungi (Shah and Pell, 2003). Each species can be used to control pests on agricultural fields based on host, landscape and field condition.

The entomopathogenic fungi are a group of fungi at least 1,000 fungal species that act as pathogen of insects (Xiao et al., 2012). These fungal are widely distributed throughout many countries such as Thailand and China (Wang and Feng, 2014), and many of these fungi are used as commercial biocontrol products. Two particular fungal species, *Metarhizium anisopliae* and *Beauveria bassiana,* are popularly used in agricultural pest control. *M. anisopliae* has been shown to kill many insect species such as the legume flower thrips (Ekesi et al., 1998), locusts (Peng et al., 2008), ticks (Leemon et al., 2008) and weevils (Shah et al.,

2008). *B. bassiana* has been reported that it can control more than 700 species of insects (Faria and Wraight, 2007).

*B. bassiana*, belongs to the phylum Ascomycota, is a pathogen of broad host range and causes white muscardine disease in insects. It is used to control many insect species such as, moth (Shelton et al., 1998), bed bug (Barbarin et al., 2012), mites (Afifi et al., 2012), caterpillar (Wang et al., 2004), whitefly (Brownbridge et al., 2001) and grasshopper (Johnson and Goettel, 1993). Nevertheless, the pathogenesis against insects is complicated and the underlying molecular mechanism of fungal-host interaction is not completely understood. Fungal infection starts upon conidial adherence, followed by germination of germ tube and appressorium (penetration structure) formation. During the penetration process, the coordination of mechanical pressure, and the production of degrading-enzymes (proteases (Donatti et al., 2008), chitinases (Fang et al., 2005) and chitosanase (Fuguet et al., 2004)) are employed to break down the insect cuticle. In our lab, we found that after 2 hour of *Spodoptera exigua* larvae infection, 10 genes were shown to be upregulated ~ up to 1.20-2.20-fold compared to the control. A catalase (*catE7*) is one of those genes which is found to be up-regulated and *catE7*-overexpressing strain showed higher virulence against *S. exigua* larvae (Chantasingh et al., 2013). Therefore, to confirm whether CatE7 indeed contribute to fungal toxicity, its activity was suppressed in this study. Gene knockdown via RNA interference has been widely used to suppress gene expression in animals and plants (Tijsterman et al., 2002) and has been reported in fungi as well (Li et al., 2010). Thus, the aim of this study is to investigate the function of this catalase, CatE7, in relation to the pathogenesis of *B. bassiana via* RNAi approach.

## Methodology

### Strains and culture media

Wild type *B. bassiana* BCC2659 was obtained from the BIOTEC culture collection, Thailand. Fungal mycelia were grown on potato dextrose agar (PDA) at 25°C for 5 days and then conidia were collected in 0.05% Triton-X100. *Escherichia coli* DH5α, used for vector construction, was cultured in Luria-Bertani (LB) media containing appropriate antibiotics. *Agrobacterium tumefaciens* EHA105, used for fungal transformation, also cultured in LB medium.

### Construction of *catE7* silencing vector

To suppress *catE7* expression in *B. bassiana* BCC2659, the catE7 silencing vector was constructed. The sense and anti-sense strands of *catE7* partial fragments were cloned in a silencing expression cassette under TrpC promoter of pSilent-1 vector (Nakayashiki et al., 2005) in order to express double-stranded RNA (dsRNA) this vector has a strong fungal promoter (TrpC) and a cutinase intron from *Magnaporthe oryzae* as a spacer for hairpin dsRNA construction. pUC vector harboring *catE7* cDNA was used as template for *catE7* partial fragments amplification using *catE7*-specific primers (Table.1) under the following PCR condition: a hot-start of 2 min at 94°C; followed by 30 sec at 94°C, 30 sec at 55°C, 90 sec at 72°C for 30 cycles and, a final 10 min at 72°C (PCR ingredients: 1X DyNAzyme II buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM primers and, 1 u DyNAzyme II DNA Polymerase). Then, these fragments were cloned into pSilent-1 vector to obtain the pSicatE7. Next, the silencing cassette was removed from the vector backbones by *Xba*I digestion. The purified fragments were blunt-ended using T4 DNA polymerase and ligated to

pPZP-anbar which was prepared by *Eco*RI digestion and also blunt-ended using T4 DNA polymerase. These cassettes and vector were ligated to obtain pPZP-SicatE7.

Table.1 Primers were used in this study.

| Name | Primer sequence | Target |
|---|---|---|
| secatE7-F | 5'-AGATCTGTCTAGTAAATCCCTAGTCAGC -3' | *catE7* sequence |
| secatE7-R | 5'-GGTACCGACATTGGTCTGAGGGTGAC-3' | *catE7* sequence |
| anticatE7-F | 5'-CTCGAGGACATTGGTCTGAGGGTGAC-3 | *catE7* sequence |
| anticatE7-R | 5'-AAGCTTGTCTAGTAAATCCCTAGTCAGC-3' | *catE7* sequence |
| Bar2013-F | 5'-ATGAGCCCAGAACGACGCCC-3' | *bar* gene |
| Bar2013-R | 5'-AGATCTCGGTGACGGGCAGG-3' | *bar* gene |
| IT-F | 5'-GTACCCACTGGAGATTTGTTGGCCATGC-3' | Intron of *cut* gene |
| P11-E7-F | 5'-GGCACCATCAACGGCAACTAC-3' | *catE7* cDNA |
| P11-E7-R | 5'-CGTGAACCTTGCCAAGGTAATC-3' | *catE7* cDNA |
| Bbtub-F | 5'-GCACTTCTGAGCTTCAGCTC-3' | *β-tub* cDNA |
| Bbtub-R | 5'-CAGTGTAGTGACCCTTGGCC-3' | *β-tub* cDNA |

*A .tumefaciens*-mediated transformation of the catE7 silencing vector into *B. bassiana*

To generate a CatE7-knockdown strain, the catE7 silencing vector (pPZP-SicatE7) was transferred into *B. bassiana* BCC2659 through *A. tumefaciens*-mediated transformation. The recombinant plasmid was transformed into *A .tumefaciens* EHA105 by cold shock method (Holsters et al., 1978) and the transformants were selected on LB agar supplemented with 25 µg/ml kanamycin. Then, bacteria harboring recombinant plasmid was cultured in the induction medium (IM) (10 mM $K_2HPO_4$, 10mM $KH_2PO_4$, 2.5 mM NaCl, 2 mM $MgSO_4$, 0.7 mM $CaCl_2$, 9 µM $FeSO_4$, 4 mM $NH_4SO_4$, 10 mM glucose, 40 mM 2-[N-morpholino] ethanesulfonic acid, pH 5.3, 0.5% glycerol (w/v), 200µM acetosyringone) at 28°C until 0.4-0.5 $OD_{660}$ and subsequently co-cultured with *B. bassiana* conidia ($1\times10^7$ spore/ml) and plated onto cellophane filters on IM plates. After 48 h, membranes were transferred onto the selective medium (MC) (2% glucose, 0.6% $NaNO_3$, 2% salt solution, 0.04% trace solution, 1.5% agar) plate containing 200 µg/ml cefotaxime and 100 µg/ml ammonium glufosinate; and further incubated at 25°C for 7 days.

Screening of CatE7-knockdown *B. bassiana* strain

After 7 days incubation, the putative fungal clones were observed on MC agar plate. For the CatE7-knockdown transformants, five fungal clones were randomly selected for DNA isolation. In order to detect the selectable marker gene (*bar* gene), PCR was performed using *bar*-specific primers (Table.1) (PCR condition: a cycle of 2 min at 94°C; followed by 30 sec at 94°C, 30 sec at 70°C, 60 sec at 72°C for 30 cycles and, 10 min at 72°C) and to detect the integrated silencing cassette PCR was also perform using ITF/secatE7-R primers (Table.1) (PCR condition: a cycle of 2 min at 94°C; followed by 30 sec at 94°C, 30 sec at 55°C, 60 sec at 72°C for 30 cycles and, 10 min at 72°C) (PCR ingredients: 1X DyNAzyme II buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM primers and, 1 u DyNAzyme II DNA Polymerase). Fungal clones that showed the expected bands, were selected for further characterization.

Catalase activity assay

A wild type *B. bassiana* BCC2659 and three CatE7-knockdown strains were cultured in 100 ml of potato dextrose broth (PDB) at 25°C for 5 days. Then, the fungal cells were harvested and 1 g of cells were subsequently ground to a fine powder in liquid nitrogen with a mortar and pestle. The powder were suspended and mixed well in 5 ml Enzhance lysis protein extraction buffer (BIOTEC, Thailand). Total protein was collected by centrifugation at 9000g for 10 min at 4°C. The protein concentration was measured using the Bradford method. The total catalase activity was determined using Goth's colorimetric method as described (Goth, 1991). Specifically, 1 mg total protein was incubated with 65 mM $H_2O_2$ in 60 mM sodium phosphate buffer, pH 7.4 at 37 °C for 1 min and then the reaction was stopped with 32.4 mM ammonium molybdate. The yellow complex was measured at 405 nm and catalase activity (U/mg) was calculated against three blanks.

Semi-quantitative RT-PCR analysis

Total RNA of wild type strain and three candidate CatE7-knockdown strains were isolated using RNeasy Plant Mini Kit (Qiagen, Germany). Then, cDNA was synthesized from 4 mg of total RNA using oligo-dT primer. The reaction was started at 70°C for 5 min and then subsequently mixed with the mixtures of 5x RT buffer, 10 mM dNTPs, 1 u of Rionuclease inhibitor at 37°C for 5 min. 200 u of RevertAid H Minus Reverse Transcriptase was added and then followed by incubation at 42°C for 60 min. The reaction was stopped at 70°C for 10 min. Multiplex PCR amplification were performed by using *catE7*-specific primers (Table.1) and *β-tubulin*-specific primers (Table.1) as internal control. The PCR condition was performed at a hot-start at 94°C for 2 min; followed by 94°C for 30 sec, 58°C for 30 sec, and 72°C for 10 min for 25 cycles (PCR ingredients: 1X DyNAzyme II buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 mM primers and, 1 u DyNAzyme II DNA Polymerase). After that, the PCR products were loaded on 2% agarose gel. The band intensity was analyzed using Scion Image program (Scion Corporation, USA).

**Results and Discussions**

Construction of CatE7-knockdown *B. bassiana* strain

To generate the CatE7-knockdown *B. bassiana* strain, the catE7 silencing vector, pPZP-SicatE7, was constructed. Then, the *catE7* silencing cassette of pSicatE7 vector was cloned into pPZP-anbar, a family of *Agrobacterium* binary vectors, in order to transfer the silencing cassette into fungal genome via *A. tumefaciens*-mediated transformation (5 transformants/µg DNA). After 7 days of incubation, 5 fungal transformants, growing on MC agar containing ammonium glufosinate, were randomly selected for DNA isolation. PCR result showed that all of five clones showed the expected band which corresponded to the *bar* gene (Fig.1A). However, only three clones (#2, #3 and, #5) showed the correct pattern of the silencing cassette (Fig.1B).

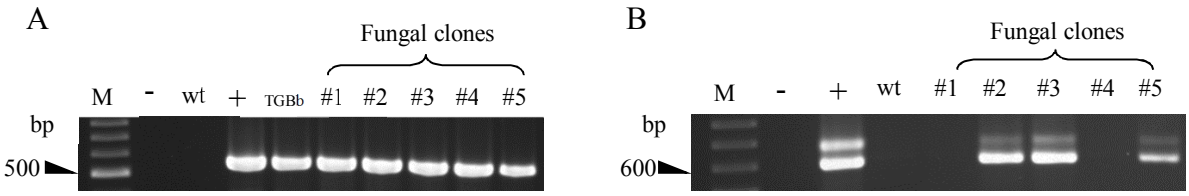


**Figure 1.** Screening of CatE7-knockdown *B. bassiana* strain. These figures showed the PCR amplification of *bar* gene (A) and the silencing cassette (B) in the nuclear genome from randomly selected *B. bassiana* transformants. M = 100 bp ladder; - = negative control; + = positive control; wt = wild type strain; TGBb = transgenic *gfp* strain.

Suppression of catE7 expression in CatE7-knockdown *B. bassiana* strain

Next, total catalase activity was characterized in the three candidate CatE7-knockdown strains, clone#2, #3 and, #5. The result showed that the activity of the three fungal clones, #2, #3 and, #5 were 24.70, 39.42 and, 14.99 U/mg protein, respectively; on the other hand, the wild type BCC2659 as expected showed the highest catalase activity of 49.47 U/mg protein (Fig.2A). To determine if this loss of catalase activity is due to the lower expression of CatE7, semi-quantitative multiplex RT-PCR was performed. The result showed that the relative expression of *catE7* gene in fungal clones (#2, #3 and #5) was decreased to 93%, 52% and, 94% respectively, when compared to the wild type (Fig2B-C). These results demonstrated that we have successfully suppressed CatE7 expression.

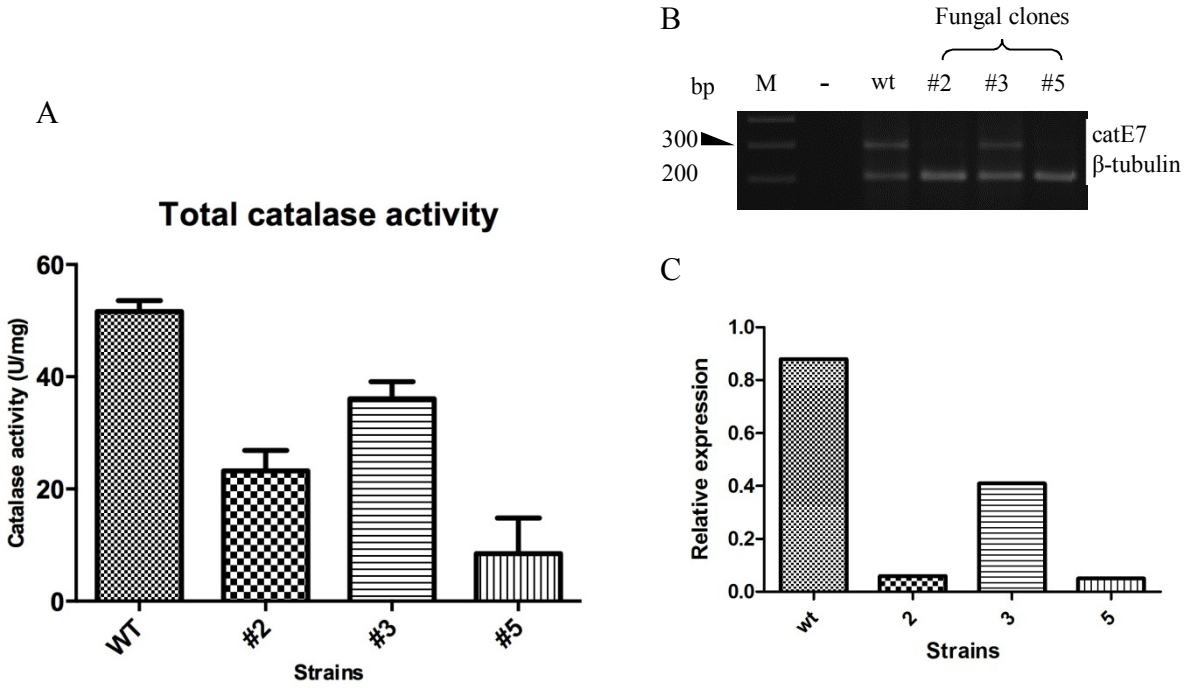


**Figure 2**. Total catalase activity and transcriptional analysis of CatE7-knockdown *B. bassiana* strain. (A) Catalase activity of three fungal clones compared to the wild type strain. (B) Agarose gel profiling of catE7 transcripts in wild type and three fungal clones (C)

Relative expression of catE7 transcripts; β-tubulin served as internal control. M = 100 bp ladder; - = negative control; wt = wild type strain.

**Conclusion**

In this study, we used RNAi technique to knockdown the catE7 expression in the filamentous fungus, *B. bassiana* BCC2659. The catE7 expression was successfully suppressed which leads to the decrease of catalase activity in the fungal transformants.

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