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Characterization of T-DNA insertion mutants with decreased virulence in the entomopathogenic fungus *Beauveria bassiana* JEF-007

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Abstract The bean bug, *Riptortus pedestris*, is a major agricultural pest that reduces crop quality and value. Chemical pesticides have contributed to pest management, but resistance to these chemicals has significantly limited their use. Alternative strategies with different modes of action, such as entomopathogenic fungi, are therefore of great interest. Herein, we explored how entomopathogenic fungi can potentially be used to control the bean bug and focused on identifying virulence-related genes. *Beauveria bassiana* (JEF isolates) were assayed against bean bugs under laboratory conditions. One isolate, JEF-007, showed >80 % virulence by both spray and contact exposure methods. *Agrobacterium tumefaciens*-mediated transformation (AtMT) of JEF-007 generated 249 random transformants, two of which (B1-06 and C1-49) showed significantly reduced virulence against *Tenebrio molitor* and *R. pedestris* immatures. Both species were used for rapid screening of virulence-reduced mutants. The two transformants had different morphologies, conidial production, and thermotolerance than the wild type. To determine the localization of the randomly inserted T-DNA, thermal asymmetric interlaced (TAIL) PCR was conducted and analysis of the two clones found multiple T-DNA insertions (two in B1-06 and three in C1-49). Genes encoding complex I intermediate-associated protein 30 (*CIA30*) and the autophagy protein (*Atg22*) were possibly disrupted by the T-DNA insertion and might be involved in the virulence. This work provides a strong platform for future functional genetic studies of bean bug-pathogenic *B. bassiana*. The genes putatively involved in fungal virulence should be experimentally validated by knockdown in future studies.

Keywords *Agrobacterium tumefaciens*-mediated transformation · *Beauveria bassiana* · *Riptortus pedestris* · Thermal asymmetric interlaced PCR · Virulence

Introduction

Bean bug *Riptortus pedestris* (Fabricius) (Hemiptera: Alydidae) causes serious damage to legumes in Asian countries. Adults appear in the field in the spring and have two or three generations per year. The first generation appears in late June to July and the second in early August to the middle of September. Adults overwinter and are commonly found in weeds or debris at the base of the crops. *R. pedestris* causes damage by sucking the bean pod and sometimes vector plant pathogenic fungi through the wound (Bae et al. 2014).

One of the ways to control bean bugs is the use of synthetic insecticides. However, the bugs are very mobile and thus avoid treatments and they have developed insecticide resistance (Maharjan and Jung 2015). Chemical pesticides can be toxic to the environment and humans. Pest resistance and environmental impacts of chemical pesticides create many opportunities for research and the development of novel and environmentally sound strategies of management.

Sihyeon Kim and Se Jin Lee contributed equally to this work

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The use of entomopathogenic fungi (Ascomycota: Hypocreales) can be an alternative pest control strategy. Over 700 different fungal species from at least 90 genera are known to be insect pathogens (Khachatourians and Sohail 2008). Entomopathogenic fungi have different modes of action from the synthetic insecticides. Most chemical pesticides disrupt the insect nervous system and/or metabolic pathways including ATP synthesis and biosynthesis of functional molecules (Klowden 2007), whereas entomopathogenic fungi invade the insect hosts through the cuticle. These fungi are environmentally sound and widely available (Roberts and Hajek 1992) and can be used against hemipteran, lepidopteran, coleopteran, and dipteran insects (Faria and Wraight 2007). Formulations of wettable powder and suspension concentrates have been developed. Various fungal-based biopesticides containing *Beauveria bassiana*, *Metarhizium anisopliae*, *Isaria*, and *Lecanicillium* spp. have been developed for use against pests in forests, fields, and greenhouses (Parker et al. 2014).

Infection of the host begins when the conidia of entomopathogenic fungi contact the insect cuticle and germinate. These conidia can penetrate directly through the cuticle by hyphal development and release of several cuticle-degrading enzymes and metabolites such as chitinases and proteases (Charnley 2003; Fang et al. 2005; Gupta et al. 1992). Once the insect cuticle has been penetrated, the fungus multiplies in the hemocoel and uses all the nutritive materials available (Charnley 2003). This results in the death of the host. Then, the fungus will exit the host and grow externally and produce conidia for epizootics (Parker et al. 2014). During the infection process, entomopathogenic fungi secrete immunosuppressive compounds, often considered to be secondary metabolites that protect the fungus against host defense mechanisms (Trienens and Rohlfs 2012).

The use of fungi as biological control agents has received much attention, but the underlying pathogenic mechanisms have not been fully explored. Several studies have been reported on screening of pathogenesis-related genes in a variety of entomopathogenic fungal isolates, especially in *B. bassiana* (Fan et al. 2011; Ying et al. 2013; Zhang et al. 2010a). Concurrently with genome sequencing of *B. bassiana*, the era of functional genomics of this species is opening, such as the identification of fungal secondary metabolites and genetic characterization (Gibson et al. 2014; Ortiz-Urquiza and Keyhani 2015; Pendrini et al. 2013; Song et al. 2015).

Many genes can be involved in the pathogenesis of entomopathogenic fungi. One of the approaches to figuring out the pathogenicity-related genes is the use of *Agrobacterium tumefaciens*-mediated transformation (AtMT). Although AtMT has been commonly used to transform plant cells (Bundock et al. 1995), it was also used to transform filamentous fungi (Groot et al. 1998). *A. tumefaciens* can transfer its T-DNA to *B. bassiana*, *Paecilomyces fumosoroseus*, and *M. anisopliae*, as to plants (Duarte et al. 2007; Lima et al. 2006; Reis et al. 2004).

In this study, we characterized the virulence of *B. bassiana* JEF isolates against bean bugs by spray and contact exposure methods and selected one highly virulent isolate, JEF-007, to determine which genes are possibly involved in the pathogenesis. AtMT of JEF-007 was performed to randomly produce *B. bassiana* transformants, and these were then subjected to thermal asymmetric interlaced (TAIL)-PCR to investigate the localization of the T-DNA to predict possible pathogenesis-related genes.

Materials and methods

Rearing of *R. pedestris*

A colony of bean bugs was provided by the Dongbu Advanced Research Center of Dongbu Farm Hannong Agrochemical Company, Korea, and reared in a plastic cage (35 × 35 × 40 cm^3) at Chonbuk National University. Two sheets of cheese cloth were suspended on each side of the cage for oviposition. Based on the results of a host plant preference test comparing green beans and peas, green beans were selected as the primary food source. Green beans (Sungwoo Seeds Co., Jeonju, Korea) were planted every 2 or 3 weeks and used to feed the bean bugs. Additionally, green bean seeds were placed in a dish in the cage. To synchronize the development of bean bugs for bioassays, eggs laid on the sheets were removed at 3 to 5 days after placing the mating adults in the cage and transferred to a fresh cage. Insects were reared at 25 ± 2 °C, 40 ± 5 % relative humidity (RH), under a 16:8 (L/D) photoperiod.

Rearing of *T. molitor*

A colony of yellow mealworms was obtained from the Rural Development Administration, Korea, and reared in a plastic cage (20 × 20 × 10 cm^3) covered with mesh for ventilation. Wheat bran was fed to *T. molitor* larvae and a piece (ca. 3 × 3 cm^2) of Chinese cabbage was provided to adults to maximize oviposition. To synchronize the development of mealworms for bioassays, mating adults were transferred to a fresh cage and kept there for ca. 5 days. The cages were held at 25 ± 2 °C and 40 ± 5 % RH with a 16:8 (L/D) photoperiod.

Microbial strains and culture media

B. bassiana ERL-1170, ERL-1050, ERL-1577, and ERL-836 were obtained from the Entomology Research Laboratory (ERL) Worldwide Collection of Entomopathogenic Fungi of the University of Vermont, Burlington, VT, USA. *B. bassiana*

JEF-105, JEF-007, JEF-013, JEF-006, and JEF-114 were isolated from soil in Korea and stored in the Jeonbuk (the prior name of Chonbuk) National University Entomopathogenic Fungal Library (JEF). Isolate, JEF-007 which was used in the gene characterization study, was deposited in the Korean Federation of Culture Collection (KFCC) (=prior name of Korean Culture Center of Microorganism (KCCM) which belongs to the WDCM in Korea (deposit number of JEF-007: KCCM11592P). The internal transcribed spacer (ITS) regions of the isolates were amplified by PCR using the following primers: ITS1-f, 5'-TCC GTA GGT GAA CCT GCG G-3', and ITS4-r, 5'-TCC TCC GCT TAT TGA TAT GC-3'. A thermal cycler (C-1000, Bio-Rad™, Hercules, CA, USA) was preheated to 95 °C for 5 min, and then PCR was performed using 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s, followed by a 10-min final extension at 72 °C, and storage at 12 °C. PCR products were analyzed by electrophoresis on 0.8 % agarose gels in 1× TBE buffer (Sambrook et al. 2001), sequenced, and blasted against the NCBI database (http://blast.ncbi.nlm.nih.gov/blast.cgi) for identification (ITS sequence of JEF-007 was submitted to GenBank (accession number: KT280275)). All isolates were stored in 20 % (*v*/*v*) glycerol at −80 °C and cultured on quarter-strength Sabouraud dextrose agar (¼SDA; Difco, Lawrence, KS, USA) in the dark at 25 ± 1 °C. Detailed information about the isolates is provided in Table S1 in the Supplementary Material.

Virulence assay

Virulence of the isolates vs. mealworm larvae and bean bug nymphs was evaluated using a contract exposure and spray. In the contact exposure bioassay, 10 isolates from the JEF library were evaluated. The 10 isolates previously showed high virulence against mealworm larvae immediately following isolation from soil. Conidial suspensions were prepared using 0.03 % (*v*/*v*) siloxane solution (Silwet, Dongbu-Hannong, Seoul, Korea) containing 1×10^7 conidia/ml and spread on ¼SDA plate. Fungal isolates were cultured on ¼SDA at 25 °C for 7 days. Ten fourth instar bean bug nymphs were placed on a cultured plate for 1 h, then transferred to a fresh Petri dish (90 × 15 mm) layered with damp filter paper. Two beans were added to each dish for nutrition, and the dishes were kept at 25 ± 2 °C and 60 % RH. To maintain high humidity conditions, 1 ml of sterile water was added to the dishes daily. Numbers of live and dead bean bugs were counted daily for 5 days post-treatment. In the spray assay, four isolates were chosen based on their virulence levels in the contact exposure assay. The isolates were cultured on ¼SDA at 25 °C for 7 days. Conidial suspensions containing 1×10^7 conidia/ml were prepared using 0.03 % (*v*/*v*) siloxane solution. Ten fourth instar bean bugs were sprayed with 3 ml of fungal suspension. Treated nymphs were placed in a screened cage ($10 \times 10 \times 4$ cm^3) with damp layered filter paper and kept at 25 ± 2 °C (60 % RH). Two beans were added to each dish for feeding. To maintain high humidity conditions, 1 ml of sterile water was added daily. Numbers of live and dead bean bugs were counted daily for 10 days post-treatment. Mycotized bean bugs with hyphal growth and sporulation were observed under a microscope (×6.7 magnification). Each treatment was replicated three times (three replicates) in the two bioassay experiments.

A. tumefaciens-mediated transformation of JEF-007

A. tumefaciens GV3101 with pCAMBIA-egfp, which was constructed by the Insect Microbiology and Biotechnology Laboratory, Chonbuk National University, Korea (Nai et al. 2015), was used for random transformation of JEF-007. *A. tumefaciens* containing pCAMBIA-egfp was inoculated in Luria-Bertani (LB; Difco, Lawrence, KS, USA) medium containing 50 μl/ml rifampicin and 50 μl/ml kanamycin and incubated at 28 °C with shaking at 150 rpm for 16–20 h. Bacterial cultures were diluted to an absorbance value of 0.15 using induction medium (IM) (10 mM K_2HPO_4, 10 mM 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*), and 200 μM acetosyringone) (final volume 20 ml) (Covert et al. 2001) and cultured at 28 °C with shaking at 150 rpm for 4–6 h until an optical density of ca. 0.4 at 660 nm was obtained. For co-cultivation of *A. tumefaciens* GV3101-pCAMBIA-egfp with JEF-007, JEF-007 conidia were collected from a 7-day-old culture on a ¼SDA plate and suspended in 0.03 % (*v*/*v*) siloxane solution to the concentration of 1×10^5 conidia/ml. The culture was mixed with an equal volume of a conidial suspension of JEF-007 (1×10^5 conidia/ml). This mixture (70 μl) was plated on co-cultivation medium (same as IM except containing 5 mM glucose instead of 10 mM glucose). Following co-cultivation at 28 °C for 2 days, the mixture was harvested with 1 ml of 0.03 % (*v*/*v*) siloxane solution for each 60 mm plate and the harvested mixture (70 μl) was then spread on ¼SDA plates containing hygromycin B (150 μg/ml) as the selection agent for fungal transformants and cefotaxime (300 μg/ml) to inhibit the growth of *A. tumefaciens* cells. These plates were incubated at 28 °C. Putative transformants were observed after 3 or 4 days. Subsequently, 249 fungal putative transformants received (coded of alphabet and numbers) were transferred to Czapek's agar supplemented with 150 μg/ml hygromycin B and sub-cultured for two generations (7 days for each generation) on Czapek's agar supplemented with 150 μg/ml hygromycin B for the experiments described below.

Screening JEF-007 random transformants with reduced virulence

To select transformants with reduced virulence, mealworm larvae were used as the first test insect. One of the reasons we used mealworms rather than bean bugs was the availability of a large number of test insects in a given period of time (to assay all 249 transformants). JEF-007 transformants were cultured on ¼SDA with 150 μg/ml of hygromycin B at 25 °C for 7 days. Wild-type JEF-007 was cultured on ¼SDA without antibiotics under the same conditions. Five fourth instar mealworms were placed on the cultured plate (five larvae/transformant) for 10 days, and the numbers of live and dead larvae were counted daily. This simple and qualitative bioassay method was used due to the large number of transformants to be investigated. After this initial screening, the transformants with reduced virulence were subjected to a second assay against mealworm larvae for re-confirmation and also against bean bug nymphs. The second screening was quantitatively conducted using the same protocol described above.

PCR-based confirmation of transformants

PCR was conducted to confirm insertion of T-DNA from GV3101-pCAMBIA-egfp into two JEF-007 transformants with reduced virulence. Genomic DNA was extracted from 7-day-old fungal mycelial masses using the quick fungal genomic DNA extraction method (Chi et al. 2009), and the presence of the T-DNA fragment was examined by PCR using AccuPower® PCR PreMix (Bioneer, Daejeon, Korea) with the following primer sets: T-DNA-f: 5'-CCC GAC CAC ATG AAG CA-3'/T-DNA-r: 5'-TGT GCC CCA GGA TGT TG-3' (Tm = 60 °C). *B. bassiana* 18S rDNA fragment served as an internal control (*Bb* 18S-f: 5'-TTA CGT CCC TGC CCT TTG TA-3'/*Bb* 18S-r: 5'-CCA ACG GAG ACC TTG TTA CG-3'). PCR amplifications were performed as follows on a C-1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA): preheating at 95 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s, followed by a 10-min final extension at 72 °C, and storage at 12 °C. PCR products were analyzed by electrophoresis on 0.8 % agarose gels in 1× TBE buffer (Sambrook et al. 2001).

Examination of conidiogenesis

The two virulence-reduced transformants were cultured on ¼SDA medium supplemented with 150 μg/ml of hygromycin by inoculating 50 μl (1×10^7 conidia/ml) of conidial suspension onto the center of an agar plate. Growth was observed by keeping the plates at 25 °C for 3, 5, 7, and 11 days. Wild-type JEF-007 was cultured on ¼SDA with/without antibiotics (hygromycin B) as controls. Conidiogenesis was observed under an optical microscope (×400), and the numbers of conidia were counted using a hemocytometer.

Conidial thermotolerance

To evaluate the thermotolerance of the transformants, 1.5 ml Eppendorf tubes (E-tubes) containing mycotized agar blocks (cultured as above for 11 days) with 1 ml of 0.03 % (*v*/*v*) siloxane solution were shaken on a vortex mixer (Vortex-Genie 2TM; VWR Scientific, New York, NY) for 30 s. To harvest the conidia, mycotized agar blocks were used so as not to disrupt the conidia as they were produced on the medium. All conidial suspensions were transferred to fresh E-tubes (500 μl per tube) and held in an incubator at 45 ± 1 °C for 30, 60, 90, and 120 min. Controls (non-exposed conidial suspensions) were kept at room temperature (25 ± 2 °C). A 10-μl sample was taken from each tube and dropped on ¼SDA medium for a germination test prior to and after exposure. After incubation (25 °C for 18 h), germination rates (%) were determined by randomly counting the number of germinated conidia among 100 counts microscopically (×400). Each treatment was replicated three times.

TAIL-PCR

Genomic DNA of transformants was extracted from 7-day-old cultured plates using the cetyltrimethyleammonium bromide (CTAB) method (Doyle and Doyle 1990). Before use, the CTAB buffer was incubated at 65 °C for 15 min and mixed with 2-mercaptoethanol (1:100, *v*/*v*). Fungal mass (ca. 1×1 cm^2) was harvested from 7-day-old cultured plates and added to the mixed solution. The fungal mass was homogenized in the liquid suspension and incubated at 65 °C for 10 min. The solution was then mixed with 400 μl of $CHCl_3$/isoamyl alcohol (24:1, *v*/*v*, stored at 4 °C) with vigorous vortexing and inverted for 2–3 min. After centrifugation at 12,000×*g* at 4 °C for 10 min, the upper layer (350 μl) was transferred to a fresh E-tube. Then, 400 μl of isopropanol (IPA) was added to the tube and mixed gently by inverting it 10 times. After incubation for 5 min at room temperature, the tube was centrifuged at 12,000×*g* and 4 °C for 5 min, and the supernatant was removed. The centrifuged pellet was dried at 30 °C for 5 min using a speed vacuum drier, and 600 μl of 1× TE buffer was added to the pellet and the solution was pipetted about 20 times to ensure complete resuspension of the pellet. Approximately 300 μl of phenol/$CHCl_3$/isoamyl alcohol (25:24:1, *v*/*v*/*v*, storage at 4 °C) was then added followed by centrifugation at 12,000×*g* at 4 °C for 5 min, and then 400 μl of the upper layer was transferred to a fresh tube. One microliter RNase A (10 mg/ml) was added to the tube, and the tube was vortexed and incubated at room temperature for 5 min. The supernatant was removed from the tube after centrifugation at 12,000×*g* at 4 °C for 5 min, 1 ml of 70 % EtOH was

added, and the supernatant removed. After washing the pellet with 70 % EtOH, it was dried at 30 °C for 5 min and dissolved in 50 μl of elution buffer (EB).

To determine the flanking regions of the inserted T-DNAs (localization of T-DNA), TAIL-PCR was performed based on the protocol of Liu et al. (1995). An arbitrarily degenerate primer (AD 3) (5'-WGT GNA GWA NCA NAG A-3') and three T-DNA border-specific primers were used per round of TAIL-PCR cycling. The T-DNA-specific primers used were as follows: LB1 (5'-TTC CTA AAA CCA AAA TCC AG- 3'), LB2 (5'-ATT CGG CGT TAA TTC AGT AC-3'), and LB3 (5'-AAA AAC GTC CGC AAT GTG TT-3'). *Bb* 18S rDNA primers were used as the internal control. All TAIL-PCR reactions were carried out on a C-1000 thermocycler. PCR reactions were performed as detailed in Table S2 in the Supplementary Material using AccuPower® PCR Premix (Bioneer, Daejeon, Korea). Specific PCR products from the tertiary TAIL-PCR reactions were extracted from agarose gels using the QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA, USA) and cloned into *Escherichia coli* using a cloning kit (All-in-One™ PCR cloning vector, Biofact, Cat. No. VT201-020, Daejeon, Korea) to minimize sequencing errors. Cloned products were sequenced by Macrogen (http://dna.macrogen.com/kor/).

Sequence analysis

Bowtie 2 (http://bowtie-bio.sourceforge.net), a memory efficient tool for aligning sequencing reads to long reference sequences, was used to analyze the flanking regions of T-DNA inserted into the fungal genome. Due to lack of the full genomic sequence of JEF-007, the *B. bassiana* ARSEF2860 genome (ADAH01.1. fsa_nt.gz 33,410 KB), which is available in GenBank (http://www.ncbi.nlm.nih.gov/genbank/), was used for this analysis. The analysis was done as follows: command of building the index (reference genome): bowtie2-build c (a main drive of a computer):\bt2_home/bb/reference/ADAH01.1. fsa_nt cba, command of aligning: bowtie2-align-f cba read_standard (sequencing data file).fq. Approximately 500 bp of TAIL-PCR products was subjected to the bowtie2 program for alignment to *Bb* genome (ARSEF2860). The genetic similarity of ARSEF 2860 and JEF-007 isolates is unknown and could have influenced the mapping results; further studies are currently underway to determine the genetic similarity of these two isolates. Based on alignment of the sequencing data to the reference genome, flanking regions, including LB3 and AD3 sequences, were identified and received GenBank accession numbers and submitted to GenBank BlastX for prediction of disrupted genes. T-DNA insertion positions were calculated by adding 44 bp (length of LB3 sequence and the rest of LB of T-DNA) to the position of LB3 of TAIL-PCR products (for example, the T-DNA insertion position in B1-06 no. 6 is 218,939 bp + 44 bp = 218,983 bp). The predicted genes reflect one flanking side of the inserted T-DNA, particularly left border of the insert DNA.

Results

Virulence of *B. bassiana* isolates against bean bug

The isolates showed high virulence against bean bugs under laboratory conditions. In the contact exposure assay, bean bug cadavers were observed 1 day after treatment. Among the 10 isolates, treatment with JEF-007, JEF-013, JEF-105, and ERL-836 resulted in 80, 75, 85, and 90 % mortality, respectively, 3 days post-treatment (Fig. 1a), and mycosis of bean bugs (sporulated bugs) was observed after 5 days (Fig. 1c). In the sporulated cadavers, hyphae were observed in the weak parts of the insect cuticles, inter-segments of legs, antenna, abdomen, and thorax, and conidiogenesis was observed on the surface of the cuticles. Spray treatment resulted in death of nymphs 3 days after treatment. Among the four isolates selected from the contact exposure assay, JEF-007 resulted in 60 % mortality at day 6 post-spray and >95 % mortality at day 10 (Fig. 1b). Compared to the contract exposure assay, the spray treatment took approximately twice as long to yield the same mortality.

Generation of JEF-007 transformants

JEF-007 showed hygromycin dosage-dependent growth, and growth was completely inhibited at 200 μg/ml hygromycin B. AtMT was conducted using a hygromycin B concentration of 150 μg/ml. No fungal growth was observed at the 150-μg/ml dosage in further assays (Fig. 2a). To construct the fungal transformation vector, the 2386-bp *egfp*-expression cassette under the *gpdA* promoter (P*gpdA*) and *trpC* terminator (T*trpC*) was digested from pBARKSI (Fungal Genetics Stock Center, USA) using *Hin*dIII and *Sac*I. The *egfp*-expression cassette was integrated into pCAMBIA (10,550 bp) that had been digested using *Hin*dIII and *Nco*I, and finally pCAMBIA-egfp (11,850 bp) was constructed (Fig. 2b). After generation of *A. tumefaciens* GV3101-pCAMBIA-egfp and fungal transformation, growth of many JEF-007 transformants was observed on ¼SDA supplemented with 150 μg/ml hygromycin (Fig. 2c). Little hyphal growth was observed when wild-type JEF-007 was inoculated; however, >200 colonies were observed in the co-culture of *A. tumefaciens* GV3101-pCAMBIA-egfp and JEF-007. Infection of bean bugs with one *egfp*-expressing transformant (not virulence-reduced transformant) resulted in the expression of green fluorescence, where the gene expression was confirmed (Fig. 2c).

(a) Contact-exposure assay

Treatment	% mortality of *R. pedestris* nymph (Mean ± SE) (days after treatment)					
	0	1	2	3	4	5
Control	0.0 ± 0.0	0.0 ± 0.0	5.0 ± 7.1	5.0 ± 7.1	5.0 ± 7.1	5.0 ± 7.1
ERL-836	0.0 ± 0.0	10.0 ± 0.0	75.0 ± 35.4	90.0 ± 14.1	100.0 ± 0.0	100.0 ± 0.0
ERL-1050	0.0 ± 0.0	20.0 ± 14.1	40.0 ± 14.1	45.0 ± 21.2	50.0 ± 14.1	65.0 ± 21.2
ERL-1170	0.0 ± 0.0	10.0 ± 0.0	35.0 ± 21.2	40.0 ± 28.2	40.0 ± 28.2	60.0 ± 28.3
ERL1575	0.0 ± 0.0	0.0 ± 0.0	35.0 ± 35.4	40.0 ± 28.2	55.0 ± 7.1	85.0 ± 7.1
ERL1578	0.0 ± 0.0	5.0 ± 7.1	40.0 ± 42.4	60.0 ± 42.4	70.0 ± 28.3	80.0 ± 14.1
JEF-006	0.0 ± 0.0	10.0 ± 0.0	45.0 ± 21.2	60.0 ± 28.3	70.0 ± 28.3	95.0 ± 7.1
JEF-007	0.0 ± 0.0	0.0 ± 0.0	70.0 ± 42.4	80.0 ± 28.3	85.0 ± 21.2	100.0 ± 0.0
JEF-013	0.0 ± 0.0	10.0 ± 0.0	45.0 ± 21.2	75.0 ± 21.2	85.0 ± 21.2	100.0 ± 0.0
JEF-105	0.0 ± 0.0	10.0 ± 0.0	50.0 ± 14.1	85.0 ± 21.2	100.0 ± 0.0	100.0 ± 0.0
JEF-114	0.0 ± 0.0	0.0 ± 0.0	20.0 ± 14.1	55.0 ± 7.1	85.0 ± 7.1	100.0 ± 0.0

(b) Spray assay

Treatment	% mortality of *R. pedestris* nymph (Mean ± SE) (days after treatment)					
	0	2	4	6	8	10
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	20.0 ± 20.0	20.0 ± 20.0	26.7 ± 23.1
ERL-836	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	6.7 ± 11.6	33.3 ± 30.6	46.7 ± 11.6
JEF-007	0.0 ± 0.0	0.0 ± 0.0	13.3 ± 11.6	60.0 ± 20.0	66.7 ± 11.6	93.3 ± 11.6
JEF-013	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	6.7 ± 11.6	33.3 ± 11.6	33.3 ± 11.6
JEF-105	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	20.0 ± 34.6	26.7 ± 30.6	26.7 ± 30.6

(c) Mycosis of *R. pedestris* nymph

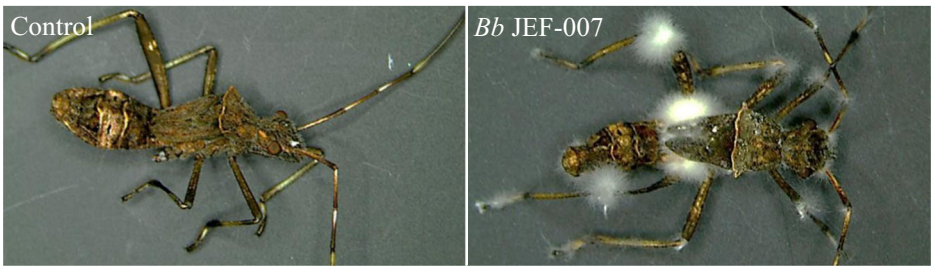


Fig. 1 Virulence of *B. bassiana* isolates against fourth instar *R. pedestris* nymphs in laboratory conditions. Cumulative mortalities of *B. bassiana*-infected bean bugs after treatment using the (**a**) fungal contact exposure method or (**b**) spraying with conidial suspensions at 1×10^7 conidia/ml. (**c**) Symptoms of infected bean bugs at 5 days after fungal treatment

Selection of virulence-reduced transformants using mealworm larvae

The virulence of the 249 transformants towards larvae was investigated, and among the transformants, 16 showed significantly lower virulence than wild type (Fig. 3a). In the second bioassay using the 16 transformants, two (B1-06 and C1-49) showed significantly lower virulence than the other selected transformants (Fig. 3b). Wild-type treatment resulted in 100 % mortality 3 days after contact exposure under Petri dish conditions. In contrast, treatment with the transformant B1-06 and C1-49 resulted in lower mortality rates of 33.33 and 3.33 %, respectively, compared to wild type ($F_{17,\ 36} = 19.6$, $p < 0.001$).

Reduced virulence of transformants against mealworms and bean bugs

The virulence of the two virulence-reduced transformants towards mealworm larvae and bean bug nymphs was reassessed. Transformants B1-06 and C1-49 were significantly less virulent than the wild type (Fig. 3c). The two transformants showed similarly low levels of virulence against mealworms ($F_{3,}$

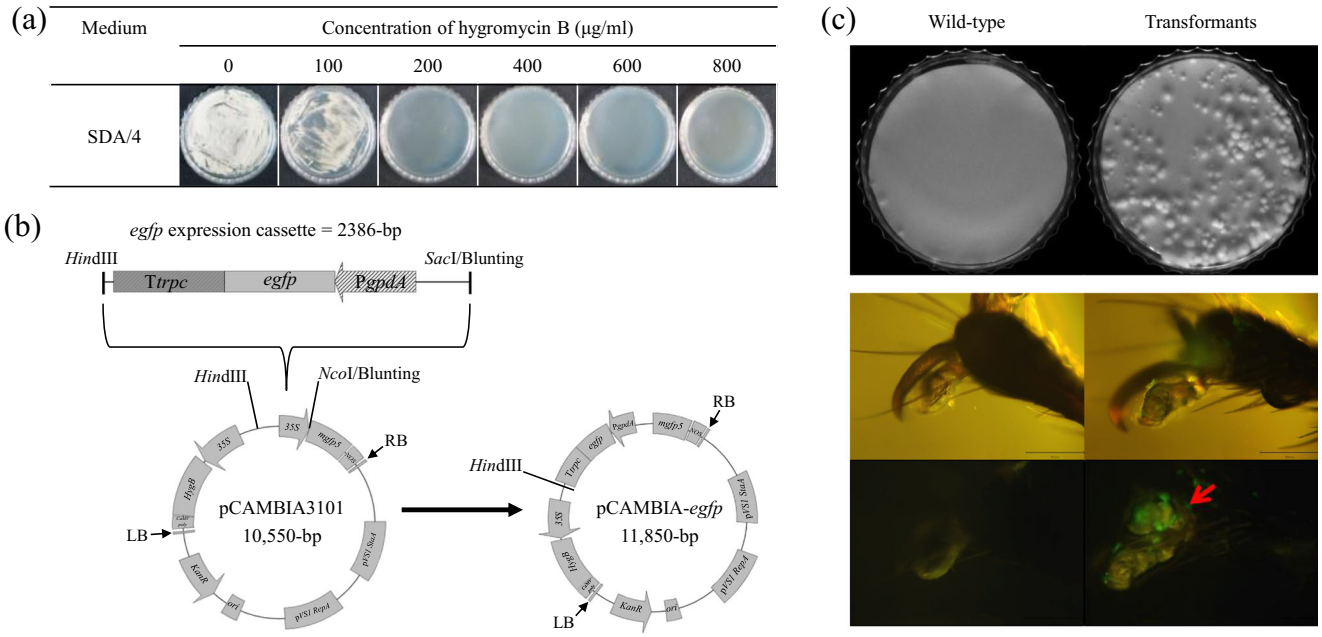


Fig. 2 Generation of *B. bassiana* JEF-007 transformants using AtMT. (**a**) Hygromycin B susceptibility testing of *B. bassiana* JEF-007. Five different concentrations of hygromycin B were used (100, 200, 400, 600, and 800 μg/ml), and fungal growth on the plates was observed at 7 days post-incubation. (**b**) Construction map of pCAMBIA-egfp used for AtMT of *B. bassiana* JEF-007. (**c**) Selection of *B. bassiana* JEF-007 transformants. Wild type and transformants were plated on ¼SDA with 150 μg/ml hygromycin B. Fungal transformants formed >100 colonies with hygromycin B resistance. In contrast, less than five colonies were observed for the wild type at 10 days after incubation. The presence of transformants was confirmed by green fluorescence in the legs of bean bugs after fungal infection

$_{12}$ = 78.1, $p < 0.001$) and bean bug nymphs ($F_{3,\ 12}$ = 53.3, $p < 0.001$) compared to the wild type. The LT_{50} (lethal time) of mealworm larvae against B1-06 and C1-49 transformants was 4.8 (4.1~5.5) days and >12 (not detectable) days, respectively, and the LT_{50} of bean bug adults against the two transformants could not be calculated due to the very low virulence.

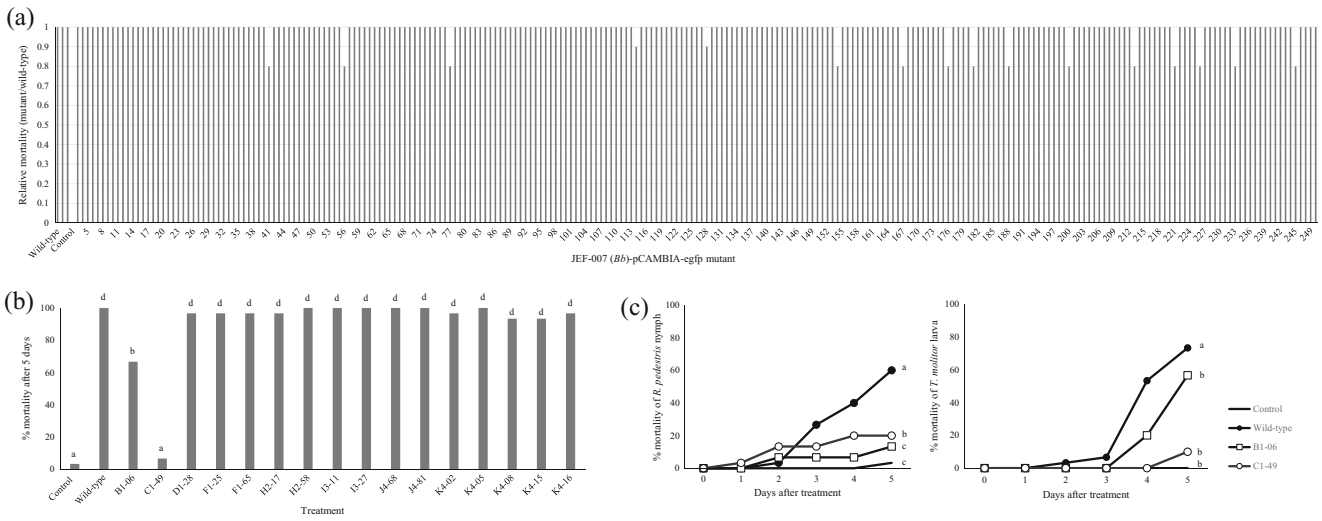


Fig. 3 Selection of virulence-reduced transformants. (**a**) Virulence assay of all 249 JEF-007(*Bb*)-pCAMBIA-egfp transformants. The virulence of each transformant was determined against *T. molitor* (*Tm*) larvae 5 days after infection. The mortality caused by the transformants was normalized to that caused by wild-type JEF-007. A total of 16 virulence-reducing transformants with a relative mortality less than 1 were identified. (**a**) B1-06; (**b**) C1-49; (**c**) D1-28; (**d**) F1-25; (**e**) F1-65; (**f**) H2-17; (**g**) H2-58; (**h**) I3-11; (**i**) I3-27; (**j**) J4-68; (**k**) J4-81; (**l**) K4-2; (**m**) K4-5; (**n**) K4-8; (**o**) K4-15 and (**p**) K4-16. (**b**) Mortality of *T. molitor* (*Tm*) larvae due to treatment with JEF-007(*Bb*)-pCAMBIA-egfp transformants on ¼SDA under laboratory conditions (10 mealworms per treatment, three repetitions). (**c**) Mortality of *T. molitor* larvae and *R. pedestris* nymphs against the two virulence-reduced transformants, B1-06 and C-49 in laboratory conditions (10 mealworms and bean bugs/treatment, three repetitions). In 5 days after the treatment, means with the same lower case letters were not significant different according to the Tukey's HSD ($p > 0.05$)

Morphological changes in the transformants

PCR of the two virulence-reduced transformants resulted in detection of the T-DNA target band (196 bp) in all PCR products except those from the wild type (Fig. 4a). *Bb* 18s rRNA PCR product (166 bp) was amplified from all transformants and the wild type. The B1-06 and C1-49 transformants had a different morphology than the wild type (Fig. 4b). The wild type grew slowly on ¼SDA supplemented with hygromycin B, and no hyphal growth was observed even 11 days after inoculation. In contrast, when the wild type was grown on ¼SDA medium without antibiotics, vigorous hyphal growth with the production of liquid forms of secondary metabolites on the hyphal mass after 7 days and conidial production after 11 days were observed. The C1-49 transformant formed yellowish colonies on ¼SDA with hygromycin B after 3 days, and growth of pink hyphae was observed at 11 days, which was significantly different to the wild type. No liquid drops were observed on the hyphal mass of C1-49. Faster growth of whitish hyphae was observed for B1-06 than the wild type on antibiotic-containing medium. The two transformants were also cultured on the nonselective medium and showed similar fungal growth to those on the selective medium (data not shown). Conidiogenesis of B1-06 was not significantly different from that of wild type, but C1-49 showed very poor conidiogenesis (Fig. 4c). In comparison with wild type (3.6×10^7 conidia/agar block (0.28 cm^2)), C1-49 produced fewer conidia (1.8×10^6 conidia/0.28 cm^2) after 11 days (Fig. 4d). When exposed to thermal stress, both transformants showed higher thermotolerance than the wild type (Fig. 4e). Of the two transformants, C1-49 had the highest germination after thermal exposure. After 60 min of exposure to 45 °C, the wild type showed 2.1 % germination while C1-49 showed 35.1 % germination, followed by B1-06 (18.0 %).

Localization of T-DNA

We purified PCR products ~500 bp in size after TAIL-PCR of the two virulence-reduced transformants followed by gel electrophoresis and cloned these bands for sequencing, and colony PCRs were conducted (Fig. 5a). In the gel electrophoresis of the colony-PCR products, the 12 colonies including B1-06 TAIL-PCR products and the 30 colonies including C1-49 TAIL-PCR products showed about 500 bp in size, but each product varied slightly in size. Sequencing data (Table S3 in the Supplementary Material) were subjected to align analysis using the Bowtie 2 program. Align analysis of the B1-06 and C1-49 TAIL-PCR products showed two (B1-06 no. 2 and no. 6) and three (C1-49 no. 15, no. 20, and no. 23) localizations of the T-DNA in the *B. bassiana* genome, respectively (Fig. 5b). In transformant B1-06, the T-DNA was inserted at position 74,123 bp in JH725175 (418,271 bp) and position 218,983 bp in JH725176 (394,139 bp). In transformant C1-49, T-DNA was inserted at position 362,999 bp in JH725151 (1,964,398 bp), position 90,631 bp in JH725165 (662,422 bp), and position 241,055 bp in JH725164 (690,658 bp). Based on the blastX, the flanking regions in transformants B1-06 and C1-49, as predicted, were possibly disrupted (Fig. 5b). In B1-06, the 218,939~219,253-bp locus (GenBank accession number: KU663176) was predicted to be a gene for complex I

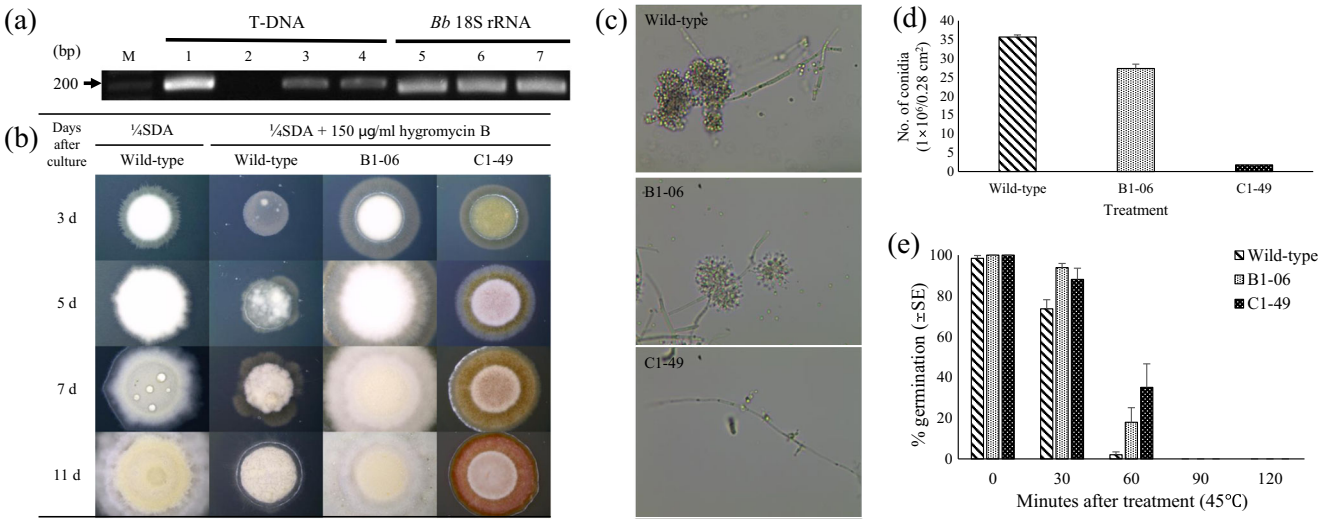
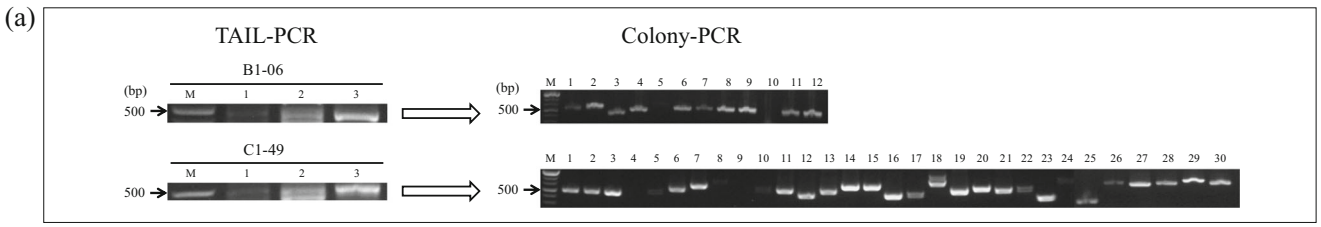


Fig. 4 Characterization of the virulence-reduced transformants, B1-06 and C1-49. (**a**) T-DNA insertions in the fungal genome were confirmed by PCR. T-DNA fragments were amplified from genomic DNA of fungal mutants. *Lane 1*, pCAMBIA-egfp plasmid as the positive control; *lanes 2 and 5*, wild type; *lanes 3 and 6*, B1-06 transformant; *lanes 4 and 7*, C1-49 transformant. *Bb 18S rRNA* was used as an internal control. (**b**) Hyphal growth of transformants and wild type was observed on ¼SDA at 3, 5, 7, and 11 days after inoculation. B1-06 and C1-49 transformants grew on ¼SDA with 150 μg/ml hygromycin B. (**c**) Conidia of transformants and wild type were observed under a microscope (×400). The fungi grew on ¼SDA and were examined at 5 days after inoculation. (**d**) Production of conidia on ¼SDA at 11 days after inoculation. (**e**) Conidial germination rate (mean ± SE) of JEF-007-pCAMBIA-egfp transformants and wild type on ¼SDA after exposure to a temperature of 45 °C



(b)

Code		Localization of TAIL-PCR product in *Bb* ARSEF2860 genome	Disrupted gene (analyzed by BlastX)	E-value	Identity (%)	Primer sequence (5'-3') for TAIL-PCR (3rd)
Transformant	Colony number					
B1-06	#2	74,079 bp (LB3) KU663175 (AD3) 74,454 bp JH725175 (418,271 bp) T-DNA insertion at 74,123 bp	-			LB3: TTT TTG CAG GCG TTA CAC AA AD3: AGA GAC GAT GAC GTG T
	#6	218,939 bp (LB3) KU663176 (AD3) 219,253 bp JH725176 (394,139 bp) T-DNA insertion at 218,983 bp	Complex I intermediate-associated protein 30 (XP_008601056)	4e-35	98%	LB3: TTT TTG CAG GCG TTA CAC AA AD3: AGT GGA GAA ACA CAG A
C1-49	#15	362,955 bp (LB3) KU663177 (AD3) 363,457 bp JH725151 (1,964,398 bp) T-DNA insertion at 362,999 bp	-			LB3: TTT TTG CAG GCG TTA CAC AA AD3: AGA GAC AAA GAA GTG A
	#20	90,587 bp (LB3) KU663178 (AD3) 91,032 bp JH725165 (662,422 bp) T-DNA insertion at 90,631 bp	Hypothetical protein BBAD15_g8425 (KGQ06260)	0.17	86%	AD3: AGT GCA GTA CCA CAG A LB3: AAC ACA TTG CGG ACG TTT TT
	#23	240,847 bp (AD3) KU663179 (LB3) 241,099 bp JH725164 (690,658 bp) T-DNA insertion at 241,055 bp	Autophagy protein (Atg22) (XP_008598965)	2e-15	93%	LB3: TTT TTG CAG GCG TTA CAC AA AD3: AGA GAC CAA GAC GTG A

Fig. 5 Prediction of disrupted genes by T-DNA localization using TAIL-PCR. **(a)** The flanking regions of T-DNA in B1-06 and C1-49 were determined using TAIL-PCR. TAIL-PCR was performed by first performing PCR with LB1 and AD3 primers (*1*), then with LB2 and AD3 primers (*2*), and finally LB3 and AD3 primers (*3*). Amplicons around 500 bp in size were extracted and cloned for sequencing. Colony PCRs of the *E. coli* clones were conducted before sequencing. In the gel electrophoresis of the colony-PCR products, the 12 colonies including B1-06 TAIL-PCR products and the 30 colonies including C1-49 TAIL-PCR products showed about 500 bp in size, but each product varied slightly in size. **(b)** The localization of the sequence flanking T-DNA insertion in the Bb ARS 2860 genome and encoded protein of disrupted genes (analyzed by BlastX). T-DNA insertions were determined using TAIL-PCR with an arbitrary degenerate primer 3 (*AD3*) and a T-DNA border specific primer (*LB3*). T-DNA insertion locations in the reference genome are indicated by *arrows*

intermediate-associated protein 30 (*e* value = 4e-35). Next, in transformant C1-49, the 90,587~91,032-bp locus (GenBank accession number: KU663178) was predicted to be a gene for hypothetical protein BBAD15_g8425 (*e* value = 0.17), and the 241,099~240,847-bp locus (GenBank accession number: KU663179) was predicted to be a gene for Autophagy protein (Atg22) (*e* value = 2e-15).

Discussion

Bioassay of *B. bassiana* isolates against bean bugs revealed a significant difference in virulence between contact exposure and spray methods. Isolates ERL-836, JEF-007, JEF-013, and JEF-105 caused bean bug mortality of 100 % in 5 days after exposure in Petri dishes. The same isolates in the spray-based bioassay had lower virulence: only JEF-007 resulted in ca. 90 % mortality after 10 days, while the rest showed <40 % virulence. One reason for the difference in mortality between the two methods could be the amount of inoculum that the insects received on the surface of their cuticles. In the contact exposure method, bean bugs were definitely inoculated with many fungal conidia, which likely resulted in multiple conidial attacks and rapid pathogenesis and death. In contrast, the spray method delivered a relatively small number of conidia to the surface of the bean bug cuticle. Nevertheless, JEF-007 treatment still resulted in higher mortality than treatment with the other isolates, suggesting that this isolate may be useful in pest management under field conditions. In terms of screening for efficacious isolates for development, the spray assay provides more realistic information than the contact exposure assay. However, the contact exposure assay could be used to screen bean bug-pathogenic isolates because of its simplicity. In the spray assay, the concentration of fungal conidia in the inoculum should be quantified.

Of the tested isolates, JEF-007 showed the highest virulence against bean bug. It was isolated from the cadaver of a lepidopteran in Korea (Lee et al. 2015). This isolate has a broad spectrum and high virulence against several agricultural pests (Lee et al. 2015). Several factors could be involved in high virulence against bean bugs. Rapid conidial germination and hyphal growth are important for penetration. Penetration of the epicuticle and pro-cuticles, including exo- and endo-cuticles, requires enzymatic degradation; JEF-007 might release relatively larger amount of virulence-related enzymes, such as a variety of lipases, proteases, and chitinases, than less virulent isolates.

JEF-007 could also potentially disrupt the host immune system by targeting insect local melanization and antimicrobial peptide synthesis though the Toll pathway (Lu and St. Leger 2016), thereby allowing it to effectively colonize the body of the insect and feed off the insect. These hypotheses require validation by using tools such as RNA-seq.

In the first screening of virulence-reduced transformants, mealworm larvae were used rather than bean bug nymphs. Many random transformants were generated, and we needed to select possible candidates with reduced virulence. It was not feasible to prepare larger numbers of bean bug nymphs for this work, which is why we used mealworm larvae. Far less time and labor were required for rearing mealworms than bean bugs. It was also much easier to handle the mealworm larvae in the bioassay. Bean bug nymphs were very active and therefore needed to be placed in a cold room or treated with CO_2 gas to reduce their mobility to enable their transferal to Petri dishes for bioassay.

The two virulence-reduced transformants showed lower virulence than wild-type JEF-007. In the second quantitative bioassay with the spraying method, the same numbers of conidia were used; thus, virulence-related genes could be knockdowned by the AtMT and resulted in the reduced virulence, not due to the tenfold decrease in conidiation. Many genes might be involved in entomopathogenic fungal pathogenicity, and some virulence-related genes appear to have been disrupted by insertion of T-DNA into the genomic DNA of JEF-007. The pathogenic mode of action of entomopathogenic fungi involves several enzymes that mainly degrade the host cuticle (Kim et al. 2010). Chitinases, lipases, proteases, and aminopeptidases can be produced by the hyphae of entomopathogenic fungi and secreted into the insect hemolymph (Butt et al. 2016). Fungal penetration through the host insect cuticle is the first step of pathogenesis (Holder and Keyhani 2005), and before the hyphal penetration, a mitogen-activated protein kinase (MAPK) signaling pathway plays an important role in recognizing extracellular signals, as described in yeast (Herskowitz 1995). The gene of MAPK *Bbmpk1* is essential for appressoria formation and penetration of the insect cuticle, and lack of *Bbmpk1* results in a relatively much lower virulence (Zhang et al. 2010b). A recent study clearly demonstrated that debilitation/slow germination is a significant factor of the virulence of conidia in insects (Faria et al. 2015). In our study, the transformant C1-49 produced fewer conidia than wild type, suggesting that conidiogenesis-related genes might be disrupted in the C1-49 transformant. Ras GTPase and mitogen-activated protein kinase are among functions responsible for conidial germination and hyphal growth of entomopathogenic fungi (Hanif 2004).

In this work, T-DNA insertions in B1-06 and C1-49 transformants were confirmed by PCR. However, the two PCR products were relatively small in intensity (lanes 3 and 4), which could be due to low copy number insertion in the fungal genome or low primer amplification efficiency, although the intensity between positive control (plasmid) and fungal genomic DNA is incomparable. The transformant C1-49 showed yellowish hyphal growth, but the other transformant, B1-06, showed white hyphal growth, similar to wild-type JEF-007. One of the reasons for the yellowish growth of C1-49 on ¼SDA with hygromycin B might be due to the inability of this transformant to move beyond the hyphal growth stage to conidiogenesis or very slow conversion to conidiogenesis. In fact, observation of C1-49 growth under a microscope revealed that this isolate produced very few conidia compared to B1-06 and wild type. Instead of generation of conidia in the C1-49 transformant, secondary metabolites, which might not be involved in the pathogenesis, could have been produced. During the initial stage of culture of C1-49, yellowish growth was observed, but after 11 days, the fungal mass had a pink color, which could be related to production of secondary metabolites. In the C1-49 transformant, some energetic resources might not be used in conidiogenesis but for the production of other non-pathogenesis-related metabolites, for example thermotolerance-related materials.

The thermotolerance of the two transformants was higher than that of wild type, and C1-49 was the most thermotolerant isolate. With regard to fungal survival in an unfavorable environment, a fungus that produces only a few conidia might require more abiotic tolerant materials than isolates that produce a larger number of conidia. Previous studies have shown that some proteins, such as for G protein signaling (RGSl) (Fang et al. 2008), formic acid-extracted (FAE) protein (Ying and Feng 2004), and heat shock protein 1 (Hamid et al. 2013), could be involved in fungal thermotolerance. However, the mechanisms by which these potential proteins mediate fungal thermotolerance are not clear.

We used the random primer AD3 in TAIL-PCR among several random primers that we examined. The efficiency of four different random primers was tested with the combination of three gene-specific primers (LB1, LB2, and LB3), but only the AD3 combination resulted in detectable PCR products (data not shown). TAIL-PCR amplified a band of around 500 bp, but direct sequencing of the PCR product revealed the presence of multiple sequences, which is why the PCR products were first cloned and then subjected to the sequencing. Alignment analysis found some unexpected sequences that contained partial gene-specific primer (LB3) sequences, partial AD3 primer sequences, or did not contain either gene-specific or AD3 primer sequences. In this work, TAIL-PCR provided some information about the regions flanking the inserted T-DNA in the genome of *B. bassiana* (two in B1-06 and three in C1-49) and how many T-DNAs were inserted into the genome (copy number).

Although we did not perform Southern blotting to determine the copy number of T-DNA, TAIL-PCR provided this type of information. Our main focus was selection of

virulence-reduced transformants among a total of 249 colonies using a simple bioassay, rather than screening for one-copy transformants, which is time-consuming work. Use of transformants with a single T-DNA insertion would provide much clearer results in terms of predicting disrupted genes involved in reduced virulence. However, given that the screening efficiency of virulence-reduced transformants was ca. 0.8 % (2 among 249 transformants) in this work, this strategy (bioassay of single copy transformants) would not have worked effectively for identification of virulence-related genes in *B. bassiana* JEF-007. In fact, based on the TAIL-PCR analysis, if Southern blot was conducted to select transformants with a single copy, no virulence-related genes would have been found.

In the alignment analysis, the *B. bassiana* ARSEF 2860 whole genome shotgun sequence from GenBank was used as the reference genome because the genome of JEF007 has not been sequenced. The success of alignment indicates that the *B. bassiana* JEF007 and ARSEF 2860 genomes are highly similar. Additional validation studies need to be conducted to confirm this, but some discussion of the roles of the above genes is merited.

In the transformant C1-49, the gene for an autophagy protein (Atg22) was disrupted. The Atg22 protein has an important self-degradation mechanism to balance energy sources in response to nutrient stress and in development as shown in yeast. After degrading cellular components, essential nutrients are recycled again for survival (Yang et al. 2006). Autophagy-related protein Atg22 is involved in vacuolar efflux for tyrosine and has an indirect effect on autophagic body breakdown at the late stage of autophagy (Yang et al. 2006). Moreover, in the *Atg22* deletion mutant, leucine is accumulated in the vacuole in *Saccharomyces cerevisiae* (Yang et al. 2006), suggesting the disruption of *Atg22* could interfere with recycling of amino acids (tyrosine and leucine) and normal functions of autophagy. Furthermore, autophagy plays an essential role in filamentous fungi for cellular development and pathogenicity, such as appressorial turgor accumulation in host penetration and damage host defense. It has a similar role of nutrient-sensing than the cyclic AMP pathway and the mitogen-activated protein kinase cascade in fungal pathogenicity (Khan et al. 2012). The molecular mechanisms of the relationships between Atg22 and conidiation, pigmentation, and virulence are interesting issues for fungal biology.

In transformant B1-06, the gene encoding protein complex I intermediate-associated protein 30 (CIA30) was possibly disrupted by the insertion of the T-DNA. Compared to the C1-49 transformant, this B1-06 transformant showed similar morphology to the wild-type JEF-007 but significantly reduced virulence against the bean bug; thus, the *CIA30* gene is more likely to be related to fungal virulence rather than morphology or development. The *CIA30* gene is a complex I (NADH-ubiquinone oxidoreductase) early assembly factor that is involved in the first step of electron transfer chain in inner mitochondrial membrane (Lazarou et al. 2009). Knockdown of the *CIA30* gene in human cells using RNA interference technique resulted in mitochondrial complex I deficiency (Vogel et al. 2005). Disruption of the fungal *CIA30* gene might reduce energy of the fungal productions and thus retard the fungal infection process. However, the functional roles of the *CIA30* gene in fungal virulence are still unclear and need further verifications.

In conclusion, we assayed the virulence of entomopathogenic *B. bassiana* JEF isolates against bean bugs under laboratory conditions and the JEF-007 isolate was selected as a possible control agent for development. To determine virulence-related genes, two random mutants with reduced virulence were generated by AtMT of bean bug-pathogenic JEF-007, and possible virulence-related genes (*CIA30* and *Atg22*) were predicted. This work provides a strong platform for further functional genetics of bean bug-pathogenic *B. bassiana* and provides insights into fungal pathogenesis. The roles of the identified genes in pathogenesis need to be validated in future experimental studies.

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Compliance with ethical standards This article does not contain any studies performed on human participants or vertebrate animals.

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Conflict of interest The authors declare that they have no competing interests.

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