

## Carboxylate Transporter Gene *JEN1* from the Entomopathogenic Fungus *Beauveria bassiana* Is Involved in Conidiation and Virulence<sup>∇</sup>

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***Beauveria bassiana* is an important entomopathogenic fungus widely used as a biological agent to control insect pests. A gene (*B. bassiana JEN1* [*BbJEN1*]) homologous to *JEN1* encoding a carboxylate transporter in *Saccharomyces cerevisiae* was identified in a *B. bassiana* transfer DNA (T-DNA) insertional mutant. Disruption of the gene decreased the carboxylate contents in hyphae, while increasing the conidial yield. However, overexpression of this transporter resulted in significant increases in carboxylates and decreased the conidial yield. *BbJEN1* was strongly induced by insect cuticles and highly expressed in the hyphae penetrating insect cuticles not in hyphal bodies, suggesting that this gene is involved in the early stage of pathogenesis of *B. bassiana*. The bioassay results indicated that disruption of *BbJEN1* significantly reduced the virulence of *B. bassiana* to aphids. Compared to the wild type,  $\Delta BbJEN1$  alkalized the insect cuticle to a reduced extent. The alkalization of the cuticle is a physiological signal triggering the production of pathogenicity. Therefore, we identified a new factor influencing virulence, which is responsible for the alkalization of the insect cuticle and the initiation of fungal pathogenesis in insects.**

Mycoinsecticides are considered promising biological control agents and alternatives or supplements to chemical pesticides (15). However, the dearth of physiological, genetic, and molecular knowledge of entomopathogenic fungi has retarded their widespread application.

For mycoinsecticide improvement, greater attention and effort have been given to elucidate the mechanisms of fungal pathogenesis (13, 14, 18, 20, 29, 49, 50, 51, 52, 53). Entomopathogenic fungi, e.g., *Metarhizium anisopliae* and *Beauveria bassiana*, invade their hosts by direct penetration of the host exoskeleton or cuticle. *M. anisopliae* and *B. bassiana* produce hydrophobic spores which contact and adhere to the insect cuticle (12). Once attached, the conidium germinates and the germ tubes differentiate into swollen infection structures called appressoria. The appressoria produce penetration pegs which penetrate the insect cuticle via cuticle-degrading enzymes (11, 19, 46) as well as mechanical pressure (24, 53). Hyphae proliferate within the hemocoel, emerge from inside the insect, and subsequently conidiate on the cadaver (15). However, much remains to be elucidated regarding the mechanisms of insect fungal pathogenesis.

To obtain detailed knowledge of the mechanisms of fungal pathogenesis, a pool of *B. bassiana* transfer DNA (T-DNA) insertional mutants had been generated through an *Agrobacterium*-mediated-transformation method (21). A mutant, designated T12, characterized by the presence of more conidia, was isolated, and its flanking sequence was obtained by T-DNA tagging. The flanking fragment contained an open read-

ing frame (ORF), which corresponded to a gene termed *JEN1*, encoding a transporter of carboxylates (<http://www.ncbi.nlm.nih.gov/Blast.cgi>). Organic acid transportation is important for the metabolism of almost all cells of multicellular organisms and unicellular microorganisms (17, 25, 26). Transport across the plasma membrane is the first step in the metabolism of these substrates, which may affect many aspects of the organism, including regulation of energy metabolism (9, 34) and acid-base equilibrium status (10).

*JEN1p* has been identified in several fungal species, e.g., *Saccharomyces cerevisiae*, *Candida albicans*, and *Kluyveromyces lactis* (9, 35, 45), which is a lactate/pyruvate symporter (1, 9, 34). The enzyme imports lactate or some short-chain monocarboxylates across the plasma membrane into cells. Then, the lactate is stereo-specifically oxidized to pyruvate. This reaction is performed by ferricytochrome *c* oxidoreductase in mitochondria (23, 33) and is tightly connected to the respiratory chain (34). *JEN1* was induced by lactic, pyruvic, acetic, and propionic acids and repressed by glucose (2, 9, 35, 45). Nevertheless, for entomopathogenic fungi, the characterization of *JEN1p* has not been investigated, and its role in infection is still a mystery.

For this paper, we studied the functions of a putative carboxylate transport gene, *JEN1*, in *B. bassiana* (*BbJEN1*). Our results demonstrated that *BbJEN1* is involved in conidiation of *B. bassiana* and that the gene is a new factor influencing virulence in entomopathogenic fungi.

### MATERIALS AND METHODS

**Strains, growth conditions, and DNA manipulations.** *B. bassiana* wild-type strain Bb0062 and its cultivation were previously described (19). For the repression and derepression study, conidia were cultivated in liquid Sabouraud's dextrose supplemented with 1% (wt/vol) yeast extract (SDY medium; pH 7.0) at 26°C on a rotary shaker at 180 rpm for about 48 h, and the hyphae were harvested ( $0.2 \pm 0.01$  g [wet weight]) and then transferred to 25-ml volumes of minimal medium (0.5% sodium nitrate, 0.03% sodium chloride, 0.03% magne-

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TABLE 1. Primers used in this work

Primer	Sequence (5'–3') <sup>a</sup>	Restriction enzyme site
P1	<u>GAATTC</u> GTGATCAGTCAGGCATGT	EcoRI
P2	<u>GCGGCCG</u> CAATTGTATGGACTATAG	NotI
P3	<u>GCGGCCG</u> CATGTTACGTCCTGT	NotI
P4	<u>GGATCCT</u> CATTGTTTGCCTC	BamHI
P5	<u>GCGGCCG</u> CTCGCCACCATGGTGAGCAA	NotI
P6	<u>GGATCCT</u> TAGGACCTGTACAGCTCG	BamHI
P7	<u>GAATTC</u> CGTGACG GCAAAAAGCAA	EcoRI
P8	<u>GAATTC</u> AAGACCATTGGGCCACTTG	EcoRI
P9	<u>TCTAGAG</u> GTCTTTCCATCGCTGTT	XbaI
P10	<u>AAGCTT</u> TGGCCTTTCCACATCCT	HindIII
P11	<u>GCGGCCG</u> CTCCCACAGTCATGGCCACT	NotI
P12	<u>GGATCCT</u> CAACTACTTCTCACCTCG	BamHI
P13	<u>CCGTCAG</u> TATCATCCAGTAAAG	
P14	<u>AGTACTT</u> AGGGGAAATA	
P15	<u>ATGGCA</u> ATGCCATTGGCCATG	
P16	<u>TTTACCT</u> CGGAAAGCTCGAC	

<sup>a</sup> Underlined sequences are restriction sites.

sium sulfate, 0.03% dipotassium hydrogen phosphate [wt/vol]) containing different sole carbon sources at hourly intervals. The substrates were 2% (wt/vol) glucose, 2% (wt/vol) acetate (pH 6.0), 2% (wt/vol) oxalate (pH 6.0), 2% (wt/vol) citrate (pH 6.0), 2% (wt/vol) lactate (pH 6.0), 2% (wt/vol) pyruvate (pH 6.0), 2% (wt/vol) pyruvate (pH 6.0) plus 2% (wt/vol) glucose, 2% (wt/vol) cicada slough, and 2% (wt/vol) cicada slough plus 2% (wt/vol) glucose. Standard procedures for DNA manipulations were followed (43). *Escherichia coli* strain DH5 $\alpha$  was used for routine bacterial transformations and maintenance of plasmids.

**Plasmid construction.** Plasmids pBT and pBFT contained the herbicide resistance gene *bar* and the herbicide resistance protein and enhanced green fluorescent protein (GFP) fusion gene *bar::egfp*, respectively.

On the basis of pBT, a vector containing the *BbJEN1p::gus* cassette was constructed. *BbJEN1p* (the *BbJEN1* promoter) and *gus* were amplified from the genomic DNA of *B. bassiana* and pBANF-bar-pAN-GUS (21), respectively, using primers P1 and P2 and primers P3 and P4, respectively (Table 1). The resultant fragments were cloned into the pUC vector (Sangon) and sequenced. The *BbJEN1* promoter, approximately 2.8 kb, was excised from the cloning vector by EcoRI and NotI and fused upstream of *gus* to form pUC-BbJEN1P-*gus*. *trpCt* (the *trpC* terminator) was recovered from pAN52-1 (21) by BamHI and XbaI digestion and ligated downstream of *gus*. pBT was excised by HindIII and XbaI, and a *BbJEN1p::gus::trpCt* fusion gene was inserted, resulting in pBT-BbJEN1P-*gus*.

On the basis of pBT-BbJEN1P-*gus*, a vector containing the *BbJEN1p::egfp* cassette was constructed. The *egfp* gene was amplified by PCR from the plasmid pEGFP-C1 (GenBank accession number U55763; BD Biosciences Clontech, CA) by using the P5 and P6 primers (Table 1) and cloned into the pUC vector. The *egfp* gene was recovered from the cloning vector by NotI and BamHI digestion and inserted into pBT-BbJEN1P-*gus*, replacing *gus* to form pBT-BbJEN1P-*egfp*.

On the basis of pBFT, vectors overexpressing *BbJEN1* and vectors disrupting *BbJEN1* were constructed. To disrupt *BbJEN1* in *B. bassiana* on the basis of homologous recombination, the herbicide resistance protein and enhanced green fluorescent protein fusion gene (*bar::egfp*) cassette was inserted into *BbJEN1*. The 5' end of *BbJEN1* was cloned by PCR with primers P7 and P8 (Table 1). The resultant PCR product was digested with EcoRI and inserted into the EcoRI site of pBFT to form pBFT-BbJEN1L. The 3' end of *BbJEN1* was amplified with primers P9 and P10 (Table 1). The PCR product was then digested with XbaI and HindIII and cloned into the corresponding sites of pBFT-BbJEN1L to form pBFT-BbJEN1L/R.

To overexpress *BbJEN1* in *B. bassiana*, the vector overexpressing *BbJEN1* was constructed. The construction of the *BbJEN1* overexpression vector pBFT-BbJEN1 was conducted as follows. Primers P11 and P12 (Table 1) were used to amplify *BbJEN1*, using *B. bassiana* genomic DNA as the template. The resultant PCR product was cloned into pGEM-T (Promega) to form pGEM-BbJEN1 and sequenced for confirmation. *BbJEN1* was excised from pGEM-BbJEN1 by NotI and BamHI digestion and inserted into pBFT with NotI and BamHI digestion to form pUC-PgpdA-BbJEN1-TrpC. The resultant plasmid was digested with the

EcoRI restriction enzyme, and the sticky ends were made blunt using T4 DNA polymerase and digested with XbaI. The 4.7-kb cleaved DNA fragment was recovered and inserted into pBFT to form pBFT-BbJEN1.

**Fungal transformation and transformant screening.** Fungal transformations were conducted as previously described (30). Genomic DNA of the *B. bassiana* wild-type strain and the transformants was extracted according to a previously described method (41). PCR analysis was performed to screen the pBT-BbJEN1P-*gus*, pBT-BbJEN1P-*egfp*, and *BbJEN1*-disrupted transformants by using primers P3 and P4, P5 and P6, and P13 and P14, respectively. Southern blot analysis was used to confirm disruption of *BbJEN1* in *B. bassiana* transformants. Reverse transcription-PCR (RT-PCR) analysis was performed to screen *BbJEN1* overexpression transformants by using primers P15 and P16. Northern blot analysis was used to confirm overexpression of *BbJEN1* in *B. bassiana* transformants.

**Determination of conidial yield.** Conidial yield was determined using the method described by Fang et al. (21). The conidia were cultivated on plates with modified Czapek's medium containing 0.5% (wt/vol) sucrose.

**Measurement of carboxylate content in hyphae.** To measure the carboxylates, hyphae were incubated for 24 h in volumes of minimal medium containing various carboxylates as sole carbon sources and harvested to be lyophilized. The lyophilized hyphae were flash frozen in liquid nitrogen and comminuted with a mortar and pestle. Samples (0.2 g [dry weight]) were transferred to 4.5 ml of distilled water and mixed well. After being kept on ice for 10 min, the samples were centrifuged at 13,000  $\times$  g for 10 min. Five hundred microliters of supernatant was used to measure the carboxylates with the P/ACE MDQ capillary electrophoresis system (Beckman & Coulter). Standard samples were from Fluka.

**$\beta$ -Glucuronidase activity assay.**  $\beta$ -Glucuronidase activity was measured as described previously (29). The protein content was determined with a previously described method (5).

**Microscopy.** To produce hyphae for microscopic analysis, the *BbJEN1p::egfp* strain was incubated in SDY medium for about 48 h, transferred to volumes of minimal medium containing different substrates, and cultivated for 12 h to detect fluorescence. For microscopic analysis, adult aphids (*Myzus persicae*) and cabbage worms (*Pieris rapae*) were inoculated with fungal conidia of the *BbJEN1p::egfp* strain. Infected insects were embedded by embedding medium (Tissue-Tek; Sakura), frozen to ensure optimal cutting temperature ( $-28^{\circ}\text{C}$ ), and sectioned (15  $\mu\text{m}$ ) on a freezing microtome (Leica CM1900, Germany). The infected aphids and the tissue of insects were directly mounted onto slides and sequentially imaged in the same microscopic field of light and fluorescence. Samples were observed and photographed with an Olympus model BX41 fluorescence microscope (Olympus, Tokyo, Japan) and an Olympus model MVX10 fluorescence stereomicroscope (Olympus, Tokyo, Japan). Both microscopes were equipped for fluorescence with a mercury lamp, with an excitation filter of 460 to 480 nm and a barrier filter of 495 to 540 nm. Light and fluorescent images were captured with a charge-coupled-device (CCD) camera (Olympus model DP71, Tokyo, Japan) connected to a computer.

**Bioassay.** To test the virulence of fungal strains, adult aphids (*M. persicae*) were used for the bioassay. The bioassay was conducted as previously described (18). Fifty percent lethal concentrations ( $\text{LC}_{50}$ s), confidence intervals, and values for other regression parameters were determined using the DPS program.

**pH determinations for infected insect cuticles.** pH determinations for infected insect cuticles were conducted as previously described (47). Cuticles from third-instar *P. rapae* worms were applied.

## RESULTS

**Molecular cloning and biochemical function validation of *BbJEN1*.** Previously, a mutant, designated T12, characterized by a higher conidial yield than that obtained with the wild-type strain, had been identified in the pool of *B. bassiana* T-DNA insertional mutants constructed by the *Agrobacterium*-mediated-transformation method (21). On the basis of the flanking sequence of T12, a 4,500-bp DNA fragment was cloned and sequenced. The fragment contained the full-length carboxylic transport protein gene, named *BbJEN1* (GenBank accession number AY187630), as well as upstream and downstream regulatory sequences. The predicted protein of *BbJEN1* contains 11 transmembrane domains and was homologous to the JEN1 protein from *Metarhizium anisopliae*, with 77% similarity. South-

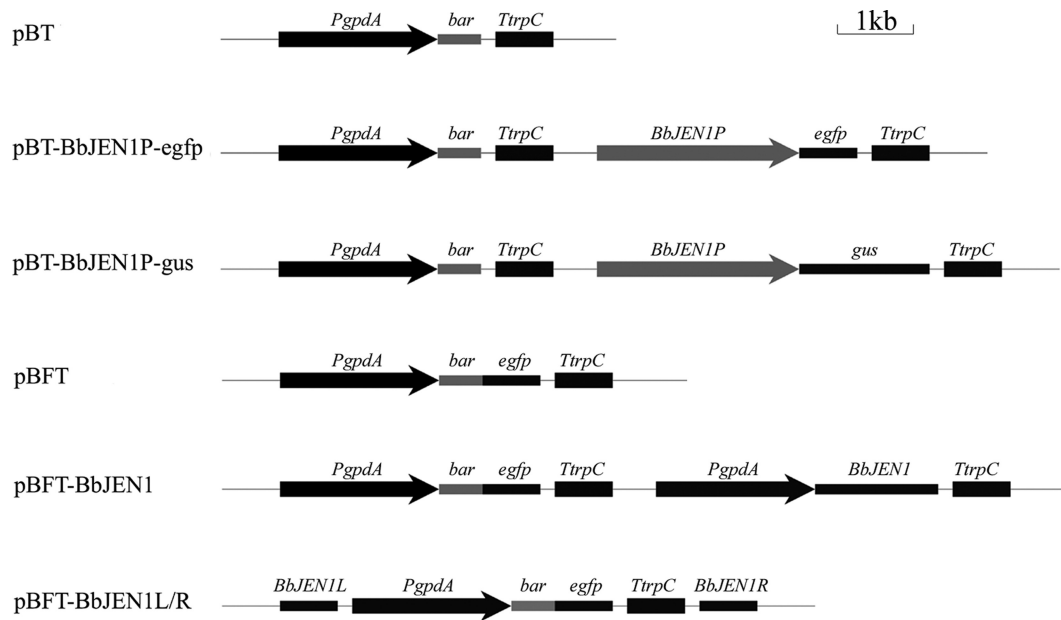


FIG. 1. Maps of pBT, pBT-BbJEN1P-egfp, pBT-BbJEN1P-gus, pBFT, the overexpression vector for *BbJEN1* (pBFT-BbJEN1), and the disruption vector for *BbJEN1* (pBFT-BbJEN1L/R). *Pgpda* is the promoter of *gpd* from *Aspergillus nidulans*, *bar* is the herbicide resistance gene, *egfp* is the enhanced green fluorescent protein gene, *gus* is the  $\beta$ -glucuronidase gene, *TrpC* is the terminator of *trpC* from *A. nidulans*, *BbJEN1* is the carboxylate transport protein gene, and *BbJEN1p* is the promoter of *BbJEN1* gene in *B. bassiana*.

ern blotting showed that *BbJEN1* existed as a single-copy gene in the *B. bassiana* genome (data not shown).

To determine the expression profile and the function of *BbJEN1*, a series of vectors were constructed (Fig. 1). The strategies of targeted gene disruption and overexpression were used to study the function of *BbJEN1*. For gene disruption, the fusion gene cassette of the herbicide resistance gene (*bar*) and the enhanced green fluorescent protein gene (*egfp*) was employed

as a visible and selectable marker and inserted into *BbJEN1* (Fig. 2A). Homologous and ectopic recombination and integration numbers were determined by Southern blot analysis (Fig. 2B). For overexpression of *BbJEN1*, the gene was placed downstream of a constitutive promoter, *gpdAp* from *Aspergillus nidulans*, and the gene cassette was delivered into *B. bassiana* by the electroporation method. Fifty herbicide-resistant colonies were obtained and analyzed. Northern blot analysis

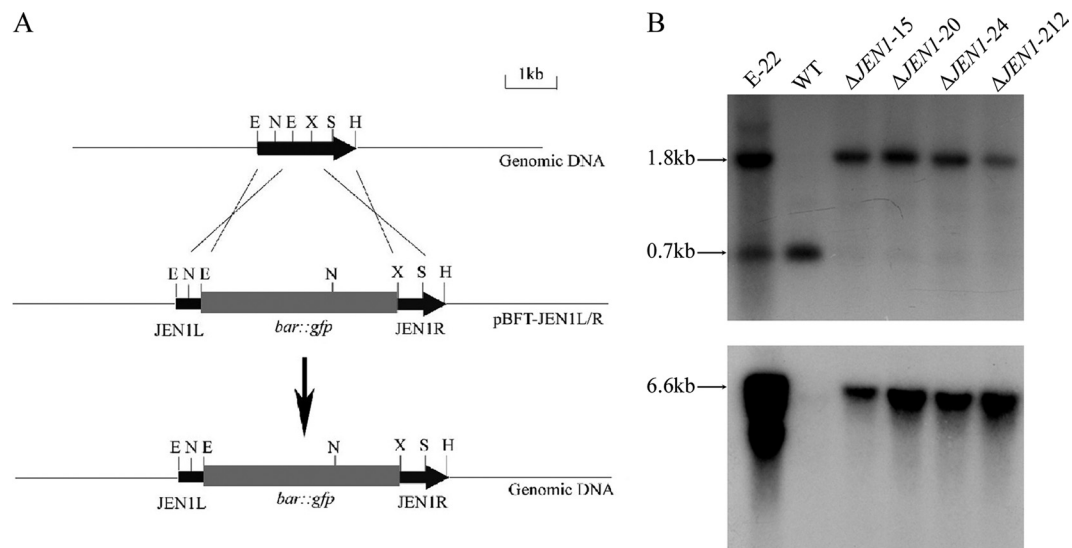


FIG. 2. Targeted disruption of the *BbJEN1* locus. (A) *BbJEN1* locus and disruption vector (pBFT-JEN1L/R). pBFT-JEN1L/R, containing the *bar::gfp* cassette, was digested with HindIII and purified by gel electrophoresis to transform *B. bassiana* Bb0062 conidia. (B) DNA gel blot analysis of *BbJEN1*-disrupted strains. Genomic DNA was digested with NcoI/SmaI (up) and DraI (down). The 550-bp *BbJEN1* fragment (up) and the 500-bp *bar* fragment (down) were labeled with [ $\alpha$ - $^{32}$ P]dCTP and used as a hybridization probe. E-22 is an ectopic transformant. "WT" represents the wild-type strain.  $\Delta$ JEN1-15,  $\Delta$ JEN1-20,  $\Delta$ JEN1-24, and  $\Delta$ JEN1-212 are *BbJEN1*-disrupted strains.

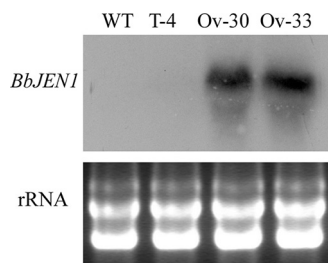


FIG. 3. Overexpression of *BbJEN1* in *B. bassiana*. Northern blot analysis was performed by probing total RNA of the *B. bassiana* wild-type strain and transformant hyphae grown in SDY (containing 4% [wt/vol] glucose to repress the expression of the native *BbJEN1* gene) with the 550-bp *BbJEN1* fragment labeled by [ $\alpha$ -<sup>32</sup>P]dCTP. Each lane represents about 30  $\mu$ g of total RNA. The bottom panel shows the loading controls, where each lane represents 20  $\mu$ g of total RNA. "WT" represents the wild-type strain. T-4 is a transgenic control. Ov-30 and Ov-33 are *BbJEN1* overexpression strains.

showed that *BbJEN1* was strongly expressed in transgenic strains holding the *gpdAp::BbJEN1* cassette (Fig. 3).

To determine the biochemical role of *BbJEN1* in the transport of carboxylates, the oxalate, citrate, pyruvate, and lactate contents in hyphae of *BbJEN1*-disrupted strains, *BbJEN1* overexpression strains, and the wild-type strain were measured by capillary electrophoresis. The amounts of the carboxylates investigated were significantly decreased in *BbJEN1* disruption hyphae, whereas they were increased greatly in *BbJEN1* overexpression hyphae. Measurements of carboxylates demonstrated approximately 2-fold-increased levels of oxalate and pyruvate in *BbJEN1* overexpression strains in comparison to

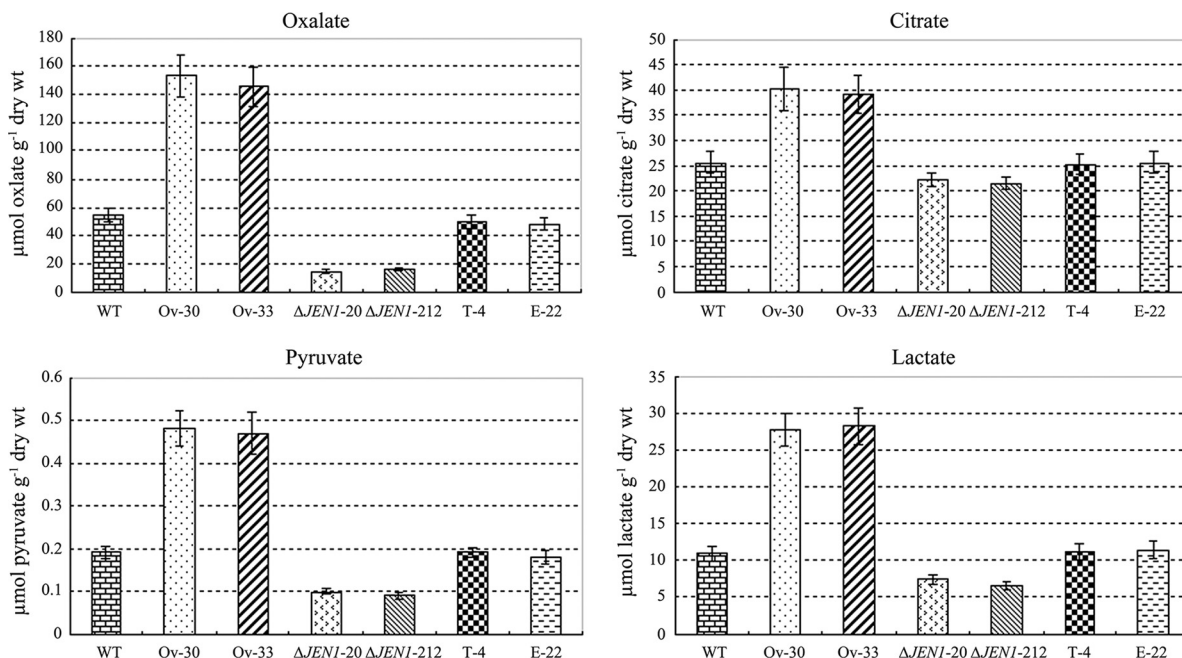


FIG. 4. *BbJEN1* could transport the carboxylates in *B. bassiana*. Hyphae were cultivated for about 2 days in SDY medium and then harvested and transferred to volumes of minimal medium supplemented with different carboxylates at concentrations of 0.2% and were incubated for 24 h. The amounts of carboxylates in hyphae were measured with capillary electrophoresis. "WT" represents the wild-type strain. T-4 is a transgenic control. Ov-30 and Ov-33 are *BbJEN1*-overexpression strains. E-22 is an ectopic transformant.  $\Delta$ *JEN1*-20 and  $\Delta$ *JEN1*-212 are *BbJEN1*-disrupted strains.

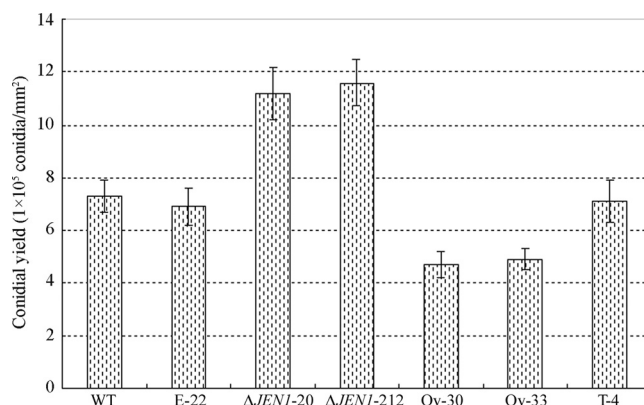


FIG. 5. *BbJEN1* influenced conidial yield in *B. bassiana*. "WT" represents the wild-type strain. T-4 is a transgenic control. Ov-30 and Ov-33 are *BbJEN1* overexpression strains. E-22 is an ectopic transformant.  $\Delta$ *JEN1*-20 and  $\Delta$ *JEN1*-212 are *BbJEN1*-disrupted strains. The conidia were cultivated on plates with modified Czapek's medium containing 0.5% (wt/vol) sucrose.

the levels for the wild-type strain, the transgenic control (T-4, holding an empty vector), and the ectopic transformant (E-22), while these two carboxylates in *BbJEN1*-disrupted strains were decreased about 1- to 2-fold over the levels for the controls. Likewise, lactate and citrate were increased in *BbJEN1* overexpression strains and were decreased in *BbJEN1*-disrupted strains to obvious degrees (Fig. 4). The results indicated that *BbJEN1* was a functional carboxylate transport protein gene in *B. bassiana*.



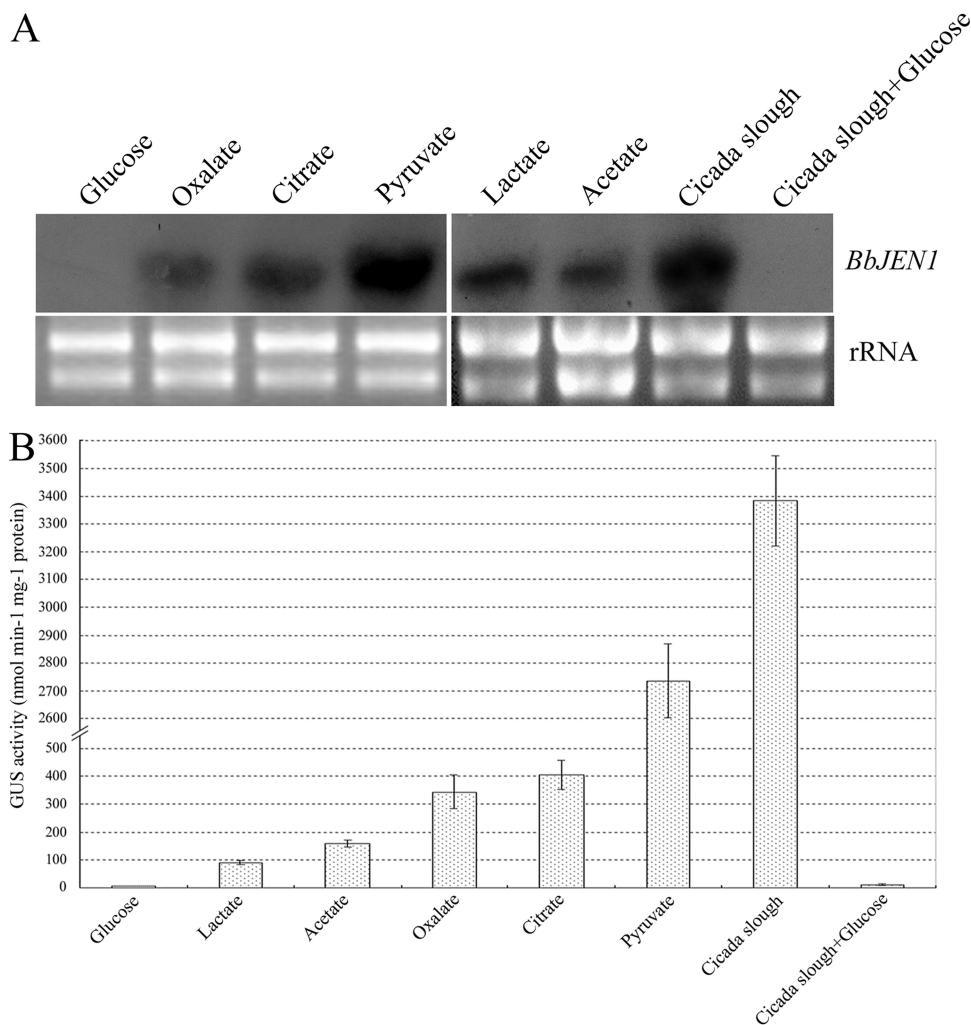


FIG. 6. *BbJEN1* expression was induced by carboxylates and cicada slough and repressed by glucose. (A) Northern analysis of *BbJEN1* transcripts from the wild-type strain induced by various substrates. Hyphae were cultivated for 48 h in SDY and then harvested, washed, and transferred to volumes of minimal medium supplemented with various substrates at concentrations of 0.2%. Total RNA was extracted at 12 h after the shift. Each lane contained about 30  $\mu\text{g}$  of total RNA. Hybridization was carried out using a *BbJEN1* fragment labeled by [ $\alpha$ -<sup>32</sup>P]dCTP. Both bottom panels represent the loading controls, where each lane represents 20  $\mu\text{g}$  of total RNA. (B) Analysis of GUS activity from the transgenic *BbJEN1p::gus* strain induced by various substrates. The hyphae of the *BbJEN1p::gus* strain were cultivated for 48 h in SDY and transferred to volumes of minimal medium culture containing various substrates at concentrations of 0.2%. After being cultivated for 12 h, hyphae were harvested and the GUS activity of the hyphae was measured.

***BbJEN1* influenced conidial yield of *B. bassiana*.** *BbJEN1* was cloned by tagging a T-DNA insertional mutant, T12, one phenotype of which showed an increase in conidial yield. When *BbJEN1* was disrupted through the homologous recombination method, the same phenomenon was observed. The gene-disrupted strains,  $\Delta JEN1$ -20 and  $\Delta JEN1$ -212, showed around 60% more conidia than the wild type. On the other hand, overexpression of the gene resulted in a significant decrease in conidial yield. Meanwhile, no obvious difference in conidial yield was found between the wild type, the transgenic control, and the ectopic transformant (Fig. 5). These results demonstrated that the expression level of *BbJEN1* influenced the conidial yield of *B. bassiana*.

***BbJEN1* expression was induced by carboxylates and the insect cuticle and was repressed by glucose.** Northern blot analysis was used to investigate the induction pattern of

*BbJEN1* transcription of *B. bassiana*. Hyphae of the wild-type strain were cultured in minimal medium containing a carboxylate (lactate, acetate, oxalate, citrate, or pyruvate), glucose, or cicada slough. All carboxylates tested could induce gene expression. However, the levels inducible by various carboxylates were different. Pyruvate induced gene expression strongly. The transcript levels of *BbJEN1* in the hyphae grown in pyruvate-containing medium were about 5-fold, 3-fold, 6-fold, and 8-fold higher than those in the oxalate-, citrate-, lactate-, and acetate-grown hyphae, respectively. Interestingly, *BbJEN1* transcript level was greatly induced by cicada slough, with a 1.5-fold increase over the level for pyruvate. However, the transcript was not detectable in hyphae grown in glucose-containing medium, even when cicada slough was added (Fig. 6A).

To confirm the induction pattern of the gene, a 2.8-kb promoter sequence of *BbJEN1* was fused to *gus* and *egfp*. Trans-

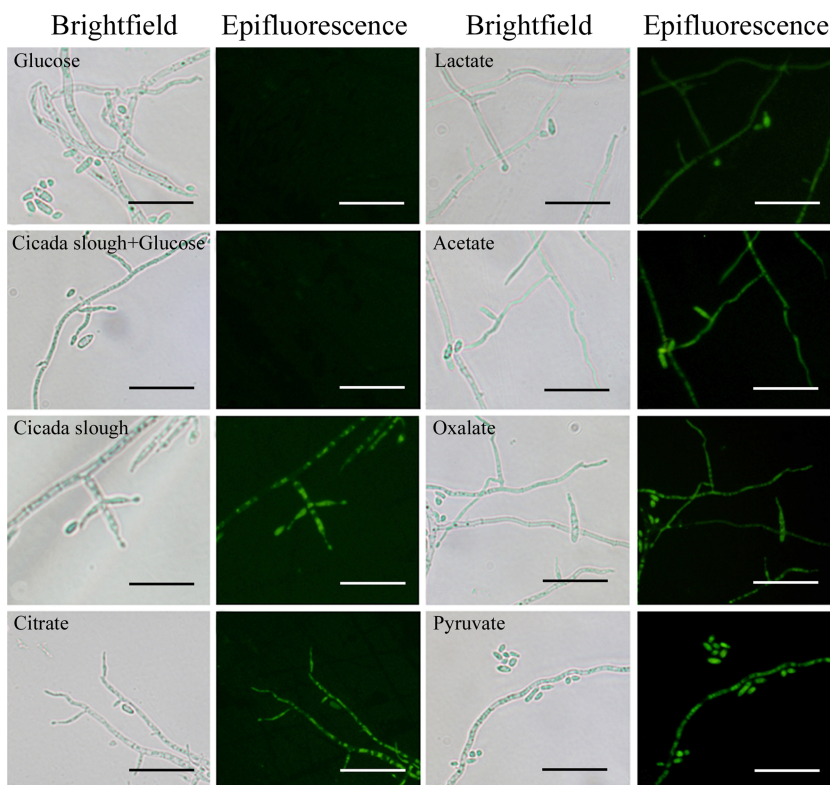


FIG. 7. GFP fluorescence was induced by carboxylates and cicada slough and repressed by glucose in the *BbJEN1p::egfp* line. Hyphae were cultivated for 48 h in SDY and transferred to volumes of minimal medium containing various substrates. After being cultivated for 12 h, hyphae were observed with epifluorescence or bright-field microscopy. Bars = 25  $\mu$ m.

genic fungi with gene cassettes of *BbJEN1p::gus* or *BbJEN1p::egfp* were generated. The fungi holding *BbJEN1p::gus* were cultured in minimal medium containing glucose, acetate, oxalate, citrate, lactate, pyruvate, cicada slough, or cicada slough plus glucose. In all transgenic hyphae, GUS levels reached the peak value at 12 h. The highest GUS activity level was detected in the hyphae grown in medium containing cicada slough, with a 1.3-fold increase over the second-highest activity level, which was induced by pyruvate (Fig. 6B).

To further investigate the induction pattern, the hyphae and conidia of fungi holding *BbJEN1p::egfp* grown in volumes of minimal medium containing different substrates were observed using a microscope. Fluorescence was detected in the fungi grown in medium supplemented with a carboxylate or cicada slough, while no fluorescence was observed when glucose was present (Fig. 7). These results were consistent with those observed in the Northern blot pattern and the GUS pattern. Taken together, these data indicate that *BbJEN1* expression was induced by the insect cuticle and carboxylates but suppressed by glucose.

***BbJEN1* expressed in the hyphae penetrating the cuticle and growing on the cadaver.** To reveal *BbJEN1* expression during infection, aphids (*M. persicae*) and cabbage worms (*P. rapae*) were inoculated with conidial suspension ( $1 \times 10^7$  conidia  $\text{ml}^{-1}$ ) of transgenic *B. bassiana* holding the *BbJEN1p::egfp* cassette. The infected insects were sectioned (15  $\mu$ m) to observe fluorescence of GFP of hyphae in insect tissues. During infection, fluorescence in the hyphae in the penetrated cuticle was ob-

served, and no fluorescence was detected in the hyphae of the wild-type strain (Fig. 8). No GFP activity was observed in the hyphal bodies in the hemolymph of the host infected by fungi holding *BbJEN1p::egfp*, indicating that the *BbJEN1* promoter directed the expression of the GFP gene during the penetration process. Furthermore, fluorescence was also obvious in the hyphae growing on the cadavers of green peach aphids (*M. persicae*) and cabbage worms (*P. rapae*) after the demise of the host, which resulted from the profuse growth of the fungi in the hemolymph. However, GFP activity was significantly inhibited when the cadavers were treated with glucose (Fig. 9).

***BbJEN1* disruption reduced the virulence of *B. bassiana*.** The fact that *BbJEN1* was strongly induced by the insect cuticle and highly expressed in the penetration process implies that *BbJEN1* may affect the virulence of the fungi. To test if deleting *BbJEN1* influences fungal virulence, we bioassayed adult aphids (*M. persicae*) treated with gene disruption strains and controls. Aphids were inoculated with fungal conidia at a concentration of  $5 \times 10^6$  conidia  $\text{ml}^{-1}$ . The relative mortality rates among adult aphids were significantly reduced with *BbJEN1*-disrupted strains  $\Delta$ JEN1-20 (43.11%) and  $\Delta$ JEN1-212 (40.32%) in comparison with the levels for the wild type (50.05%) and the ectopic transformant E-22 (50.83%) at 8 days after inoculation (Fig. 10A). Meanwhile, the  $\text{LC}_{50}$ s (concentrations needed for the pathogen to kill 50% of aphids) of  $\Delta$ JEN1-20 and  $\Delta$ JEN1-212 were nearly 1-fold higher than those of the wild-type strain and the ectopic transformant (E-22) (Tukey's post hoc tests;  $F_{3,8} = 27.426$ ;  $P < 0.01$ ) (Fig. 10B). The  $\text{LC}_{50}$

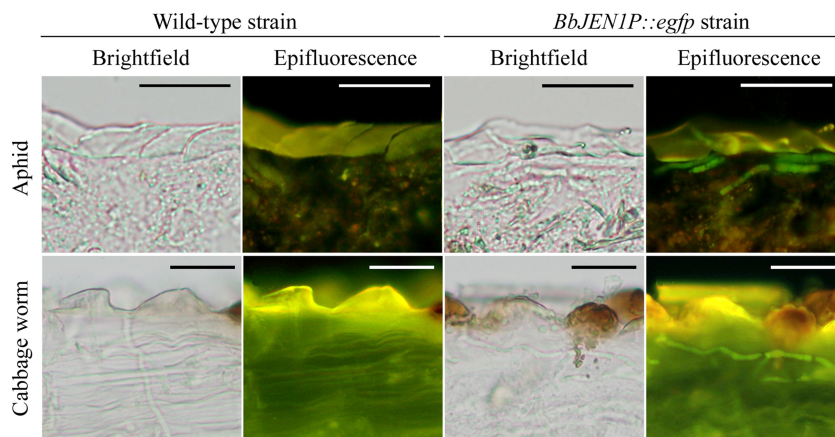


FIG. 8. *BbJEN1* expressed during penetration of the insect cuticles. Green peach aphids (*M. persicae*) and cabbage worms (*P. rapae*) were inoculated with the wild-type strain and the *BbJEN1p::egfp* transformant. After 48 h, the infected insects were sliced into 15- $\mu$ m sections with a Leica microtome and observed with epifluorescence or bright-field microscopy. Bars = 25  $\mu$ m.

was based on the efficiency of infection (54). The increases in  $LC_{50}$  and the decreases in mortality rate induced by the mutants suggested that disruption of *BbJEN1* impaired the infection efficiency of *B. bassiana* in aphids. However, overexpression of *BbJEN1* induced no significant change in the virulence of the pathogen (data not shown).

Ambient pH influences the virulence of several pathogenic fungal species during infection (7, 31, 32, 47). To illustrate the

possible connection between *BbJEN1* expression and the ambient pH values of insect cuticles, we measured the pH values in cuticles of third-instar *P. rapae* worms infected by the *BbJEN1*-disrupted strain, the *BbJEN1* overexpression strain, and the wild-type strain. The natural pH in the *P. rapae* cuticle was  $6.50 \pm 0.05$ . After infection with the wild-type strain, the pH in cuticles rose to  $7.82 \pm 0.06$ . Interestingly, the pH in the cuticle infected by the *BbJEN1*-disrupted strain was lower

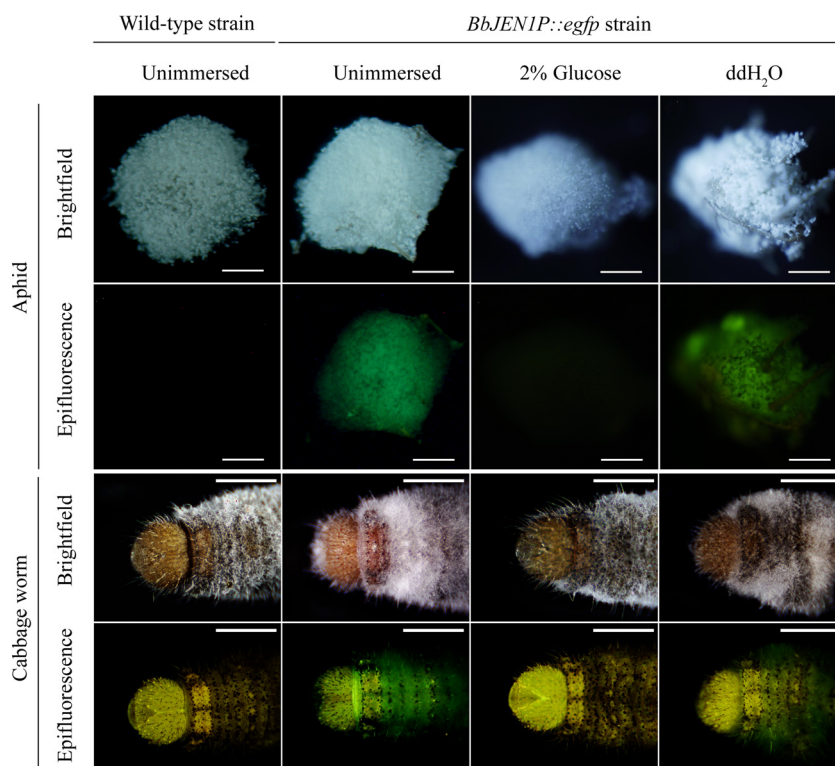


FIG. 9. *BbJEN1* expressed in the hyphae growing on the cadaver. Green peach aphids (*M. persicae*) and cabbage worms (*P. rapae*) were inoculated with the wild-type strain and the *BbJEN1p::egfp* transformant, respectively. After 7 days, some infected insects were immersed in 2% (wt/vol) glucose and distilled water for 3 h. The infected insects were mounted onto slides to detect the fluorescence of the hyphae under a fluorescence microscope. The bars correspond to 1 mm (aphid) or 2 mm (cabbage worm).



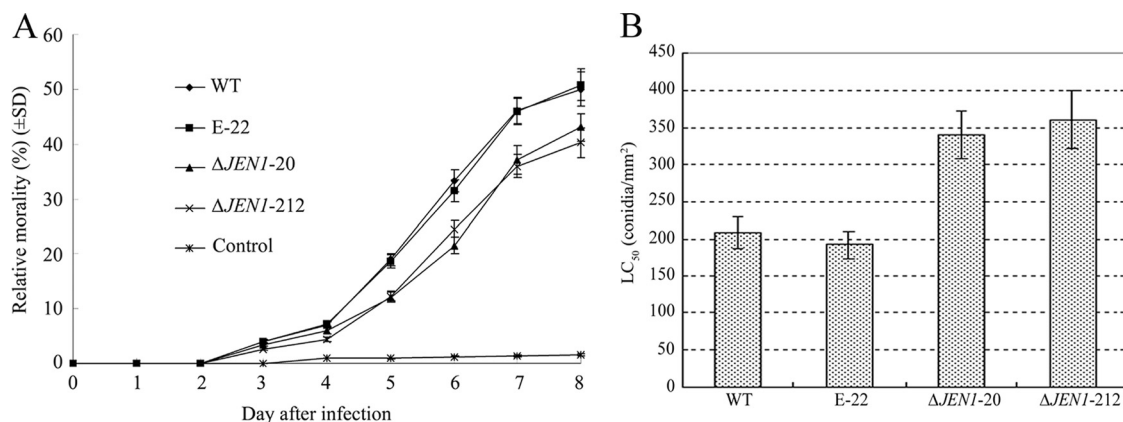


FIG. 10. *BbJEN1* disruption reduced the virulence of the fungi. (A) Relative mortality rates among adult aphids (*M. persicae*). Aphids were inoculated with fungal conidia at a concentration of  $5 \times 10^6$  conidia  $\text{ml}^{-1}$  by using a Potter precision laboratory spray tower (Burkard Manufacturing Co., Ltd., England). Control aphids were treated with water plus 0.05% (vol/vol) Tween 80. (B)  $\text{LC}_{50}$ s of *BbJEN1*-disrupted strains and the wild-type strain for *M. persicae* at 7 days after inoculation. Aphids were inoculated with fungal conidia by using a Potter precision laboratory spray tower (Burkard Manufacturing Co., Ltd., England). Five conidial suspensions ( $5 \times 10^5$ ,  $1 \times 10^6$ ,  $5 \times 10^6$ ,  $1 \times 10^7$ , and  $5 \times 10^7$  conidia  $\text{ml}^{-1}$ ) were used for inoculation.

( $7.65 \pm 0.03$ ), while the value in the cuticle infected by *BbJEN1* overexpression strain was higher ( $8.02 \pm 0.05$ ) than that in the cuticle infected by the wild-type strain ( $7.82 \pm 0.06$ ) (Tukey's post hoc tests;  $F_{2,6} = 44.1$ ;  $P < 0.01$ ). It was deduced that *BbJEN1* disruption decreased the transfer of organic acid from the cuticle into cells of the fungi, resulting in lower ambient pH values in infected cuticles, and consequently, the virulence of the fungi was impaired.

## DISCUSSION

The current work demonstrated that *BbJEN1* encodes a functional JEN1 protein, which transports carboxylates into cells. In yeast, JEN1p is an important transporter of carboxylates (2, 9, 45). We measured the amounts of carboxylates in hyphae of *BbJEN1*-disrupted and *BbJEN1* overexpression strains and demonstrated that BbJEN1p could transport not only monocarboxylates but also some dicarboxylates and tricarboxylates into *B. bassiana*.

*BbJEN1* was cloned through tagging of the T12 mutant, which produced more conidia than the wild type (21). When *BbJEN1* was disrupted through the homologous recombination method, the same phenomenon was observed, whereas when the gene was overexpressed, the conidial yield was significantly decreased, suggesting that the conidial yield is relative to *BbJEN1* expression. For fungi, the carbon source is considered an important nutrition factor for conidial yield (28, 42, 44). BbJEN1p can transfer carboxylates into cells. Disruption of carboxylate transport may affect conidial yield through nutritional alteration.

Northern analysis showed that *BbJEN1* was strongly induced by cicada slough. To investigate the expression pattern of *BbJEN1* during infection of the fungi, we employed GFP as a marker controlled by the *BbJEN1* promoter and monitored the marker protein activities. It was demonstrated that *BbJEN1p::egfp* was highly expressed during the penetration of the insect cuticles by the fungi. However, we had not found *BbJEN1p::egfp* expression in the hemolymph of the cabbage

worm. This implies that *BbJEN1* may be involved in infection and that its expression level may affect the virulence of the fungi.

To test this hypothesis, we performed the bioassay by infecting aphids with *BbJEN1*-disrupted fungi. Significant reductions in relative mortality rate among adult aphids and significant increases in  $\text{LC}_{50}$  were observed. The  $\text{LC}_{50}$  increase reflects the decrease in infection efficiency (54). The  $\text{LC}_{50}$  increases obtained with *BbJEN1*-disrupted strains demonstrated that *BbJEN1* disruption reduced the virulence of *B. bassiana* to insects. Why does *BbJEN1* disruption decrease the virulence of the fungi? An explanation is that *BbJEN1* disruption impairs the importation of organic acids, such as oxalic acid, produced during penetration of the insect cuticles, into fungal cells, resulting in alteration of the ambient pH condition during invasion. Extracellular pH is considered a major determinant in expression of genes essential to the growth, differentiation, and virulence in fungal pathogens of humans, plants, and insects (22, 37, 40, 47, 55, 56). Gene expression in fungi induced by ambient pH is regulated via a conserved signaling cascade (3, 4, 27, 32, 39). Among the genes controlled by ambient pH are those encoding extracellular enzymes (31, 36, 47), components of secondary metabolite biosynthetic pathways (6, 16), and cell wall biosynthesis proteins (8, 38). Some of these pH-regulated genes have been implicated in fungal pathogenesis. Alkalinization of the insect cuticle, possibly by the fungus itself, is a physiological signal that triggers the production of pathogenicity factors (12), and alkalinization of the cuticle during infection increased cuticle-degrading protease production (48). It is reported that the transport of acids would change the extracellular acid-base equilibrium (10). BbJEN1p helps the fungi in transporting carboxylates into cells. Consequently, a decrease of extracellular organic acids will lead to alkalinization of the infected cuticles. Thus, when *BbJEN1* was disrupted, the pH value was lowered by the accumulated organic acids in the infected cuticles, and the lower pH may result in impairment of the virulence of the pathogen.



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