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Increased Insect Virulence in *Beauveria bassiana* Strains Overexpressing an Engineered Chitinase▽

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Entomopathogenic fungi are currently being used for the control of several insect pests as alternatives or supplements to chemical insecticides. Improvements in virulence and speed of kill can be achieved by understanding the mechanisms of fungal pathogenesis and genetically modifying targeted genes, thus improving the commercial efficacy of these biocontrol agents. Entomopathogenic fungi, such as *Beauveria bassiana*, penetrate the insect cuticle utilizing a plethora of hydrolytic enzymes, including chitinases, which are important virulence factors. Two chitinases (Bbchit1 and Bbchit2) have previously been characterized in *B. bassiana*, neither of which possesses chitin-binding domains. Here we report the construction and characterization of several *B. bassiana* hybrid chitinases where the chitinase Bbchit1 was fused to chitin-binding domains derived from plant, bacterial, or insect sources. A hybrid chitinase containing the chitin-binding domain (BmChBD) from the silkworm *Bombyx mori* chitinase fused to Bbchit1 showed the greatest ability to bind to chitin compared to other hybrid chitinases. This hybrid chitinase gene (Bb*chit1*-Bm*ChBD*) was then placed under the control of a fungal constitutive promoter (*gpd*-Bb*chit1*-Bm*ChBD*) and transformed into *B. bassiana*. Insect bioassays showed a 23% reduction in time to death in the transformant compared to the wild-type fungus. This transformant also showed greater virulence than another construct (*gpd*-Bb*chit1*) with the same constitutive promoter but lacking the chitin-binding domain. We utilized a strategy where genetic components of the host insect can be incorporated into the fungal pathogen in order to increase host cuticle penetration ability.

Entomopathogenic fungi are considered alternatives or supplements to chemical pesticides. However, mycoinsecticides constitute a very small percentage of the total insecticide market. A major consideration in the development of mycoinsecticides has been that, compared to chemical insecticides, they require a longer time after application for insect control, during which time infected insects can cause serious damage to crops (26). Improvements in the virulence of entomopathogenic fungi can be achieved by understanding mechanisms of pathogenesis and genetically modifying targeted virulence factors.

One of the advantages of entomopathogenic fungi is that the host insect need not ingest them. Entomopathogenic fungi, such as *Beauveria bassiana*, infect the host insect by penetrating the insect cuticle. The fungal conidium attaches to the cuticle by nonspecific hydrophobic mechanisms (4) and subsequently germinates. The insect cuticle consists primarily of a chitin matrix embedded with proteins (7). Entomopathogenic fungi produce extracellular proteases and chitinases that degrade these proteinaceous and chitinous components, allowing hyphal penetration through the cuticle and access to the nutrient-rich insect hemolymph (6). Proteases, particularly the subtilisin-like protease called Pr1 (16), and the chitinase Bbchit1 (9) are important virulence factors for *B. bassiana*.

Chitinases are widely distributed and are found in viruses, bacteria, fungi, plants, and animals. Some of these chitinases contain a chitin-binding domain linked to the catalytic site via an intermediate region (2). Fungal chitinases belong to class III chitinases and are part of family 18 of glycosyl hydrolases (11, 12). Some fungal chitinases contain a chitin-binding domain (17, 23), while others do not (10, 23). The chitin-binding domain facilitates chitinase binding, thus allowing efficient degradation of chitin but not soluble substrates (3, 14). The chitin binding ability of the chitin-binding domain is also dependent on the position and type of certain amino acid residues. Amino acid substitutions within the chitin-binding domain of *Bacillus circulans* chitinase A1 resulted in a change from an irreversible chitin binding pattern to a reversible one (30). Two chitinase genes, Bb*chit1* (9) and Bb*chit2* (GenBank accession number AY147011), have been cloned from *B. bassiana*; however, neither has a chitin-binding domain.

Chitin is an important component of insect cuticle, which is the primary barrier against pathogens. Overexpression of Bbchit1 was able to increase the ability of *B. bassiana* to digest insect cuticle, resulting in increased virulence against insects (9). Here we are interested in fusing a chitin-binding domain to Bbchit1 to form an engineered chitinase, which may have higher chitinolytic ability and, consequently, increased virulence of *B. bassiana* toward insects. Five chitin-binding domains from different sources were individually fused to Bbchit1 to produce hybrid chitinases. The hybrid chitinases were expressed in *Pichia pastoris* and tested for binding efficiencies to chitin and chitinase activity. The hybrid chitinase which contained the chitin-binding domain from the silkworm *Bombyx*

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TABLE 1. Source of chitin-binding domains used to produce *B. bassiana* (Bbchit1) hybrid chitinases

GenBank accession no.	Family no. of carbohydrate-binding module	Source	Chitin-binding domain (amino acid residue positions)	Hybrid chitinase	Method used	Oligonucleotides[a]
AF273695	14	*Bombyx mori*	BmChBD (Thr^{434}-Ala^{543})	Bbchit1-BmChBD	PCR	P1, P2
AY098731	14	*Choristoneura fumiferana*	CfChBD (Thr^{433}-Ala^{557})	Bbchit1-CfChBD	PCR	P11, P12
DQ407723	18	*Momordica charantia*	McChBD (Glu^{21}-Pro^{65})	Bbchit1-McChBD	PCR	P13, P14
M57601	12	*Bacillus circulans*	BcChBD (Val^{642}-Gln^{699}) DmChBD (Pro^{269}-Arg^{338})	Bbchit1-BcChBD	Synthesis	P15–P20
NM140936	14	*Drosophila melanogaster*	mBmChBD	Bbchit1-DmChBD	PCR	P21, P22
AF273695		Mutant of BmChBD[b]		Bbchit1-mBmChBD	Site-directed mutagenesis	P1, P3–P6

[a] See Table 2 for oligonucleotide sequences.
[b] Mutant from *B. mori*. See Fig. 1 for amino acid alterations.

mori fused to Bbchit1 showed the greatest binding efficiency to chitin and the greatest chitinolytic activity. The hybrid chitinase gene was linked to a constitutive fungal promoter, and a *B. bassiana* transformant showed higher levels of virulence compared to the nontransformed strain or to a strain transformed with Bb*chit1* with the constitutive promoter.

MATERIALS AND METHODS

Fungal and bacterial strains. *B. bassiana* Bb0062 single spore isolate was previously described (8). Cultures were grown on Sabouraud's dextrose agar (Difco Laboratories, Detroit, MI) supplemented with 1% (wt/vol) yeast extract for 14 days at 26°C with a 15-h light/9-h dark cycle. *Escherichia coli* DH5α was employed for DNA manipulations.

Sources of chitin-binding domains. Five chitin-binding domains derived from a plant, a bacterium, and insects, which are distributed within different families of carbohydrate-binding modules according to the CAZy database (http://afmb.cnrs-mrs.fr/CAZY/), were selected to be fused to *B. bassiana* chitinase Bbchit1. Of these five chitin-binding domains, three were from insects and in the same carbohydrate-binding module family. The *Drosophila* putative chitin-binding domain was derived from the peritrophic matrix (29). The details of these chitin-binding domains and their assigned names and cloning methods are described in Table 1. All chitin-binding domains were cloned or synthesized into pMD-T vector (TaRaKa, Dalian, People's Republic of China).

Several amino acid residues within the chitin-binding domain of the *Bombyx mori* chitinase, BmChBD, were genetically altered to form mBmChBD. Consensus amino acid residues, potentially important for chitin binding, were targeted by comparing chitin-binding domains in chitinases from five insects: *Choristoneura fumiferana*, *Spodoptera litura*, *B. mori*, *Hyphantria cunea*, and *Manduca sexta*. From this evaluation, aromatic residues and several cysteine residues in the wild-type *B. mori* chitin-binding domain (BmChBD) were changed to alanine and glycine, respectively, to form mBmChBD (Fig. 1). Primers P3, P4, and P5 (Table 2) were synthesized and used to construct mBmChBDC (C terminus of the chitin-binding domain where the mutant amino acid residues exist) by PCR amplification. The N terminus of the chitin-binding domain of the chitinase gene from *B. mori*, BmChBDN, was cloned by PCR using primers P1 and P6 (Table 2). The DNA fragment (mBmChBD) containing mBmChBDC and BmChBDN was obtained by extension with DNA polymerase on the basis of the overlapping sequences. Using this PCR mixture (1 μl) as the template, primers P1 and P5 (Table 2) were used to obtain mBm*ChBD* by PCR for cloning. mBm*ChBD* was then cloned into pMD-T and verified by sequencing.

Construction and expression of hybrid chitinases in *Pichia pastoris*. Primers P8 and P9 (Table 2) were used to clone Bb*chit1* by PCR using genomic DNA from *B. bassiana*. The resultant PCR product was cloned into pMD-T (TaKaRa, Dalian, People's Republic of China) and sequenced. The chitin-binding domains and the mutant chitin-binding domain (mBmChBD) described above were fused onto the C terminus of Bbchit1 via introduced restriction enzyme sites in the primers (Table 2). All six hybrid chitinases were constructed in the following way. The Bb*chit1* released from pMD-T vector with EcoRI and XbaI and a chitin-binding domain released from the respective pMD-T vectors with SpeI and NotI and EcoRI/NotI-digested pPIK9K were ligated to form an expression vector for yeast (*P. pastoris*). Yeast transformation and the overexpression of all hybrid chitinases were conducted according to the manufacturer's instructions (Invitrogen, San. Diego, CA). Overexpression of hybrid chitinases was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on the basis of protein sizes and Western blotting analysis using anti-Bbchit1 (9). In order to purify the yeast-expressed hybrid chitinases, supernatants from yeast cultures were concentrated against PEG 20000 and subsequently applied to filtration chromatography Hiload26/60 (Amersham, NJ). The eluant was assayed for chitinase activity and protein concentration (5), and fractions containing chitinase activity were collected and analyzed by SDS-PAGE.

Binding efficiency of chitinase to chitin. Powdered α-chitin from crab (32 μg; diameter of ≤0.25 mm) (Shanghai Chemicals Co., Shanghai, People's Republic of China) was washed with 10 mM potassium phosphate buffer (pH 6.0) and mixed with 70 μg chitinase in 500 μl of 20 mM potassium phosphate buffer (pH 6.0). After incubation for 1 h at 4°C with shaking (200 rpm), the mixture was centrifuged at 12,000 rpm at 4°C for 10 min. The supernatant was collected, and chitinase in the eluant was assayed by SDS-PAGE. In order to recover the chitinase bound to chitin, 500 μl of 200 mM NaCl and 8 M urea were used, sequentially, to wash the pellet. The supernatants from each step were analyzed by SDS-PAGE. The final pellet was suspended in 500 μl of 1% SDS and boiled for 10 min, and the supernatant was analyzed by SDS-PAGE. The SDS-polyacrylamide gel was scanned using an ImageMaster VDS-CL system (Total Lab 1D software) (Amersham, NJ), and protein amounts in the gel were calculated relative to band density.

The binding efficiencies of chitinases to chitin were also tested directly by fluorescein isothiocyanate (FITC) (Sigma-Aldrich, Shanghai, People's Republic of China). To label chitinase with FITC, a mixture containing 1 mg chitinase and 0.02 mg FITC in 0.25 M sodium bicarbonate buffer (pH 9.2) was incubated at 140 rpm at 25°C for 3 h. The labeling mixture was then desalted in a column (Amersham, NJ) with 20 mM potassium phosphate buffer (pH 6.0) at 2 ml/min. The protein fractions were pooled, dialyzed against 20 mM potassium phosphate buffer (pH 6.0), and concentrated against PEG 20000. The ratio of FITC to chitinase (F/P) was determined by the following formula: $F/P = (2.87 \times OD_{495})/[OD_{280} - (0.35 \times OD_{495})]$, where OD_{495} is the optical density at 495 nm.

FITC-labeled chitinases were bound to chitin as previously described for unlabeled chitinases. FITC-labeled chitinases bound to chitin were observed microscopically with an Olympus BX41 microscope (Olympus, Tokyo, Japan). The absorbance wavelength was 490 nm (emission wavelength, 520 nm).

FIG. 1. Comparison of amino acid residues between the chitin-binding domain of chitinase from *B. mori* (BmChBD) and the corresponding altered chitin-binding domain (mBmChBD). Aromatic amino acid residues and several cysteine residues of BmChBD were substituted with alanine or glycine in mBmChBD. The mBmChBD included the following substitutions: Y496A, P498A, C503G, Y506A, W507A, F517A, C519A, P521A, F525A, C532G, D533A, W534A, and P535A.

TABLE 2. Primers used in this study

Primer	Sequence (5′-3′)[a]	Restriction enzyme site(s)
P1	ACTAGTCACAACCACCACCACCGTG	SpeI
P2	GCGGCCGCCCGGGTTAATCTA GAGAACATTCCGGTCTGTC	NotI, SmaI, and XbaI
P3	GGTAGCAAGGCTGCGCGATGTGTGAACGGCGAGGGAGTTCAGGCGTCGGCGCAAGCGGG	
P4	AGCAGCAGCGCCAACGTTAAGTTTCACGTTAGCGATTGTCCCCGCTTGCGCCGACGC	
P5	CCCGGGCGGCCGCTTACGAACATTCCGGTCTGTCTGTATTTTCAGCAGCAGCGCCAAC	SmaI and NotI
P6	TCGCGCAGCCTTGCTACCCTCTTTCTTGTCTGCTATGGCGTCGTCCTCAGAGTT	
P7	GAATTCATGACAACCACCACCACCGTGAAA	EcoRI
P8	GAATTCGCCGGCACCTGCGCCACCAAAG	EcoRI
P9	GCGGCCGCCCGGGCTAGTCTAGAGCAGTCCCCAAAGTCCCCTTG	NotI, SmaI, and XbaI
P10	GAATTCATGGCTCCTTTTCTTCAAACC	EcoRI
P11	ACTAGTCACCACTATAGCCAAGCCAAAG	SpeI
P12	GCGGCCGCCCGGGTTAATCTAGAGACGCCAGACAGTCTTCTC	NotI, SmaI, and XbaI
P13	ACTAGTCGAGCAATGTGGGCGGCAGGCC	SpeI
P14	GCGGCCGCCCGGGTTAATCTAGAGA TGTAGGAGTTTGGCCGCC	NotI, SmaI, and XbaI
P15	ACTAGTCGTGAAGACAGCCGCCGAAACGACAAATCCTGGTGTATCCGCTTG	
P16	TATATGTGACCAATTGTCCCGCAGTATAAGCTGTGTTGACCTGCCAAGCGGATACACCA	
P17	AATTGGTCACATATAACGGCAAGACGTATAAATGTTTGCAGCCCCACACCTCCTTGGCA	
P18	CTATTGAAGCTGCCACAAGGCAGGAACGTTGGATGGTTCCCATCCTGCCAAGGAGGTGT	
P19	ACTAGTCGTGAAGACAGCCGCCGAAAC	SpeI
P20	GCGGCCGCCCGGGTTAATCTAGAGATTGAAGCTGCCACAAGGCAG	NotI, SmaI, and XbaI
P21	ACTAGTCCCCACCACAGTGGTGTGCACC	SpeI
P22	GCGGCCGCCCGGGTTAATCTAGATCCCGGCCATCGCAACACTT	NotI, SmaI, and XbaI
P23	GCGGCCGCATGAGTAAAGGAGAAGAACT	
P24	GGATCCTCATTTGTATAGTTCATCCATGCC	

[a] Underlined sequences are restriction sites.

Chitinase activity assay. One soluble substrate and three insoluble substrates were used to assay chitinase activity. The three insoluble substrates were (i) the powdered α-chitin previously described, (ii) colloidal chitin (24), and (iii) silkworm cuticle. For the preparation of silkworm cuticle, ecdysal molts of fourth-instar silkworms were comminuted (diameter of ≤0.25 mm) and subsequently used as an insoluble substrate for chitinase activity assays (20). The soluble substrate was 4-methylumbelliferyl-*N*′,*N*′,*N*″-β-D-triacetylchitotrioside [4-MU-(GlcNAc)3] (Sigma-Aldrich, St. Louis, MO), and chitinase activity was assayed with a 10-min incubation at 37°C (29).

Thin-layer chromatography (TLC) analysis of chitinase hydrolysis products. Chitin oligosaccharides were kindly provided by Chien-Jui Huang of National Taiwan University. Chitin hydrolytic products were analyzed by TLC (13).

Kinetic analysis of chitinases. Kinetic analysis (K_m and V_{max}) was performed by the method of Arakane et al. (1) with modifications. The reaction mixture (700 μl) contained 250 μl of 0.1 M potassium phosphate buffer (pH 6.4), 250 μl enzyme solution (0.2 μM), and 200 μl colloidal chitin at concentrations ranging from 1 mg/ml to 5 mg/ml.

Scanning electron microscopy. Fourth-instar aphids (*Myzus persicae*) were frozen at −20°C for 1 h and treated with 1.7 μM chitinase in 20 mM potassium phosphate buffer (pH 6.0) at 37°C for 12 h. Aphids were fixed with 1% (wt/vol) osmium tetroxide for 1 h. The samples were rinsed with distilled water, treated with 2% (wt/vol) tannic acid for 14 h, and then rinsed with distilled water. Postfixation was carried out in 1% (wt/vol) osmium tetroxide for 1 h. After the samples were dehydrated in a series of 50% to 100% ethanol and *t*-butyl alcohol-acetonitrile solutions, they were sputter coated with gold. The surfaces of the aphids were observed using a Hitachi S-3000N scanning electron microscope (Hitachi, Tokyo, Japan).

Blotting analysis. Western blotting was performed according to the manufacturer's instructions (Opti-4CN Western blot kit; Bio-Rad, Philadelphia, PA). Rabbit Bbchit1 antibody (anti-Bbchit1) was previously described (9).

Northern blotting was conducted with 20 μg total RNA for each sample (21). RNA was prepared using RNeasy minipreparation kit (QIAGEN, Valencia, CA). The DNA probe (1,047-bp-long open reading frame of Bbchit1) was labeled with [α-^{32}P]dCTP using a labeling kit (Amersham, NJ).

Chitinase vector construction. Plasmid pBARGFP (Fig. 2) contained the herbicide resistance gene *bar* and enhanced green fluorescent protein gene *egfp*.

On the basis of pBARGFP, four vectors overexpressing chitinases or the chitin-binding domain were constructed (Fig. 2). The construction of Bbchit1 overexpres-

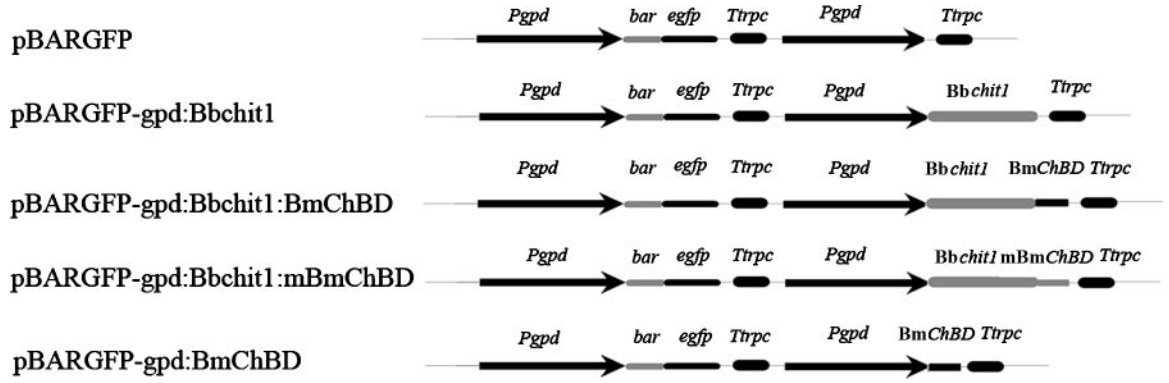

FIG. 2. Maps of pBARGFP, overexpression vector for Bbchit1 (pBARGF-gpd:Bbchit1), Bbchit1-BmChBD (pBARGF-gpd:Bbchit1:BmChBD), Bbchit1-mBmChBD (pBARGF-gpd:Bbchit1:mBmChBD), and BmChBD (pBARGF-gpd:BmChBD). *Pgpd* is the promoter of *gpd* from *Aspergillus nidulans*, *bar* is the herbicide resistance gene, *egfp* is the enhanced green fluorescent protein gene, *Ttrpc* is the terminator of *trpc* from *A. nidulans*, Bb*chit1* is the chitinase gene, Bm*ChBD* is a chitin-binding domain of a chitinase from *B. mori*, and the altered chitin-binding domain is mBm*ChBD*.

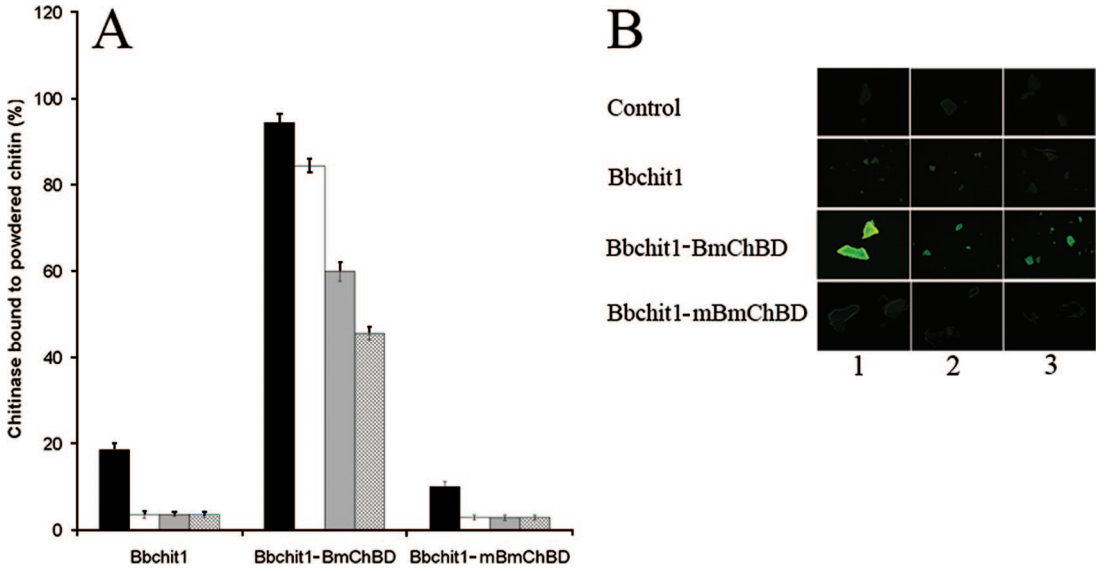

FIG. 3. (A) Chitin binding ability of Bbchit1, Bbchit-BmChBD, and Bbchit-mBmChBD to powdered α-chitin. Purified chitinases were bound to powdered chitin. After an incubation for 1 h at 4°C, the mixture was centrifuged, the supernatant (unbound protein) was subjected to SDS-PAGE analysis, and the amount of protein bound to the powdered chitin (black) was recorded as the difference between input protein and unbound protein (in the supernatant). The protein bound to chitin after being washed sequentially with 200 mM NaCl (white) and 8 M urea (gray) and finally suspended in 1% SDS and boiled for 10 min (hatched) is shown. All experiments were repeated three times with three replicates for each repeat. Standard errors (error bars) are shown. (B) Chitin-binding assay of FITC-labeled Bbchit1, Bbchit1-BmChBD, and Bbchit1-mBmChBD. For a control, powdered chitin treated with 20 mM potassium phosphate buffer (pH 6.0) was used. Panels 1, powdered chitin bound to chitinase. Panels 2, powdered chitin with chitinases washed with 200 mM NaCl. Panels 3, powdered chitin with chitinases washed with 8 M urea.

sion vector pBARGFP-gpd:Bbchit1 was conducted as follows. (i) Primers P9 and P10 (Table 2) were used to amplify Bb*chit1* using *B. bassiana* genomic DNA as the template. The resultant PCR product was cloned into pMD-T (TaRaKa, Dalian, People's Republic of China) to form pMD-Bbchit1 and sequenced for confirmation. (ii) Bb*chit1* was excised from pMD-Bbchit1 by EcoRI and SmaI digestion and inserted into the corresponding sites of pBARGFP. For the construction of the vector to overexpress the hybrid chitinase Bb*chit1*-Bm*ChBD* and the vector for Bb*chit1*-mBm*ChBD* overexpression, Bm*ChBD* and mBm*ChBD* were first fused to the C terminus of Bb*chit1*, respectively. The resultant hybrid chitinase genes were subsequently inserted into EcoRI and SmaI sites of pBARGFP. The fourth vector, pBARGFP-gpd:BmChBD (Fig. 2), was constructed to overexpress the chitin-binding domain from *B. mori* chitinase. The construction of pBARGFP-gpd:BmChBD was performed exactly by the procedures for the construction of pBARGFP-gpd:Bbchit1. BmChBD was first cloned by PCR with primers P2 and P7 (Table 2) and then inserted into the EcoRI and SmaI sites of pBARGFP.

Fungal transformation and transformant screening. Fungal transformations were conducted as previously described (22). Transformants were initially selected on the basis of herbicide (glufosinate ammonium; Sigma-Aldrich, St. Louis, MO) resistance and green fluorescent protein (GFP) expression. Mitotically produced single spore isolates of each transformant were obtained. Chitinase activity from the transformants grown in basal salt medium (0.03% NaCl [wt/vol], 0.03% $MgSO_4$ [wt/vol], 0.03% K_2HPO_4 [wt/vol]) supplemented with 2% glucose (to suppress the expression of native Bbchit1) was assayed as previously described (9). Chitinase overexpression was confirmed by Northern and Western blotting analyses.

The insertion of target genes in the transformants was further confirmed by PCR. Primers P23 and P24 (Table 2) were used to confirm transformants with Bb*chit1* insertion. Primers P2 and P10 (Table 2) were used to confirm transformants with Bb*chit1*-Bm*ChBD* and Bb*chit1*-mBm*ChBD*. Primers P2 and P7 were used to confirm the insertion of Bm*ChBD*.

Bioassay. Adult aphids (*M. persicae*) (0 to 2 days) reared on cabbage in a greenhouse were obtained by the method of Vandenberg et al. (28) and used for bioassays. Aphids were inoculated with fungal conidia at a concentration of 1×10^7 conidia/ml using a Potter precision laboratory spray tower (Burkard Manufacturing Co. Ltd., England). Infected aphids were transferred to an HPG-280H artificial climate cell (Donglian Electronic Company, Harbing, People's Republic of China) at 22 to 24°C and fed cabbage, and mortality was monitored at 12-h intervals. Control aphids were treated with water plus 0.05% (vol/vol) Tween 80. Three replicates were performed for each treatment and each replicate contained 30 aphids. Fifty percent lethal time (LT_{50}), confidence interval, and other regression parameters were determined using the SPSS program.

RESULTS

Binding efficiency of hybrid chitinases to chitin. Chitin-binding domains from insects (*B. mori*, *C. fumiferana*, and *Drosophila melanogaster*), a plant (*Momordica charantia*), and a bacterium (*B. circulans*) were fused to Bbchit1, resulting in hybrid chitinases Bbchit1-BmChBD, Bbchit1-CfChBD, Bbchit1-DmChBD, Bbchit1-McChBD, and Bbchit1-BcChBD, respectively. These hybrid chitinases were successfully expressed in yeast (*P. pastoris*), which was confirmed by SDS-PAGE and Western blotting analysis (data not shown). The hybrid chitinases accounted for more than 65% of the yeast extracellular proteins. Hybrid chitinases were subsequently purified by chromatography through a gel filtration column and used for chitin-binding assays.

Chitin binding efficiencies of hybrid chitinases were dependent on chitin-binding domains. Bbchit1-BmChBD, Bbchit1-CfChBD, and Bbchit1-BcChBD had significantly higher binding efficiencies to chitin than wild-type Bbchit1 did ($t = 5.528$, 4.32, and 3.96, respectively; $P < 0.05$; df = 3). Chitin bound most efficiently to Bbchit1-BmChBD (93.8% ± 1.7% binding, $n = 3$), Bbchit1-CfChBD (73.9% ± 2.1% binding, $n = 3$), and Bbchit1-BcChBD (65.1% ± 1.2% binding, $n = 3$), whereas only 19.5% ± 2.9% ($n = 3$) of Bbchit1 was bound to chitin. The addition of a chitin-binding domain from *D. melanogaster* or *M. charantia* to Bbchit1 had no significant effect on the chitin binding efficiency (Bbchit1-

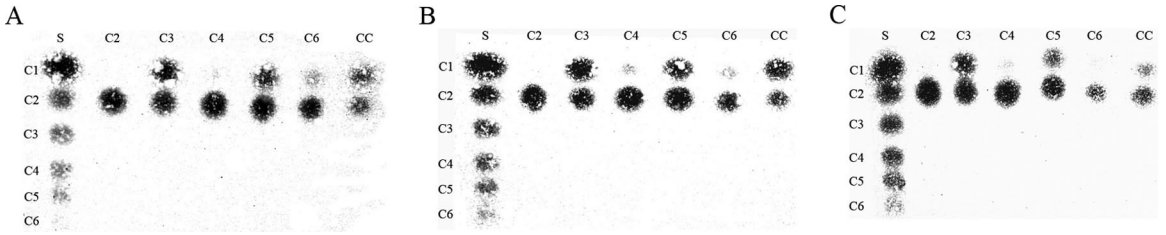

FIG. 4. TLC analysis of chitinase hydrolytic products from respective chitin oligosaccharides and colloidal chitin as substrates. (A) Bbchit1, (B) Bbchit1-BmChBD, and (C) Bbchit1-mBmChBD. The chitin oligosaccharide hydrolytic products are shown vertically. C1 through C6 refer to GlcNAc1 through GlcNAc6, respectively. Substrates, except S (the chitin oligosaccharide standards) are shown horizontally. CC, colloidal chitin.

DmChBD [23.2% ± 3.6% binding; $n = 3$] and Bbchit1-McChBD [24.1% ± 3.2% binding; $n = 3$]).

With Bbchit1-BmChBD, 49.7% of the chitinase was still bound to chitin after being washed with a urea solution and boiling in SDS solution. However, when this chitin-binding domain was altered, the chitin binding efficiency of Bbchit1-mBmChBD (11.5% ± 1.1% binding; $n = 3$) was lower than that of the wild-type Bbchit1. A NaCl solution was sufficient to release Bbchit1 and Bbchit1-mBmChBD from chitin (Fig. 3A). The greater binding efficiency of Bbchit1-BmChBD was also shown by the increased fluorescence of the FITC-labeled hybrid chitinase adsorbed onto chitin after being washed by 8 M urea, while the fluorescent intensities of Bbchit1 and Bbchit1-mBmChBD were relatively weaker after the washings (Fig. 3B). Therefore, the addition of a chitin-binding domain, BmChBD, resulted in higher chitin binding ability of Bbchit1, and certain amino acid residues within the chitin-binding domain were critical for binding.

Chitinase biochemical analysis. The addition of a chitin-binding domain altered the kinetics of Bbchit1. The K_m value of Bbchit1-BmChBD (0.61 g/liter) was lower than that of Bbchit1 (2.62 g/liter), indicating that Bbchit1-BmChBD had a higher affinity to colloidal chitin. However, the V_{max} of Bbchitl-BmChBD (0.19 μmol/min/mg) was significantly lower than that of Bbchit1 (0.39 μmol/min/mg). A similar inconsistency was observed by Arakane et al. (1). The actual reaction rate of an enzyme is related to K_m and V_{max} at a certain substrate concentration; therefore, V_{max}/(molar weight of enzyme)/K_m was used to evaluate substrate turnover. Bbchitl-BmChBD exhibited a V_{max}/(molar weight of enzyme)/K_m (14.98) which was 2.95 times greater than Bbchit1(5.07), indicating that Bbchit1-BmChBD possessed greater chitin hydrolytic ability.

Bbchit1-BmChBD and Bbchit1 had similar optimum pH and temperature activities. Each showed chitinase activity from pH 4.0 to 9.0 with an optimum of pH 5.0 (data not shown). Chitinase activity with Bbchit1-BmChBD and Bbchit1 was optimal at 40°C (data not shown).

Chitin oligosaccharides of various lengths and colloidal chitin were used as substrates for chitinolytic activity whose products were assessed by TLC. Bbchit1 was unable to degrade GlcNAc2 within 4 h (Fig. 4A), suggesting that it did not possess *N*-acetylglucosaminidase activity. GlcNAc2 was the major product when GlcNAc3-6 or colloidal chitin was used as the substrate, indicating that Bbchit1 had chitobiosidase activity. However, the presence of GlcNAc when GlcNAc4, GlcNAc6, or colloidal chitin was used as a substrate suggested that GlcNAc3 was an intermediate product that was subsequently hydrolyzed to GlcNAc1 and GlcNAc2.

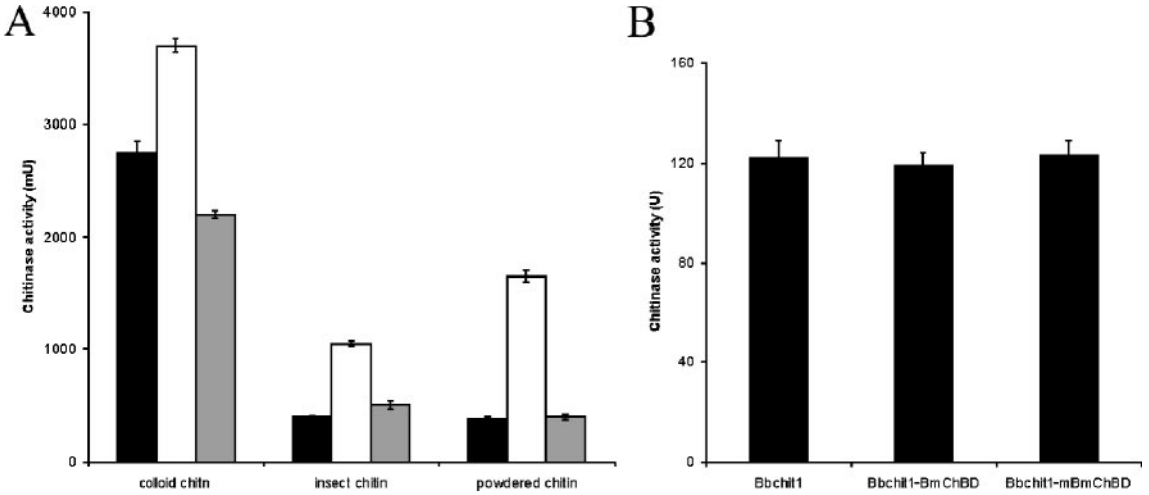

FIG. 5. Chitinase activity on soluble substrate as well as different insoluble substrates, colloidal chitin, insect cuticle, and powdered chitin. Chitinase (2 nM) was used in the chitinase activity assays. (A) Chitinase activity against insoluble chitin. Bbchit1 (black), Bbchit1-BmChBD (white), and Bbchit1-mBmChBD (gray) are shown. One unit of chitinase activity was defined as the amount of enzyme required to release 1 μmol *N*-acetylglucosamine (GlcNAc) per hour. (B) Chitinase activity against soluble chitin [4-MU-$(GlcNAc)_3$]. One unit of chitinase activity was defined as the amount of enzyme required to release 1 nM MU per minute.

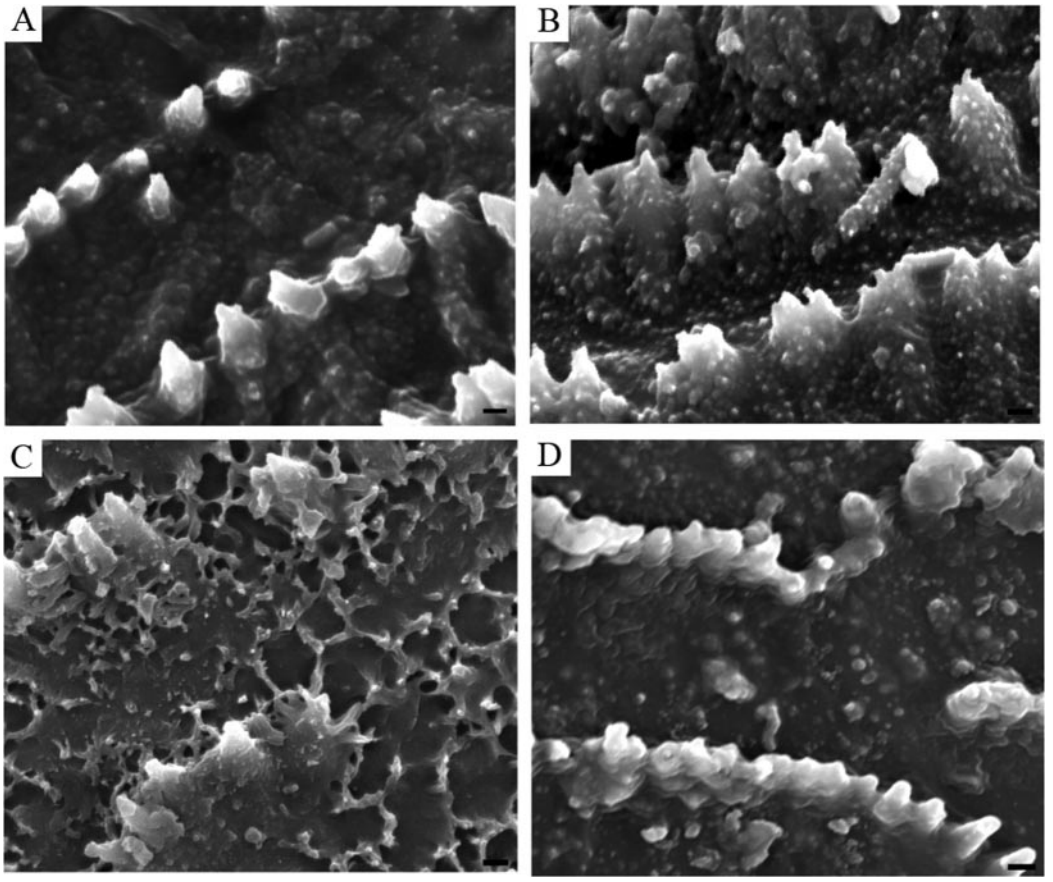

FIG. 6. Scanning electron microscopy of the hydrolytic effects of Bbchit1, Bbchit1-BmChBD, and Bbchit1-mBmChBD on the gross morphological structure of aphid (*Myzus persicae*) cuticles. Chitinases were incubated with cuticle for 12 h at 37°C. (A) Control (20 mM potassium phosphate buffer [pH 6.0] without chitinase), (B) Bbchit1, (C) Bbchit1-BmChBD, (D) Bbchit1-mBmChBD. Bars = 1 μm.

Therefore, Bbchit1 is an endochitinase with chitiobiosidase activity. Bbchit1, Bbchit1-BmChBD, and Bbchit1-mBmChBD had similar hydrolytic products (Fig. 4B and C), showing that the addition of BmChBD did not alter the chitin digestion patterns of Bbchit1.

Enzyme activity of hybrid chitinases. Using colloidal chitin, silkworm cuticle, or powdered chitin as substrates, Bbchit1-BmChBD had, respectively, 1.3 ($P < 0.05$), 1.9 ($P < 0.001$), and 5.5 ($P < 0.001$) times greater chitinase activity than Bbchit1. Bbchit1-mBmChBD had similar chitinase activities to those of Bbchit1 on insoluble chitin substrates. The efficiency of chitin hydrolysis by the hybrid chitinases and Bbchit1 varied according to the type of chitin. With colloidal chitin as the substrate, chitinase activities were the highest (Fig. 5A). Bbchit1-BmChBD, Bbchit1-mBmChBD, and Bbchit1 had similar chitinase activities with the soluble chitinase substrate, 4-MU-$(GlcNAc)_3$ (Fig. 5B). Therefore, the introduced chitin-binding domain enhanced chitin binding efficiency, resulting in the increased activity against insoluble chitin but not a soluble substrate.

Gross morphological changes on aphid (*M. persicae*) cuticular surfaces were observed by scanning electron microscopy after treatment with chitinases. Ridge-like structures could be observed on the control aphid cuticular surface (treated with 20 mM potassium phosphate buffer, pH 7.0). Cuticles treated with Bbchit1-BmChBD showed a degradation of the ridge-like structures and the appearance of hexagonal surface features. Cuticles treated with Bbchit1 or Bbchit-mBmChBD showed no distinguishable differences in surface features compared to control cuticle (Fig. 6). The alteration of cuticular surface features by Bbchit-BmChBD and not the other chitinases is suggestive of the increased chitinolytic capacity of the hybrid chitinase on chitin within intact cuticles.

Construction and characterization of *B. bassiana* transformants overproducing an engineered chitinase. Bb*chit1*-Bm*ChBD*, Bb*chit1*, Bb*chit1*-mBm*ChBD*, and Bm*ChBD* were each placed under a fungal constitutive promoter, *Pgpd*, to form four vectors, which were then used to transform *B. bassiana*. The plasmid pBARGFP was also transformed into *B. bassiana*. Transformants overexpressing Bbchit1-BmChBD, Bbchit1, and Bbchit1-mBmChBD were obtained, and two of each transformant were randomly selected by single spore isolation. Transformations of *B. bassiana* with *gpd*-Bb*chit1*-Bm*ChBD*, *gpd*-Bb*chit1*, *gpd*-Bb*chit1*-mBm*ChBD*, and *gpd*-Bm*ChBD* were confirmed by PCR analysis (data not shown). Overexpression of Bbchit1-BmChBD, Bbchit1, and Bbchit1-mBmChBD was assessed by Northern blotting analysis (Fig. 7A) and Western blotting analysis using Bbchit1 antibody (Fig. 7B). Chitinase activities in Bbchit1-BmChBD, Bbchit1, Bbchit1-

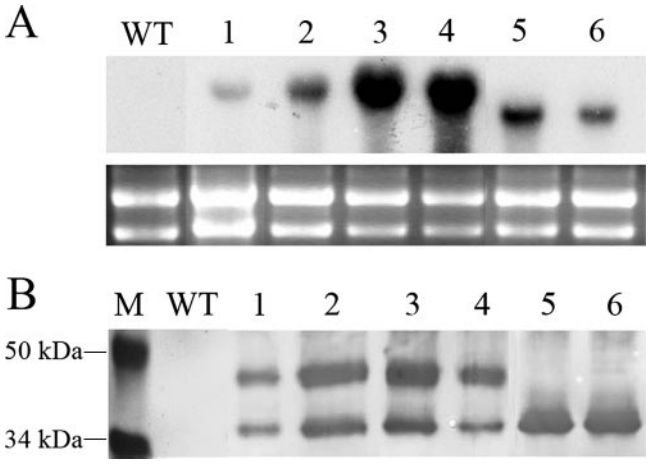

FIG. 7. Northern blotting and Western blotting analysis of wild-type *B. bassiana* and transformants overexpressing chitinase. (A) Top panel; Northern blotting analysis. Bottom panel; loading controls where each lane contained ca. 20 μg total RNA. (B) Western analysis. Each lane contained 15 μg protein. WT, *B. bassiana* wild-type; 1 and 2, representative transformants Bbchit1-mBmChBD-2 and Bbchit1-mBmChBD-5, overexpressing Bbchit1-mBmChBD; 3 and 4, representative transformants Bbchit1-BmChBD-19 and Bbchit1-BmChBD-42, overexpressing Bbchit1-BmChBD; 5 and 6, representative transformants Bbchit1-8 and Bbchit1-26, overexpressing Bbchit1; M, prestained protein standards (Bio-Rad, Philadelphia, PA).

mBmChBD, BmChBD, and pBARGFP transformants were assessed (Table 3).

Bioassays. Overexpression (under the control of *gpd*) of Bbchit1, Bbchit-BmChBD, or Bbchit-mBmChBD transformants resulted in increased virulence compared to the virulence of the wild-type *B. bassiana* (Table 3). Overexpression of the chitin-binding domain of *B. mori* chitinase alone (without the addition of Bbchit1) did not alter the virulence of *B. bassiana* (Table 3). The virulence of the transformant with pBARGFP was similar to that of the wild-type strain. Compared to the wild-type strain Bb0062, the LT_{50} of one transformant overexpressing Bbchit1-BmChBD (Bbchit1-BmChBD-19) was reduced by 23.0%. The LT_{50} of a transformant overexpressing Bbchit1 (Bbchit1-28) was reduced by 18.3%. The virulence in the transformant with overexpression of Bbchit1-mBmChBD was similar to that of a transformant overexpressing Bbchit1.

DISCUSSION

Two chitinase genes, Bb*chit1* (9) and Bb*chit2* (GenBank accession number AY147011), have been cloned from *B. bassiana*. However, neither chitinase has a chitin-binding domain. Here we utilized a chitin-binding domain from an insect, the silkworm moth (*B. mori*), to produce an engineered Bbchit1 in the insect pathogen *B. bassiana*. The engineered chitinase showed increased kinetic properties in chitin hydrolysis than those of the wild-type chitinase. Furthermore, when the engineered chitinase was overexpressed in *B. bassiana*, the genetically modified fungus showed an increase in virulence over a strain with a constitutively expressed native chitinase gene, Bb*chit1*. Insects produce chitinases to digest their cuticles during the molting process. These chitinases usually possess a chitin-binding domain, which facilitates chitinase binding and efficient degradation of chitin (1). By adding an insect chitinase chitin-binding domain to the chitinase of a fungal insect pathogen, we utilized a unique strategy where genetic components of the host insect were incorporated into the fungal pathogen in order to increase virulence against the host insect.

The chitin-binding domain of chitinases has previously been found to be an important feature for efficient binding to insoluble chitin and hydrolysis of insoluble chitin. The removal of the substrate-binding domain from chitinases or cellulases decreases their activities on insoluble substrates but not on soluble substrates (19). Deletion of the chitin-binding domain from a *Streptomyces griseus* chitinase resulted in an inability to bind to powdered chitin, with a resultant decrease of 50% in hydrolytic activity compared to the wild type (15). Limon et al. (18) engineered a hybrid chitinase by fusing a chitin-binding domain of ChiA from *Nicotiana tabacum* to a chitinase from *Trichoderma harzianum*. This hybrid chitinase had greater in vitro binding efficiency to powdered chitin and to fungal cell walls. Chitin-binding domains in plant chitinases may contribute to resistance against fungal pathogens. A chitinase, RSC-a, produced by rye (*Secale cereale*) seeds possesses a typical chitin-binding domain and has greater binding efficiency to powdered chitin and stronger inhibition against *Trichoderma* spp. than chitinase RSC-c, which has no chitin-binding domain (27).

The alteration of amino acid residues in a chitin-binding domain from *Bacillus circulans* resulted in some mutants with greater chitin binding efficiencies, some with less binding, and others with no change (30). We found that changes in aromatic amino acid and cysteine residues in the chitin-binding domain (i.e., BmChBD → mBmChBD) resulted in reduced chitin

TABLE 3. Chitinase activity analysis in *B. bassiana* transformants[a]

Strain[b]	Chitinase activity (mU/ml)[c]	Bioassay[d]		
		Slope	LT_{50} (h) (95% confidence interval)	r^2
Wild-type strain Bb0062	0.77 ± 0.08	7.13	151.4 (149.1–153.7)	0.98
Bbchit1-8	9.56 ± 0.76	7.83	135.7 (133.3–138.1)	0.96
Bbchit1-26	18.34 ± 0.85	8.07	123.7 (121.7–125.7)	0.96
Bbchit1-BmChBD-19	18.29 ± 0.56	6.13	116.8 (114.2–119.4)	0.95
Bbchit1-BmChBD-42	8.64 ± 0.35	7.50	118.2 (116.4–120.0)	0.98
Bbchit1-mBmChBD-2	12.06 ± 1.22	7.62	137.5 (134.2–140.8)	0.98
Bbchit1-mBmChBD-5	18.15 ± 0.59	7.33	135.2 (133.0–137.4)	0.98
BmChBD	0.86 ± 0.08	7.32	150.6 (148.5–152.7)	0.97
BARGFP	0.73 ± 0.02	7.19	152.1 (148.9–155.3)	0.94

[a] Mycelia of each strain (2 g) were transferred from SDY broth into basal salt medium with 2% glucose to suppress the expression of native Bbchit1.

[b] Bbchit1, transformants overexpressing Bbchit1; Bbchit1-BmChBD, transformants overexpressing Bbchit1-BmChBD; Bbchit1-mBmChBD, transformants overexpressing Bbchit1:mBmChBD; BmChBD, transformant overexpressing chitin-binding domain from *B. mori*; BARGFP, transformant with pBARGFP integrated.

[c] One unit of chitinase activity was defined as the amount of enzyme required to release 1 mol of *N*-acetylglucosamine per hour.

[d] Insect bioassays were conducted with aphids (*M. persicae*). Each treatment had three replicates ($n = 30$). The experiment was repeated three times. A χ^2 test showed that there were no significant differences in the values of the three repeated experiments ($P < 0.05$). Therefore, values in the table were calculated from data collected from all experiments.

binding. Moreover, the addition of the mutant chitin-binding domain to Bbchit1 (Bbchit1-mBmChBD) reduced its chitin binding efficiency. Possible explanations for this reduction might be that the amino acid alterations changed the tertiary structure or surface charge of Bbchit1.

The major constituents within insect cuticle are protein and chitin (7). Insect pathogenic fungi, such as *B. bassiana* and *Metarhizium anisopliae*, produce extracellular proteases and chitinases directed at hydrolyzing these cuticular components (6, 9, 16, 25). Constitutive expression of Pr1A by the insect pathogen *M. anisopliae* in infected insects resulted in a 25% increase in virulence and a concomitant decrease in host food consumption (26). The effectiveness of the strains with overexpression of Pr1A and, in this study, the engineered Bbchit1 strains create an opportunity to produce a strain with overexpression of Pr1 and Bbchit1, which may result in synergy of the two gene products.

Given the unique strategy of using genetic components derived from insects, one can envision the potential utility of several other ligands derived from insects in order to increase the effectiveness of transgenic entomopathogens. The chitin-binding domain could be fused onto Pr1A or other hydrolytic enzymes such as lipases. Insect tissue-specific ligands could be engineered onto fungal toxins directed toward the insect nervous system. There is an enormous array of metabolites produced by fungi that can be manipulated with specific ligands derived from the host insect itself. These engineered genes can be transformed into biocontrol fungi or genetic manipulation for crop protection.

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