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Tvbgn3, a β-1,6-Glucanase from the Biocontrol Fungus *Trichoderma virens*, Is Involved in Mycoparasitism and Control of *Pythium ultimum*▽

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Even though β-1,6-glucanases have been purified from several filamentous fungi, the physiological function has not been conclusively established for any species. In the present study, the role of Tvbgn3, a β-1,6-glucanase from *Trichoderma virens*, was examined by comparison of wild-type (WT) and transformant strains in which Tv*bgn3* was disrupted (GKO) or constitutively overexpressed (GOE). Gene expression analysis revealed induction of Tv*bgn3* in the presence of host fungal cell walls, indicating regulation during mycoparasitism. Indeed, while deletion or overexpression of Tv*bgn3* had no evident effect on growth and development, GOE and GKO strains showed an enhanced or reduced ability, respectively, to inhibit the growth of the plant pathogen *Pythium ultimum* compared to results with the WT. The relevance of this activity in the biocontrol ability of *T. virens* was confirmed in plant bioassays. Deletion of the gene resulted in levels of disease protection that were significantly reduced from WT levels, while GOE strains showed a significantly increased biocontrol capability. These results demonstrate the involvement of β-1,6-glucanase in mycoparasitism and its relevance in the biocontrol activity of *T. virens*, opening a new avenue for biotechnological applications.

Filamentous fungi of the genus *Trichoderma* have long been recognized as agents for the biocontrol of plant diseases. *Trichoderma* spp. can directly impact mycelia or survival propagules of other fungi through production of toxic secondary metabolites, formation of specialized structures, and secretion of cell wall-degrading enzymes (6, 50, 54). This mycoparasitic activity of *Trichoderma* spp. against phytopathogenic fungi and oomycetes due to lytic activity of cell wall-degrading enzymes has been widely studied (59). In addition to mycoparasitism, other mechanisms have been proposed to account for biocontrol of plant disease by *Trichoderma* spp., including the induction of resistance in the host plant and competition for nutrients and potential infection sites (20). All of these mechanisms have been shown to be employed effectively by *Trichoderma virens*. This biocontrol agent has been recognized as an aggressive mycoparasite capable of competing ecologically when colonizing potential sites of infection (21, 43). Different strains have been shown to induce phytoalexin production (19, 22) and systemic resistance (12). *T. virens* produces secondary metabolites, including gliotoxin, gliovirin, and peptaibols with known antimicrobial activities (23, 62), that have been shown to act synergistically with lytic enzymes to enhance the destruction of host cell walls (11).

During mycoparasitism, the ability of *Trichoderma* to sense a potential fungal host has been demonstrated, and key elements in the signaling transduction pathways and regulatory sequences in the promoter regions of mycoparasitism-related genes have been identified (7, 38, 64). Recently, the production of several extracellular proteins and corresponding genes by *Trichoderma* has been reported to be regulated during early interaction with plants (12, 57, 58). For example, the presence of plant roots induced the production in *T. virens* of a hydrophobin-like elicitor, Sm1, able to trigger systemic resistance in the plant (12), and two aspartyl proteases were found to be induced during root colonization by *Trichoderma asperellum* (58). In another symbiotic plant-fungus system, a β-1,6-glucanase was identified as being secreted into the apoplast of the host grass by its endophytic fungus, *Neotyphodium* sp. (40). Multiple functions for the enzyme were proposed, including the degradation of the cell walls of other fungi that may be encountered within the host plant. However, there are no data available on the regulation or potential involvement of β-1,6-glucanases secreted during *Trichoderma*-plant interactions.

Chitinases and glucanases are the most extensively studied hydrolytic enzymes, since chitin and β-1,3-glucan are the main fungal wall structural components (5). However, the cell walls of plant-pathogenic oomycetes, such as *Pythium ultimum*, are composed mainly of β-1,3- and β-1,6-glucans and cellulose instead of chitin (5). Furthermore, less-abundant structural components, such as β-1,6-glucan, have been found in *Saccharomyces cerevisiae* as the link between cell wall proteins and the main β-1,3-glucan/chitin polysaccharide (24, 33). Thus, β-1,6-glucanase may contribute to the efficient disorganization and further degradation of the host cell walls that occur during mycoparasitism. The lytic activity of β-1,6-glucanases against yeast (63) and cell walls of filamentous fungi in combination with other lytic enzymes has been described (9, 39). Recently, a β-1,6-glucanase has been shown to be a virulence factor in the interaction between the mycopathogen *Verticillium fungicola* and its host, *Agaricus bisporus* (1).

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Even though β-1,6-glucanases have been purified from several filamentous fungi, including *Penicillium brefeldianum* (51), *Acermonium* sp. (37)., and *Trichoderma harzianum* (8, 9, 39), their physiological function has not been conclusively established. Current knowledge of β-1,6-glucanases is limited to studies of biochemical and lytic properties of purified enzymes (9, 37, 39). We have previously cloned a gene, Tv*bgn3*, encoding a β-1,6-glucanase from *T. virens* (25). In this study, we assessed the physiological and ecological role of this enzyme in *T. virens* using a functional genomics approach. A series of transformants in which Tv*bgn3* was disrupted or constitutively overexpressed were compared to the wild type (WT) to elucidate the role of this enzyme. Our results provide direct in vivo evidence of the involvement of β-1,6-glucanases in biocontrol. To our knowledge, this is the first report of deletion of β-1,6-glucanase in any fungal biocontrol agent.

MATERIALS AND METHODS

Fungal materials. Two strains of *T. virens* were used in this study: the wild-type strain, Gv29-8, and an arginine-auxotrophic strain, Tv10.4, the recipient for fungal transformation (4). The strains were routinely maintained on potato dextrose agar (PDA) (Difco). The isolate of the seedling pathogen, *Pythium ultimum*, was kindly provided by C. Howell (USDA-Agricultural Research Service, Southern Plains Agricultural Research Center, College Station, TX). *Rhizoctonia solani* was isolated from a diseased cotton seedling in a production field from College Station, TX.

Isolation of high-molecular-weight Tv*bgn3* bacterial artificial chromosomal (BAC) clone and Southern analysis. After probing a *T. virens* BAC library (18) with the complete 1.29-kb open reading frame (ORF) of the β-1,6-glucanase cDNA clone from *T. virens* (25), one of the positive clones (4L19) was further digested with several restriction enzymes. A 7-kb ClaI fragment containing Tv*bgn3* ORF was subcloned into pBluescript II SK(+/−), obtaining the vector pSZD4, which was used for sequencing and further analysis. Nucleotide sequencing was performed by a primer-walking strategy (49). All sequencing reactions were performed at the Gene Technologies Laboratory (Texas A&M University). DNA sequences were analyzed by DNA Strider 1.2 (35) and Sequencher 4.1 (GCC, Ann Arbor, MI). The QIAprep Spin Miniprep kit (QIAGEN, Valencia, CA) was used for plasmid DNA purification. For gene copy number determination, the 1.29-kb ORF of the β-1,6-glucanase cDNA clone (25) was used as a probe in a standard Southern blot procedure (49). *T. virens* genomic DNA was isolated as previously described (62).

Tv*bgn3* gene expression analysis. To assess nutritional regulation of Tv*bgn3*, Vogel's minimal medium (60) without a carbon source (VM) or supplemented with either 1.5% glucose (VMG) or 0.5% fungal cell walls from *Rhizoctonia solani* (VMR) or *Pythium ultimum* (VMP) was used (46). Regulation of Tv*bgn3* expression by nitrogen was examined with VM with glucose (VMG-N) or *R. solani* cell walls (VMR-N) as a carbon source in the absence of a nitrogen source. After 2 days of growth in VMS (1.5% sucrose) medium (46), the mycelia were rinsed with sterile water and transferred to fresh media described above. Mycelia were collected after 1, 6, and 24 h and frozen in liquid nitrogen, and total RNA was extracted as previously described (12).

Expression of β-1,6-glucanase under different nutritional conditions was assessed by semiquantitative reverse transcriptase analysis (sqRT-PCR). To define the optimal number of PCR cycles for linear amplification of each gene, a range of PCR amplifications was performed. Subsequently, PCR products were electrophoresed and stained with ethidium bromide, and band signals were quantified by Scion image software (http://www.scioncorp.com/). The Tv*bgn3* gene-specific primers were as follows: forward, 5′-ATTACAGGCGAGTGGAGCA T-3′ (Sd6); reverse, 5′-GCGTTCGTTGGGATGTAGTT-3′ (Sd5). These primers amplified a 200-bp PCR product from both genomic and cDNA. Therefore, the negative control for reverse transcription-PCR (RT-PCR) was DNase treated and cleaned RNA reactions without reverse transcription. PCR amplification of Tv*bgn3* fragments comprised of 30 cycles (each cycle, 30 s at 94°C, 20 s at 57°C, and 20 s at 72°C). Actin-specific primers (designed based on *Trichoderma reesei* actin gene; GenBank accession no. X75421), used as an internal standard, were as follows: forward, 5′-AAGAAGTTGCTGCCCTCGT-3′ (ActF); reverse, 5′-GCTCAGCCAGGATCTTCATCATC-3′ (ActR). Actin PCR amplification with ActF-ActR consisted of 25 cycles (each cycle, 30 s at 94°C, 30 s at 58°C, and 40 s at 72°C). The sizes of the PCR products are 550 bp and 1 kb in cDNA and genomic DNA, respectively. PCR was performed according to the manufacturer's instructions using an Invitrogen (Carlsbad, CA) *Taq* DNA polymerase kit. PCR products were electrophoresed on 2% agarose gels, and band intensities compared within each experiment after ethidium bromide staining.

Analysis of Tvbgn3 protein levels in the presence of a host plant. A hydroponic growth system (12) was used to analyze possible changes in the production of Tvbgn3 in the presence of a host plant. Briefly, *T. virens* was grown in liquid medium in the presence or absence of cotton seedlings. Proteins in the culture filtrate were collected, concentrated, and analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as described below.

Construction of β-1,6-glucanase deletion and overexpression transformants. The β-1,6-glucanase disruption vector (pSZD12) was constructed by replacing a 2-kb fragment (1.35-kb Tv*bgn3* ORF and a 650-bp upstream region) with a 3.0-kb SmaI/EcoRV fragment of the *T. virens arg2* gene (4). The resulting vector contained 1.2-kb and 3.8-kb noncoding upstream and downstream Tv*bgn3* sequences from pSZD4 flanking the selectable *arg2* gene. The 3.8-kb fragment was PCR amplified with a forward primer containing an engineered BamHI restriction site starting directly after the Tv*bgn3* stop codon. Following amplification, the 3.8-kb PCR product was restricted with BamHI and ligated into the vector containing 1.2 kb flanking the *arg2* gene to obtain the gene replacement vector (pSZD12) (see Fig. 3A). The 8-kb ApaI/NotI deletion cassette was isolated from pSZD12 and used for fungal transformation. An overexpression vector (pJCC6) was constructed by placing the 1.29-kb ORF of the Tv*bgn3* cDNA clone between the promoter and the terminator regions of the *T. virens gpd* (*glyceraldehyde-3-phosphate dehydrogenase*) gene (3). This vector includes a 3.0 kb SmaI/EcoRV fragment containing the *arg2* gene as a selectable marker. For fungal transformation, a circular Tv*bgn3* overexpression vector was used in our standard transformation protocol (4).

Transformation and screening of transformants. Preparation of protoplast- and polyethylene glycol-mediated transformation of Tv10.4 with selection for arginine prototrophy was performed as previously described (4). Stable prototrophic transformants were selected by consecutive transfer of single colonies to VMS, PDA, and VMS.

Screening of deletion strains was first performed by PCR. For deletion transformants (GKO), β-1,6-glucanase-specific primers, Sd6 and Sd5, were used to amplify the 200-bp PCR product from the WT genomic DNA. Transformants yielding no product following PCR amplification were further selected for Southern blotting analysis.

Screening of potential transformants for overexpression of Tv*bgn3* (GOE) was initially performed by enzymatic activity assays of culture filtrates. Culture filtrates were obtained by inoculating 12-well MULTIWELL tissue culture plates (Becton Dickinson and Co., Franklin Lakes, NJ) containing 3 ml per well of VMM (1.5% mannose) with a conidial suspension of the appropriate fungal strain. Plates were incubated on a rotary shaker with slight agitation for 2 days at 23°C. Culture filtrates were collected by filtration through a 10-μm NITEX nylon cloth (TETKO, Inc., Depew, NY), and β-1,6-glucanase activity was assayed as described below.

Confirmation of transformants. To confirm effective disruption or overexpression of Tv*bgn3*, expression levels of the gene under inducing conditions was assessed. For that, conidia of the WT, GKO, and GOE strains were inoculated in VMS and transferred to VMR as described above. After 18 h, mycelia were collected and RNA isolated. Fifteen micrograms of total RNA per sample was analyzed by Northern blotting with hybridization performed at 42°C using Ultrahyb (Ambion). Hybridization signals were detected with a FUJIFILM BAS-1800 Phosphor-Imager and analyzed by using ScienceLab'98 Image Gauge ver. 3.4X image software. Transformants were further verified by detection of Tvbgn3 by SDS-PAGE and Western blotting and quantification of β-1,6-glucanase activity in culture filtrates after 5 days of fungal growth in VMS.

Protein extraction, SDS-PAGE, and Western blot analysis. Proteins in the culture filtrates were precipitated by 80% ammonium sulfate. Pellets were resuspended in 50 mM sodium acetate and dialyzed against the same buffer (10-kDa molecular mass cutoff; Pierce, Rockford, IL). Protein concentrations were determined by using the Bio-Rad Bradford microassay with bovine serum albumin as a standard. Proteins were separated by 15% SDS-PAGE. One gel followed by Coomassie staining was used as a control, whereas another one was electroblotted to a nitrocellulose membrane (Osmonics Inc., Gloucester, MA). Tvbgn3 polyclonal antibodies (dilution, 1:1,000) were used in a standard Western blot procedure (49).

β-1,6-glucanase activity assay. Activity of β-1,6-glucanase in the culture filtrates was determined by liberation of reducing sugars from pustulan solution (20

mg/ml) (*Umbilicaria papullosa*; Calbiochem, San Diego, CA) after 40 min of incubation at 37°C (9, 41). An enzyme unit was defined as the amount of enzyme that catalyzes the release of reducing sugar groups equivalent to 1 μmol of glucose for 1 min under the described assay conditions. Transformants with the highest enzymatic activities were selected for Southern blotting analysis.

N-terminal sequencing and antibody production. Protein extracts of the *T. virens* overexpression strain GOE53, obtained as described above, were analyzed by SDS-PAGE. The protein band corresponding to Tvbgn3 was excised and electroeluted in the Electroeluter 422 (Bio-Rad, CA). To confirm the identity of the eluted protein, N-terminal sequencing was performed by automated Edman chemistry on a Hewlett Packard G1005A Protein Sequencher (Protein Chemistry Laboratory, Texas A&M University). The electroeluted protein was used to produce a polyclonal antibody in rabbits (Sigma, The Woodlands, TX).

Phenotypic analysis of transformants. Cultures of selected transformants were compared to that of the WT strain for colony morphology, radial growth, spore formation and production, root colonization, and gliotoxin synthesis. Agar discs from actively growing colonies were inoculated in the center of VMS, PDA, or water agar (WA) plates. Formation of aerial hyphae and phialides, colony pigmentation, and colony growth patterns were visually discerned over 7 days. Hyphal extension on these three media was recorded at 24 and 48 h of growth at 27°C. The border of the hyphal extension was marked each day, each plate was photographed, and the surface area of growth was determined using ImageJ software (http://rsb.info.nih.gov/ij/). Each treatment contained four repetitions, and each experiment was repeated at least twice. The growth patterns of expanding hyphae from an agar disc centrally placed on a microscope slide coated with PDA or VMS was observed at 24 and 48 h. The numbers of conidia were determined by removing agar discs (three per plate; five repetitions) from 10-day-old PDA or VMS that had been uniformly inoculated with a conidial suspension. Discs were placed in sterile water containing 0.01% Tween 80 and vortexed for 1 min, and conidia were counted with a hemacytometer. To assess chlamydospore production, each strain was grown in a molasses medium (61) for 14 days, harvested by vacuum filtration, biomass air-dried overnight, and ground in a Wiley mill (40 mesh). Each preparation (0.1 g) was then suspended in 0.01% Tween 80 solution and counts of chlamydospores determined with a hemacytometer. The abilities of strains to colonize cotton roots were determined by first coating cotton seeds with chlamydospore preparations (61), planting seeds in plastic containers (3.81 by 20.9 cm), and incubating the seedlings in a growth chamber (EGC) at 25°C for 10 days. Five plants per strain were harvested and root systems washed, weighed, and homogenized in a Waring blender for 30 s. Serial dilutions were assayed by determining numbers of CFU on GVSM (43). The experiment was repeated twice. The synthesis of gliotoxin was assessed by measuring the zone of inhibition on antibacterial indicator plates using *Bacillus cereus* as the indicator organism (44). Each strain was replicated on five different plates, with the experiment repeated. Quantitative data were analyzed by analysis of variance (ANOVA) and Fisher's protected least significant difference (PLSD) test ($P < 0.05$) (Statview v. 5.0.1; SAS Institute, Cary, NC).

Fungal growth inhibition assay. To assess the biological function of Tvbng3, two representatives from each set of GKO and GOE transformants, selected based on their Tvbgn3 protein and activity levels, were compared with the WT. Growth inhibition of *P. ultimum* by extracellular proteins of the *T. virens* WT and transformants was examined in 12-well MULTIWELL tissue culture plates as previously described (3). Extracellular proteins were obtained by filtering 4-day VMS culture filtrates of each treatment through a 10 μm NITEX nylon cloth and then a 0.45-μm filter and concentrating them by using a 10-kDa-cutoff Millipore Amicon Ultra centrifugal filter device (Bedford, MA). Following concentration, proteins were added to the cooled autoclaved PDA in sufficient volume to result in a 1× or 2× concentration. The 1× concentration represented the concentration of the original culture filtrate. Agar plugs (diameter, 5 mm) of actively growing cultures of *P. ultimum* were placed on the middle of the wells and incubated at 27°C. When the hyphae in any of the treatments reached the edges of the well, the incubation was considered complete. Growth area of the fungus was marked, and the images were processed using ImageJ software.

Plant bioprotection assay. Cotton seeds (*Gossypium hirsutum* cv. Deltapine 451 B/RR) were coated with a latex sticker (Rohm and Haas, Philadelphia, PA), followed by coating with fine granules of chlamydospore preparations (61). The inoculum of *P. ultimum* was prepared as previously described (21). The coated seeds were planted in test tubes, each containing 10 g of a soil-sand mixture (60% Lufkin fine sandy loam and 40% fine sand) infested with *P. ultimum* oospores. Negative (C−) and positive (C+) controls consisted of tubes containing uninfested soil in which untreated seeds were planted or infested soil in which untreated seeds were planted, respectively. Each treatment, consisting of 10 tubes, was replicated 3 times, and the experiment was repeated twice. The tubes were incubated for 7 days in a growth chamber at 25°C and with a 14-h photoperiod. After incubation, the number of healthy surviving seedlings was counted.

Computational analysis. The URLs of the databases used in this study are as follows: ExPASy proteome server, Swiss Institute of Bioinformatics, http://us.expasy.org; EMBOSS, European Bioinformatics Institute, http://www.ebi.ac.uk/emboss; and GenBank, National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov. Multiple sequence alignments were performed using ClustalW (Kyoto University Bioinformatics Center [http://clustalw.genome.jp]). The previously published GenBank accession number of *T. virens* Tvbgn3 is AF395757 (25).

RESULTS

Reisolation of Tv*bgn3* and sequence analysis. To obtain the flanking regions of the Tv*bgn3* ORF for construction of deletion strains and analysis of putative regulatory motifs, a clone with a high-molecular-weight insert was isolated from the *T. virens* BAC library (18) using the 1.29-kb ORF of the β-1,6-glucanase cDNA (25) as a probe. From the 7-kb BAC subclone (pSZD4), we generated a 3.9-kb sequence containing 1.35 kb of the Tv*bgn3* coding region, 1.91 kb of 5′ flanking sequence, and 0.64 kb of the 3′ flanking sequence. The Tv*bgn3* copy number was determined by Southern analysis of *T. virens* WT genomic DNA. The restriction pattern indicates that Tv*bgn3* is a single-copy gene in the *T. virens* genome (data not shown).

The Tv*bgn3* ORF, interrupted by a single intron, encodes a 429-amino-acid polypeptide with a predicted molecular mass of 48.1 kDa and a pI of 5.56 (25). Analysis of the 740-bp region upstream of Tv*bgn3* (Fig. 1) revealed putative TATA boxes at positions −16, −91, and −540 and putative CAAT boxes at −23, −104, −114, −445, and −478 upstream of the ATG codon. Additionally, several putative regulatory motifs in the promoter region of Tv*bgn3*, previously found in other fungal genes, including those of *Trichoderma*, were identified. An AceI (5′ AGGCA 3′) motif, involved in the induction of cellobiohydrolase genes in response to cellulose (2, 48), was found at position −681. Two stress response sites, STRE (CCCCT) (7, 34), were identified at −461 and −667. Some of the mycoparasitism response elements (MYRE1 through MYRE4) described for *ech42* and *prb1* of *Trichoderma atroviride* (7) and Tv*sp1* of *T. virens* (46) and the newly identified MYRE5 motif (CP4-CP1, CX3-CX1, or PX2-PX1) in the promoter region of the *Trichoderma hamatum chit42*, *prb1*, and *xbg1.3-110* genes (55) were also found in the Tv*bgn3* promoter. These include MYRE3 (GGGCAC) at −502, PX2 (ATTTAAG) at −102, and CP2 (AACGTT) at −144. In contrast to other mycoparasite-related genes (7, 46), neither CreA-binding motifs (5′ SYGGRG 3′) involved in catabolite repression (26, 53) nor a HGATAR site, a putative binding consensus for nitrogen regulation (47), was found in the Tv*bgn3* promoter region.

N-terminal analysis of the deduced amino acid sequence of Tv*bgn3* using SignalP and the hydropathy plot calculated by the method of Kyte and Doolittle (27) indicated that a putative cleavage site between the signal peptide and the mature, secreted protein is located between residues 17 and 18. However, N-terminal sequencing of the partially purified Tvbgn3 protein (See Materials and Methods) yielded the seven-residue sequence FEPSLAS (Fig. 1). The mature protein therefore starts at amino acid 41. The presence at positions 39 to 40 of the KR sequence, a target sequence for the specific protease Kex2, further supports the additional processing of the preprotein

FIG. 1. Analysis of putative regulatory motifs in promoter region of Tv*bgn3* and protein processing. Only the sequence upstream of Tv*bgn3* and the N terminus of the gene are presented, since the Tv*bgn3* ORF was published previously (25). The predicted amino acid sequence of Tvbgn3 is shown below the nucleotide sequence. The peptide sequence with dashed underline corresponds to that obtained by N-terminal sequencing of the protein. The intron sequence (at positions 176 to 236) is marked in lowercase. The consensus sequences for 5′ and 3′ intron splicing sites are marked in bold. The signal sequence cleavage and the proenzyme cleavage sites are indicated by dashed and solid arrows, respectively. Sites of putative regulatory motifs correspond to the numbers in Results and are indicated as follows: TATA, clear boxes; CAAT, dotted boxes; AceI (AGGCA), light gray box; STRE (CCCCT), dark gray boxes; MYRE3 (GGGCAC), dashed box; PX2 (ATTTAAG), shaded light gray; and CP2 (AACGTT), shaded dark gray.

(17). Thus, the mature protein contains 389 amino acids, a predicted molecular mass of 43.6 kDa, and a pI of 5.31. Several potential posttranslational modifications are predicted from the sequence (ScanProsite), including an N-glycosylation site (residue 29), an amidation site (residue 37), and multiple phosphorylation and N-myristoylation sites.

Tv*bgn3* gene expression analysis. The basal level of expression of Tv*bgn3* under noninducing conditions was very low and could not be detected by standard Northern blot analysis (data not shown). Thus, sqRT-PCR was performed to assess the regulation of Tv*bgn3* by different starvation conditions or diverse carbon sources at different time points. A range of PCRs was performed to determine the optimal number of PCR cycles for linear amplification of the gene.

sqRT-PCR analysis confirmed very low basal levels of expression of Tv*bgn3* in VM, VM supplemented with glucose and nitrogen (VMG), or VM supplemented with glucose but depleted of nitrogen (VMG-N) (Fig. 2). When *R. solani* or *P. ultimum* cell walls were used as a carbon source, expression of Tv*bgn3* was found to be induced, regardless of the presence or absence of a nitrogen source (VMR and VMR-N). Tv*bgn3* induction in VMR was first observed at the 6-h time point, and the expression level remained similar after 24 h of incubation. A stronger induction was observed in the presence of *Pythium* cell walls. The expression of Tv*bgn3* in VMP was detected as early as 1 h after the transfer of mycelia to the fresh medium, with the maximum level of expression observed at 24 h (Fig. 2).

Tvbgn3 protein levels during interaction with cotton roots. In addition to regulation by the fungal host, we examined if Tv*bgn3* is regulated during WT interaction with plants using a hydroponic system (12). Proteins secreted into the hydroponic growth medium containing only mycelia of the WT or mycelia interacting with cotton roots after 48 h were concentrated and analyzed by Western analysis. No Tvbgn3 could be detected in hydroponic media of the WT growing in the presence or absence of the cotton seedlings (data not shown).

Identification of β-1,6-glucanase deletion and overexpression transformants. The vector for gene replacement, pSZD12, was constructed to replace the entire Tv*bgn3* ORF with a selectable marker (Fig. 3A). A total of 90 stable transformants were tested by PCR for a Tv*bgn3* gene disruption. Six PCR-selected candidates were further analyzed by Southern hybridization to

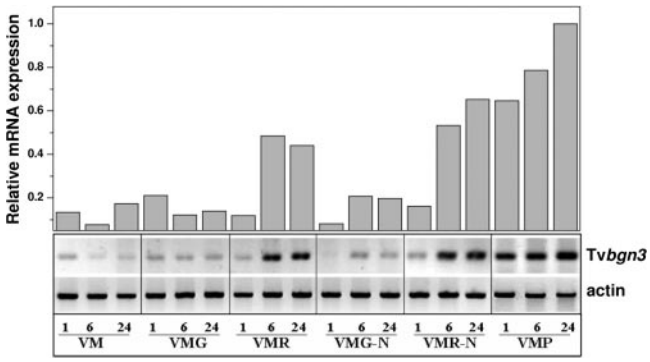


FIG. 2. Semiquantitative RT-PCR analysis of Tv*bgn3* expression during growth of *T. virens* in submerged culture under different nutritional conditions. Media used were the following: Vogel's minimal medium without carbon source (VM) or supplemented with glucose (VMG), *R. solani* (VMR), or *P. ultimum* cell walls (VMP); or VM lacking nitrogen but supplemented with glucose (VMG-N) or *R. solani* cell walls (VMR-N). Listed in the bottom panel are hours after transferring mycelia to the indicated media. Bottom panel, amplification of a 200-bp product using Tv*bgn3*-specific primers; fungal actin was amplified as a control for equal amounts of cDNA, yielding a 550-bp fragment from cDNA. Intensities of bands were quantified using Scion Image software, and the data were expressed as arbitrary units of Tv*bgn3* expression normalized to actin (bars).

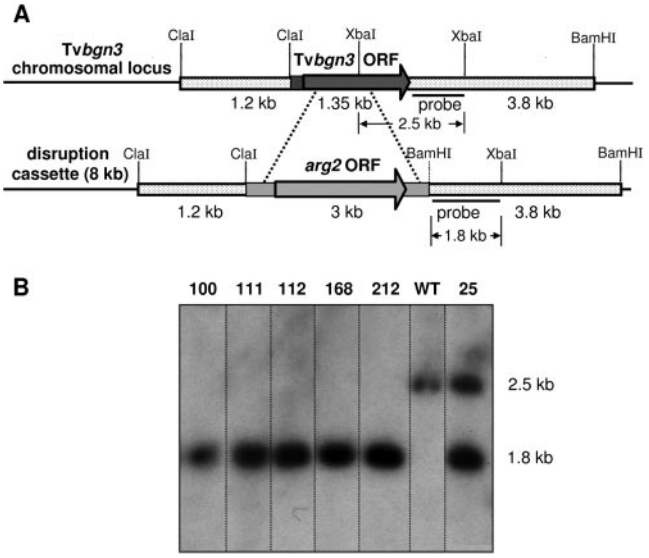


FIG. 3. Southern analysis and confirmation of Tv*bgn3* disruptants. (A) Scheme of the gene deletion strategy. The indicated probe (1.8-kb BamHI/XbaI fragment) was obtained by digestion of a 3.8-kb insert from pSZD12 (note: the BamHI restriction site, indicated by dashed line, was engineered to the forward primer for PCR amplification of 3.8 kb; therefore, it is not present in the WT genome). The expected sizes for native (2.5 kb) and deletion (1.8 kb) events are indicated. The dimensions are not drawn to scale. (B) Southern analysis of the *T. virens* WT strain and Tv*bgn3* deletion transformants (GKO100, GKO111, GKO112, GKO168, and GKO212). Autoradiograph of DNA gel blot hybridized with [^{32}P]dCTP-labeled probe indicated in A. Fifteen micrograms of genomic DNA was digested with BamHI/XbaI and loaded per lane.

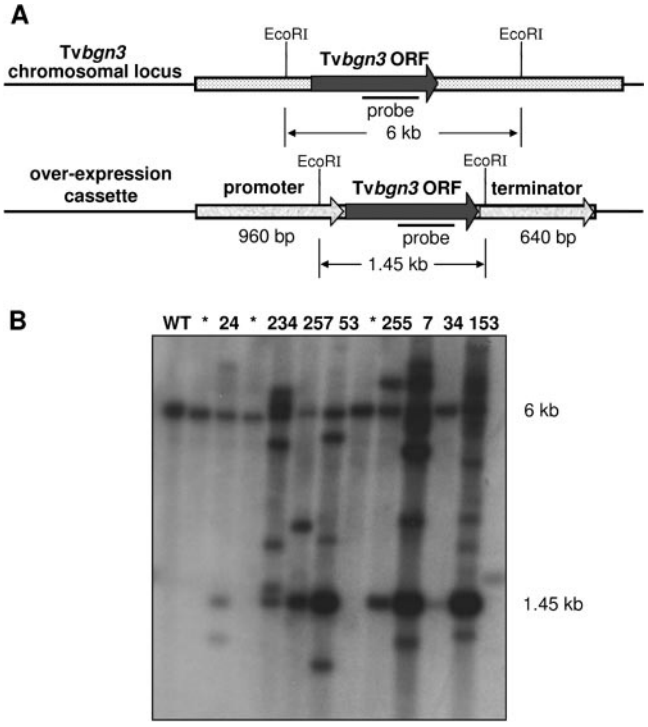


FIG. 4. Confirmation of Tv*bgn3* overexpression transformants. (A) Gene overexpression strategy. The expected sizes in native (6 kb) and overexpression (1.45 kb) events are indicated. The dimensions are not drawn to scale. (B) Southern analysis of *T. virens* WT strain and Tv*bgn3* overexpression transformants (GOE24, GOE234, GOE257, GOE53, GOE255, GOE7, GOE34, and GOE153). Fifteen micrograms of genomic DNA was digested with EcoRI and probed with a [^{32}P]dCTP-labeled 829-bp SalI/PstI fragment of Tv*bgn3*. Asterisks indicate strains not containing the overexpression cassette.

verify the gene disruption. As represented in Fig. 3A, after double digestion of the genomic DNA with BamHI/XbaI and hybridization with the 1.8-kb BamHI/XbaI fragment immediately downstream of Tv*bgn3* ORF, a positive disruption event was expected to yield a 1.8-kb band versus a 2.5-kb band in the WT. Five strains, GKO100, GKO111, GKO112, GKO168, and GKO212, were clearly disrupted for the gene, while strain 25 contained both 1.8-kb and 2.5-kb bands, indicating ectopic integration of pSZD12 (Fig. 3B).

The overexpression vector pJCC6 was constructed for constitutive overproduction of the Tv*bgn3* gene (Fig. 4A). Screening of stable putative Tv*bgn3* overexpression transformants was initially performed by measuring β-1,6-glucanase activity (9, 41). The transformants with highest enzymatic activity were selected for Southern analysis. Out of 72 putative GOE strains, 11 candidates were selected for Southern blotting analysis. Genomic DNA of putative β-1,6-glucanase overexpression strains was digested with EcoRI. A positive Tv*bgn3* overexpression event was expected to yield a 1.45-kb versus 6-kb band for the WT after hybridization with the gene-specific probe (Fig. 4A). Eight β-1,6-glucanase overexpression transformants were identified (GOE24, GOE234, GOE257, GOE53, GOE255, GOE7, GOE34, GOE153) (Fig. 4B). Most of the GOE transformants had additional bands indicating various integration events with genomic rearrangements. The intensity of the 1.45-kb band in some of the transformants, such as GOE7, GOE53, and GOE153, suggested integration of multiple copies of the construct (Fig. 4B).

β-1,6-glucanase expression, protein production, and enzymatic activity of transformants. To confirm successful disruption of the Tv*bgn3* gene and that the overexpression transformants were functional, i.e., showing elevated levels of transcription compared to the WT, Northern analysis was performed. Figure 5 shows Northern analysis of selected transformants grown under inducing conditions (VM supplemented

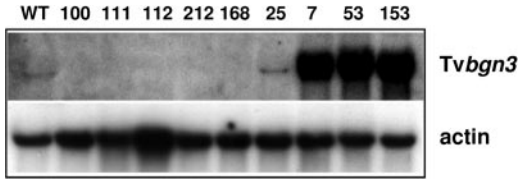
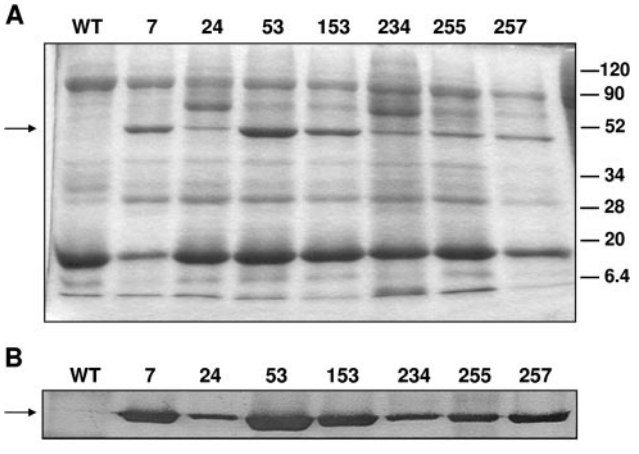


FIG. 5. Northern analysis of Tv*bgn3* expression in deletion and overexpression strains. Total RNA was extracted from the wild type (WT), deletion strains (GKO100, GKO111, GKO112, GKO168, and GKO212), a strain with ectopic integration of the construct (25), and selected overexpression strains (GOE7, GOE53, and GOE153) cultured in VM medium supplemented with 0.3% *R. solani* cell walls for 18 h. Fifteen micrograms of total RNA was separated on a formaldehyde-agarose gel, transferred to a Hydrobond-N$^+$ nylon membrane, and hybridized with a 538-bp XbaI/PstI Tv*bgn3* fragment (top panel). Bottom panel, the same membrane was hybridized with a 550-bp actin fragment PCR amplified from *T. virens* WT cDNA as a control for even loading.



C

T. virens strains	β-1-6-glucanase activity (U/mg)
WT	4.2
GOE7	46.1
GOE24	9.0
GOE53	87.9
GOE153	35.0
GOE234	5.5
GOE255	10.5
GOE257	8.8

FIG. 6. Tvbgn3 production and activity in overexpression strains. (A) Coomassie-stained pattern of SDS-PAGE analysis of protein extracts from *T. virens* WT and overexpression strains grown for 5 days in VMS. Eight micrograms of total protein was loaded per lane. Lanes, from left to right: *T. virens* (WT); overexpression strains (GOE7, GOE24, GOE53, GOE153, GOE234, GOE255, and GOE257); molecular masses, in kilodaltons, are indicated on the right. The Tvbgn3 protein is indicated by an arrow. (B) Immunoblot analysis of protein extracts; samples and SDS-PAGE are as in A. Mature 43.6-kDa Tvbgn3 is indicated by an arrow. (C) Total β-1,6-glucanase activity in protein extracts from 5-day-old VMS culture filtrates of wild-type and overexpression strains, measured as released reducing sugars from pustulan as the substrate (see Materials and Methods). Values are an average for two repetitions and are expressed as units of activity per milligram of protein.

with *R. solani* cell walls). No transcripts of Tv*bgn3* were detected in any of the GKO transformants (GKO100, GKO111, GKO112, GKO168, and GKO212), confirming the disruption of the gene by the selectable marker (Fig. 5). In agreement, the Tvbgn3 protein was absent in GKO strains as confirmed by Western analysis (data not shown). Among overexpression strains, Tv*bgn3* transcript levels were much higher in three selected mutants (GOE7, GOE53, GOE153) than in the WT, indicating the constitutive expression of the construct (Fig. 5). Increased expression levels in selected GOE strains correlated with protein production, since these transformants produced the largest amount of Tvbgn3 after 5 days of growth in VMS medium (Fig. 6). The other four transformants (GOE24, GOE234, GOE255, and GOE257) produced less Tvbgn3 than GOE7, GOE53, and GOE153 but more than the WT, as confirmed by both SDS-PAGE and Western analysis (Fig. 6A and B). Furthermore, we confirmed that the constitutively synthesized protein was correctly processed and functional, since the amount of the Tvbgn3 protein produced (Fig. 6A and B) correlated with the levels of the total β-1,6-glucanase activity detected using pustulan as the substrate (Fig. 6C). Transformants GOE7, GOE53, and GOE153 displayed 10.9-, 20.8-, and 8.3-fold-higher β-1,6 glucanase activity levels (expressed in U/mg) than the WT, whereas the activities of GOE24, GOE234, GOE255, GOE257 were in a range of 1.30- to 2.5-fold higher than those of the WT (Fig. 6C). Protein and enzymatic activity assay levels for transformants (Fig. 6) correlated with the results from the Southern analysis (Fig. 4). The selected GOE strains, showing the highest integration of multiple copies of the overexpression construct (GOE7, GOE53, and GOE153), also displayed the highest protein level and enzymatic activity.

Phenotypic analysis of transformants. A number of phenotypic assays were conducted with the gene disruption, overexpression, and WT strains to ensure that the strains were comparable in many developmental and life cycle events. Similarity among the mutant and WT strains for these events provided confidence that the results from the biocontrol assays were a function of genetic manipulations in Tv*bgn3* rather than the transformation procedure. There were no evident changes in cultures of GKO or GOE strains compared to the WT with respect to colony morphology and pigmentation, phialide formation, the presence of aerial hyphae, or the growth pattern of hyphae on solid media. No differences among the strains were found for conidial abundance on complex (PDA) or minimal (VMS) medium or in chlamydospore production under fermentation conditions. Fungal growth was assessed for three GKO strains (GKO111, GKO112, and GKO212), three GOE strains (GOE7, GOE53, and GOE153) and the WT under different nutritional conditions (VMS, WA, and PDA). There was no statistically significant difference in the mycelial surface area among the strains after 1 or 2 days growth on any of the media (Table 1). Zones of inhibition on antibacterial indicator plates resulting from the presence of gliotoxin in culture filtrates were similar among all strains tested. Finally, there was no significant difference in the ability of the strains to colonize roots of cotton seedlings (expressed as CFU/g root tissue).

TABLE 1. Growth analysis of WT, GKO, and GOE strains

Strain	Growth (%) on medium[a]					
	PDA		VMS		WA	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
WT	8.9	28.5	7.1	25.2	12.8	64.0
GKO111	8.1	28.5	6.9	26.3	10.7	58.6
GKO112	8.6	29.2	6.6	24.5	11.4	58.6
GKO212	7.8	27.4	7.1	26.3	9.8	54.9
GOE7	7.7	26.5	7.0	24.9	9.3	54.2
GOE53	8.1	28.8	6.8	24.9	10.8	58.0
GOE153	7.3	24.8	6.1	22.7	9.3	54.6

[a] Surface area of growth of the WT, GKO strains, and GOE strains in PDA, VMS, or WA plates, recorded for 2 days. The data are represented as the ratio of fungal growth area to the total area of the plate in percentages. Each column represents the mean growth area of four replicates from two independent experiments. Based on ANOVA ($P < 0.05$), there was no significant differences in growth area among these strains on any of the media tested.

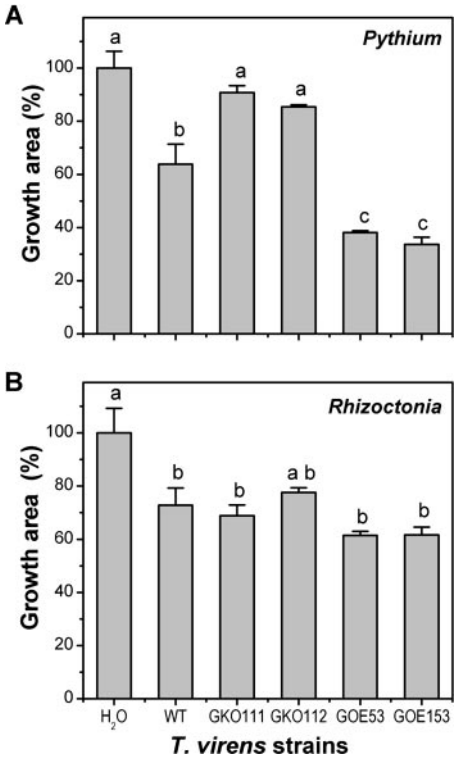


FIG. 7. Growth inhibition of *P. ultimum* (A) and *R. solani* (B) by protein extracts obtained from *T. virens* WT, GKO strains, and GOE strains at 2× concentration. Data are represented as the ratio of the pathogen growth area in medium containing *Trichoderma* protein extracts compared to the growth area in the water control. Each column represents mean growth area of two replicates from two independent experiments with standard error bars. Columns with a letter in common did not differ significantly according to Fisher's PLSD test at the significance level of 5%.

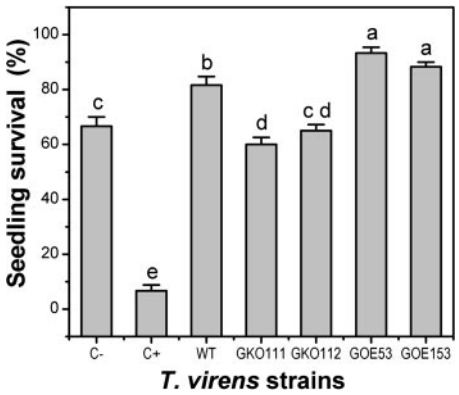


FIG. 8. Plant biocontrol assay. Each bar represents the total number of healthy plants after 7 days of incubation of *Trichoderma*-coated seeds in soil infested with *Pythium ultimum*. Treatments are as follows: C−, negative control (no pathogen, no *Trichoderma*); C+, positive control (pathogen present, no *Trichoderma*); *T. virens* Gv29-8 (WT), Tv*bgn3* deletion (GKO111 and GKO112), and overexpression (GOE53 and GOE153) strains. Each bar represents mean seedling survival of three replicates with 10 plants each from 2 independent experiments with standard error bars. Columns with a letter in common did not differ significantly according to Fisher's PLSD test at the significance level of 5%.

Growth inhibition of phytopathogens by WT and transformants. The abilities of *T. virens* Tv*bgn3* transformants and the WT to inhibit the growth of the fungal pathogens *P. ultimum* and *R. solani* were compared. The pathogen was inoculated onto medium containing the protein extracts of the WT, GKO, or GOE strain, and following incubation, hyphal extension of the pathogen was recorded and the growth area calculated for each treatment.

The average growth rate of *P. ultimum*, given as a rate of increase in the radius of the fungal growth area during 7 h on unamended PDA, was 1.12 mm/h. Despite such a high growth rate, *P. ultimum* was inhibited by the extracellular protein extracts of *T. virens*. The inhibition resulted from the fraction of proteins with molecular masses greater than 10 kDa, since that was the size of the membrane used for protein concentration. This excludes toxic effects of culture filtrates due to the presence of antibiotic compounds (gliotoxin and gliovirin) known to be produced by *T. virens*. When the original concentration of culture filtrates was used, there was a noticeable but not significant ($P < 0.05$) inhibition of *Pythium* growth by GOE strains (data not shown). However, when 2× concentrations were used, the growth of *P. ultimum* was significantly reduced by GOE strains (GOE53, 38.1%; GOE153, 33.7%) compared to that of the WT (63.8%). The effect of Tv*bgn3* deletion on growth inhibition was clear, since GKO strains had a significantly reduced ability to inhibit *Pythium* growth (GKO111, 90.8%; GKO112, 85.4%) compared to that of the WT (63.8%). The growth of *P. ultimum* on medium containing extracellular proteins from GKO strains did not differ significantly from that on the water-amended medium (Fig. 7A). These results suggest a major role of Tvbgn3 in the growth inhibition of *P. ultimum*.

Rhizoctonia solani showed as lower growth rate than *P. ultimum*. Hyphal growth, measured after 17 h of incubation, yielded an average rate of 0.6 mm/h. However, *R. solani* was significantly inhibited by all *Trichoderma* strains at both concentrations. Regardless of the concentration of proteins applied, the inhibition patterns of the WT and of transformants were similar. A slight increase in the inhibition rate by GOE strains compared over that of the WT was observed at a 2× concentration, but the difference was not statistically significant (at a significance level of 5%) (Fig. 7B).

Plant biocontrol assay. To assess the relevance of this enzyme in the biocontrol ability of *T. virens* against *P. ultimum*, the protection of cotton seedlings against this pathogen by the different transformants was compared (Fig. 8). While 66.7% of seedlings survived in noninfested soil (C−), infestation of soil with *Pythium* inoculum reduced seedling survival to 6.7% (C+). Seed treatment with any of the *Trichoderma* strains resulted in a significant improvement of seedling survival. Treatment with the WT resulted in 81.7% survival. GKO and GOE strains showed significantly reduced (GKO111, 60%; GKO112, 65%) or enhanced (GOE53, 93.3%; GOE7, 88.3%) biocontrol activity, respectively, compared to those for the WT. The results of the in vivo plant bioassay correlated with those of the in vitro growth inhibition assay and confirmed the involvement of Tvbgn3 in the biocontrol of *P. ultimum* by *T. virens*.

DISCUSSION

Tv*bgn3* from *T. virens* encodes an extracellular β-1,6-glucanase. The protein shares a high similarity to β-1,6-glucanases from other filamentous fungi, including biological control agents, endophytes of grasses, and mycoparasites. Its relationship to glycosyl hydrolase family 5, including the predicted catalytic residues (32), was recognized previously (25, 29). Protein sequence analysis indicates that the gene codes for a preproprotein. The first 17 residues correspond to a signal peptide probably promoting secretion of the enzyme into its immediate environment, a factor considered to be essential in the parasitic ability of mycopathogens (25). The amino acids immediately preceding the experimentally determined N-terminal residues of Tvbgn3 are Lys-Arg (KR), which constitute a recognition site for a subtilisin-like endoproteinase (Kex2) known to processes secreted proteins in different organisms (14, 17, 56). A Kex2 recognition site has been detected in a β-1,6-glucanase (Bgn16.2) from *T. harzianum* (29), as well as in other hydrolytic enzymes from *Trichoderma*, such as the chitinase CHIT42 (15) and the proteases Prb1 (16) and Tvsp1 (46). Since this proteolytic processing is required for protein activation (36) and the Kex2 sites were identified in proteins demonstrated to have biological activity, it would appear that the same mechanism of processing plays an important role in biological activation of Tvbgn3.

The mycoparasitism in *Trichoderma* species is proposed to be regulated by catabolite repression (30). Since the soil is generally considered a nutritionally sparse environment, when carbon is deprived, alternative pathways are induced in response to environmental stimuli, such as the presence of a potential fungal host. Genes involved in mycoparasitism from *Trichoderma* species have been shown to be differentially regulated in a carbon/nitrogen-dependent-fashion and in the presence of a fungal host (30, 31, 42, 46). In this study, gene expression analysis demonstrated the induction of Tv*bgn3* by the presence of fungal cell walls, a condition often regarded as a simulation of mycoparasitism. In the presence of *Rhizoctonia* cell walls, there was a relatively moderate level of induction of Tv*bgn3* expression. Remarkably, earlier and higher expression of Tv*bgn3* was observed in the presence of *P. ultimum* cell wall fractions. This stronger induction by *Pythium* cell walls is coherent with its high content of β-1,6-glucans, characteristic of oomycetes, in contrast to *R. solani* cell walls, which are mainly based on chitin and β-1,3-glucans (5). The increase of Tv*bgn3* transcripts seems to correspond to the inducer stimulus and not to starvation conditions, since incubation in media lacking carbon or nitrogen resulted in very low levels of transcription. The described gene expression pattern is consistent with the regulatory motifs found in the Tv*bgn3* promoter region. No *cis*-acting elements involved in carbon or nitrogen regulation were found, yet several MYRE elements, proposed to be involved in mycoparasitism (7, 55), were identified. Recently, Montero et al. (39) described a similar pattern of expression for another β-1,6-glucanase (Bgn16.3) purified from *T. harzianum*. Bgn16.3 was not detected under chitin induction but did specifically and greatly accumulate in the presence of fungal cell walls, suggesting the involvement of this enzyme in mycoparasitism (39). The presence of several other putative regulatory sequences in the promoter region of Tv*bgn3*, such as those for cellulose (2, 48) and stress response elements (7, 34), suggests a complex transcriptional regulation in response to environmental stimuli and deserves further experimental analysis. To assess whether Tvbgn3 is regulated during the interaction of *T. virens* with plant roots, the levels of Tvbgn3 secreted by the WT growing alone or with cotton roots were compared in a hydroponics system. Although we cannot rule out Tvbgn3 being regulated during the interaction with a host plant but expressed below our detection limits, our results indicate that expression of Tvbgn3 is mainly regulated by the presence of a fungal host and not during the interaction with plants.

The Tv*bgn3* gene regulation pattern suggests a role of Tvbgn3 in degrading glucan polymers of the fungal host and providing *Trichoderma* a nutritional carbon source. As described for other secreted hydrolytic enzymes, β-1,6-glucanases may also play a role in fungal development, including hyphal growth and branching, sporulation, or autolysis, after exhaustion of the external carbon source (reviewed in reference 45). To assess the biological function of the enzyme in *T. virens*, transformant strains were constructed in which the gene was successfully deleted or constitutively expressed. When these strains (two independently derived GOE and GKO transformants and the wild type) were assessed for a number of phenotypic traits (conidiation, chlamydospore production, gliotoxin synthesis, growth rate, hyphal growth patterns, colony morphology, and root colonization), we found the strains to be similar. Thus, our data do not support any relevant role for Tvbgn3 in normal fungal development. However, these data do support the validity of comparing these mutant strains to determine the role of Tv*bgn3* during the interaction of *T. virens* and seedling pathogens.

Beta-1,6-glucanases purified from *Trichoderma* (8, 9, 39) were shown to have little or no effect on degradation of cell walls of filamentous fungi alone but significantly improved hydrolytic activity in combination with other cell wall-degrading enzymes (9). Protein extracts of our transformants lacking or overexpressing Tv*bgn3* were compared with the WT for their ability to inhibit the growth of fungal plant pathogens. The compounds, such as gliotoxin or peptaibols, known to enhance the activity of hydrolytic enzymes (11, 52) were excluded to solely test the inhibition activity of secreted enzymes. The effect of protein extracts on the growth of *P. ultimum* was more prominent than that for *R. solani*. This was somewhat anticipated, since *P. ultimum* cell walls are composed of β-1,3- and β-1,6-glucans and *R. solani* cell walls consist mainly of chitin (5), and it is consistent with the gene regulation pattern described above. Differences in the relative hydrolytic activities of β-1,6-glucanases on different cell walls depending on their β-1,6-glucan content have been demonstrated (9, 39). Remarkably, in our study, *Pythium* inhibition by the GOE strains was almost twofold higher than the WT level, while the GKO strains displayed 1.6-fold lower ability to inhibit pathogen growth (Fig. 7A). Lack of a significant difference in growth of *P. ultimum* between the untreated control and the treatment with proteins from the disruptant strains suggests a very important role of β-1,6-glucanase in mycoparasitic activity of *T. virens* against this pathogen. Similar findings were obtained when the gene encoding β-1,6-glucanase from the pathogenic

fungus *V. fungicola* was disrupted; the mycoparasitic ability with *A. bisporus* was markedly reduced (1).

To confirm the biological relevance of the antifungal activity observed in the in vitro assay, the levels of bioprotection achieved by the different strains against *P. ultimum* were tested in plant bioassays. The results of the plant protection assays clearly demonstrate an effect of deleting or overexpressing Tv*bgn3*. The disruption of the gene resulted in significantly reduced levels of disease protection compared to those for the WT. Accordingly, seedling survival significantly increased when seeds were treated with the GOE strains (Fig. 8). Constitutive overexpression of other cell wall-degrading enzymes, such as chitinase, β-1,3-glucanase, or protease, has been shown to improve the activity of biological agents (3, 13, 28, 46). In the present work, we successfully overproduced a β-1,6-glucanase in *Trichoderma* by overexpression of Tv*bgn3* in a wild-type background. Previous attempts to overproduce β-1,6-glucanase in *Trichoderma* did not result in an increase of extracellular protein under most conditions, probably due to degradation by acidic proteases or instability at low pH (10). The transformants overexpressing Tv*bgn3* were significantly better in disease control than the WT, confirming the relevance of β-1,6-glucanase in biocontrol. Previously, in vitro lytic activity of purified β-1,6-glucanases from *Trichoderma* has been demonstrated (8, 9, 39). Our study reports for the first time the biological significance of such an enzyme in vivo using a plant-pathogen-biocontrol agent bioassay.

In conclusion, the data presented in this study elucidated the role of β-1,6-glucanase in *T. virens* physiology and ecology. Targeted gene disruption provided the opportunity to study the importance of Tvbgn3 in *T. virens* physiology. To our knowledge, this is the first report of deletion of β-1,6-glucanase in any fungal biocontrol agents. We demonstrated that deletion of the gene had no evident effect on the fungal metabolism and growth. Instead, the involvement of β-1,6-glucanase in mycoparasitism and plant protection was shown. Our results shed additional insights into *Trichoderma* mycoparasitism and biocontrol processes and open new opportunities for biotechnological applications. Indeed, successful overproduction of Tv*bgn3* in *T. virens* strains expressing other mycoparasitism-related genes has been shown to achieve greater protection against a broader range of plant pathogens (S. Djonovic and C. M. Kenerley, unpublished data).

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REFERENCES

1. **Amey, R. C., P. R. Mills, A. Bailey, and G. D. Foster.** 2003. Investigating the role of a *Verticillium fungicola* β-1,6-glucanase during infection of *Agaricus bisporus* using targeted gene disruption. Fungal Genet. Biol. **39:**264–275.
2. **Aro, N., A. Saloheimo, M. Ilmen, and M. Penttila.** 2001. ACEII, a novel transcriptional activator involved in regulation of cellulase and xylanase genes of *Trichoderma reesei*. J. Biol. Chem. **276:**24309–24314.
3. **Baek, J. M., C. R. Howell, and C. M. Kenerley.** 1999. The role of an extracellular chitinase from *Trichoderma virens* Gv29-8 in the biocontrol of *Rhizoctonia solani*. Curr. Genet. **35:**41–50.
4. **Baek, J. M., and C. M. Kenerley.** 1998. The *arg2* gene of *Trichoderma virens*: cloning and development of a homologous transformation system. Fungal Genet. Biol. **23:**34–44.
5. **Bartnicki-Garcia, S.** 1968. Cell wall chemistry, morphogenesis, and taxonomy of fungi. Annu. Rev. Microbiol. **22:**87–108.
6. **Benitez, T., A. M. Rincon, M. C. Limon, and A. C. Codon.** 2004. Biocontrol mechanisms of *Trichoderma* strains. Int. Microbiol. **7:**249–260.
7. **Cortes, C., A. Gutierrez, V. Olmedo, J. Inbar, I. Chet, and A. Herrera-Estrella.** 1998. The expression of genes involved in parasitism by *Trichoderma harzianum* is triggered by a diffusible factor. Mol. Gen. Genet. **260:** 218–225.
8. **de la Cruz, J., and A. Llobell.** 1999. Purification and properties of a basic endo-β-1,6-glucanase (BGN16.1) from the antagonistic fungus *Trichoderma harzianum*. Eur. J. Biochem. **265:**145–151.
9. **de la Cruz, J., J. A. Pintor-Toro, T. Benitez, and A. Llobell.** 1995. Purification and characterization of an endo-β-1,6-glucanase from *Trichoderma harzianum* that is related to its mycoparasitism. J. Bacteriol. **177:**1864–1871.
10. **Delgado-Jarana, J., J. A. Pintor-Toro, and T. Benitez.** 2000. Overproduction of β-1,6-glucanase in *Trichoderma harzianum* is controlled by extracellular acidic proteases and pH. Biochim. Biophys. Acta **1481:**289–296.
11. **Di Pietro, A., M. Lorito, C. K. Hayes, R. M. Broadway, and G. E. Harman.** 1993. Endochitinase from *Gliocladium virens*—isolation, characterization, and synergistic antifungal activity in combination with gliotoxin. Phytopathology **83:**308–313.
12. **Djonovic, S., M. J. Pozo, L. J. Dangott, C. R. Howell, and C. M. Kenerley.** 2006. Sm1, a proteinaceous elicitor secreted by the biocontrol fungus *Trichoderma virens* induces plant defense responses and systemic resistance. Mol. Plant-Microbe Interact. **19:**838–853.
13. **Flores, A., I. Chet, and A. Herrera-Estrella.** 1997. Improved biocontrol activity of *Trichoderma harzianum* by over-expression of the proteinase-encoding gene *prb1*. Curr. Genet. **31:**30–37.
14. **Fuller, R. S., A. Brake, and J. Thorner.** 1989. Yeast prohormone processing enzyme (Kex2 gene-product) is a Ca^{2+}-dependent serine protease. Proc. Natl. Acad. Sci. USA **86:**1434–1438.
15. **Garcia, I., J. M. Lora, J. de la Cruz, T. Benitez, A. Llobell, and J. A. Pintor-Toro.** 1994. Cloning and characterization of a chitinase (CHIT42) cDNA from the mycoparasitic fungus *Trichoderma harzianum*. Curr. Genet. **27:**83–89.
16. **Geremia, R. A., G. H. Goldman, D. Jacobs, W. Ardiles, S. B. Vila, M. Vanmontagu, and A. Herrera-Estrella.** 1993. Molecular characterization of the proteinase-encoding gene, *prb1*, related to mycoparasitism by *Trichoderma harzianum*. Mol. Microbiol. **8:**603–613.
17. **Goller, S. P., D. Schoisswohl, M. Baron, M. Parriche, and C. P. Kubicek.** 1998. Role of endoproteolytic dibasic proprotein processing in maturation of secretory proteins in *Trichoderma reesei*. Appl. Environ. Microbiol. **64:**3202–3208.
18. **Grzegorski, D.** 2001. The construction and characterization of three genomic libraries of *Trichoderma virens* strain Tv29-8. Masters Thesis. Texas A&M University, College Station, Tex.
19. **Hanson, L. E., and C. R. Howell.** 2004. Elicitors of plant defense responses from biocontrol strains of *Trichoderma virens*. Phytopathology **94:**171–176.
20. **Harman, G. E., C. R. Howell, A. Viterbo, I. Chet, and M. Lorito.** 2004. *Trichoderma* species—opportunistic, avirulent plant symbionts. Nat. Rev. Microbiol. **2:**43–56.
21. **Howell, C. R.** 2002. Cotton seedling preemergence damping-off incited by *Rhizopus oryzae* and *Pythium* spp. and its biological control with *Trichoderma* spp. Phytopathology **92:**177–180.
22. **Howell, C. R., L. E. Hanson, R. D. Stipanovic, and L. S. Puckhaber.** 2000. Induction of terpenoid synthesis in cotton roots and control of *Rhizoctonia solani* by seed treatment with *Trichoderma virens*. Phytopathology **90:**248–252.
23. **Howell, C. R., R. D. Stipanovic, and R. D. Lumsden.** 1993. Antibiotic production by strains of *Gliocladium virens* and its relation to the biocontrol of cotton seedling diseases. Biocontrol Sci. Technol. **3:**435–441.
24. **Kapteyn, J. C., R. C. Montijn, E. Vink, J. de la Cruz, A. Llobell, J. E. Douwes, H. Shimoi, P. N. Lipke, and F. M. Klis.** 1996. Retention of *Saccharomyces cerevisiae* cell wall proteins through a phosphodiester-linked β-1,3-/β-1,6-glucan heteropolymer. Glycobiology **6:**337–345.
25. **Kim, D. J., J. M. Baek, P. Uribe, C. M. Kenerley, and D. R. Cook.** 2002. Cloning and characterization of multiple glycosyl hydrolase genes from *Trichoderma virens*. Curr. Genet. **40:**374–384.
26. **Kulmburg, P., M. Mathieu, C. Dowzer, J. Kelly, and B. Felenbok.** 1993. Specific binding-sites in the *Alcr* and *Alca* promoters of the ethanol regulon for the *CreA* repressor mediating carbon catabolite repression in *Aspergillus nidulans*. Mol. Microbiol. **7:**847–857.
27. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
28. **Limon, M. C., J. A. Pintor-Toro, and T. Benitez.** 1999. Increased antifungal activity of *Trichoderma harzianum* transformants that overexpress a 33-kDa chitinase. Phytopathology **89:**254–261.
29. **Lora, J. M., J. de la Cruz, A. Llobell, T. Benitez, and J. A. Pintor-Toro.** 1995. Molecular characterization and heterologous expression of an endo-β-1,6-

glucanase gene from the mycoparasitic fungus *Trichoderma harzianum*. Mol. Gen. Genet. **247:**639–645.
30. **Lorito, M., R. L. Mach, P. Sposato, J. Strauss, C. K. Peterbauer, and C. P. Kubicek.** 1996. Mycoparasitic interaction relieves binding of the Cre1 carbon catabolite repressor protein to promoter sequences of the *ech42* (endochitinase-encoding) gene in *Trichoderma harzianum*. Proc. Natl. Acad. Sci. USA **93:**14868–14872.
31. **Mach, R. L., and S. Zeilinger.** 2003. Regulation of gene expression in industrial fungi: *Trichoderma*. Appl. Microbiol. Biotechnol. **60:**515–522.
32. **Mackenzie, L. F., G. S. Brooke, J. F. Cutfield, P. A. Sullivan, and S. G. Withers.** 1997. Identification of Glu-330 as the catalytic nucleophile of *Candida albicans* exo-β-(1,3)-glucanase. J. Biol. Chem. **272:**3161–3167.
33. **Magnelli, P., J. F. Cipollo, and C. Abeijon.** 2002. A refined method for the determination of *Saccharomyces cerevisiae* cell wall composition and β-1,6-glucan fine structure. Anal. Biochem. **301:**136–150.
34. **Marchler, G., C. Schuller, G. Adam, and H. Ruis.** 1993. A *Saccharomyces cerevisiae* UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. EMBO J. **12:**1997–2003.
35. **Marck, C.** 1988. 'DNA strider'—a 'C' program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. Nucleic Acids Res. **16:**1829–1836.
36. **Markaryan, A., J. D. Lee, T. D. Sirakova, and P. E. Kolattukudy.** 1996. Specific inhibition of mature fungal serine proteinases and metaloproteinases by their propeptides. J. Bacteriol. **178:**2211–2215.
37. **Martin, K. L., S. E. Unkles, B. M. McDougall, and R. J. Seviour.** 2006. Purification and characterization of the extracellular β-1,6-glucanases from the fungus *Acremonium* strain OXF C13 and isolation of the genes encoding these enzymes. Enzyme. Microb. Technol. **38:**351–357.
38. **Mendoza-Mendoza, A., M. J. Pozo, D. Grzegorski, P. Martinez, J. M. Garcia, V. Olmedo-Monfil, C. Cortes, C. Kenerley, and A. Herrera-Estrella.** 2003. Enhanced biocontrol activity of *Trichoderma* through inactivation of a mitogen-activated protein kinase. Proc. Natl. Acad. Sci. USA **100:**15965–15970.
39. **Montero, M., L. Sanz, M. Rey, E. Monte, and A. Llobell.** 2005. BGN16.3, a novel acidic β-1,6-glucanase from mycoparasitic fungus *Trichoderma harzianum* CECT 2413. FEBS J. **272:**3441–3448.
40. **Moy, M., H. J. M. Li, R. Sullivan, J. F. White, and F. C. Belanger.** 2002. Endophytic fungal β-1,6-glucanase expression in the infected host grass. Plant Physiol. **130:**1298–1308.
41. **Nelson, N. J.** 1957. Colorimetric analysis of sugars. Methods Enzymol. **3:** 85–86.
42. **Olmedo-Monfil, V., A. Mendoza-Mendoza, I. Gomez, C. Cortes, and A. Herrera-Estrella.** 2002. Multiple environmental signals determine the transcriptional activation of the mycoparasitism related gene *prb1* in *Trichoderma atroviride*. Mol. Genet. Genomics **267:**703–712.
43. **Park, Y. H., C. M. Kenerley, and J. P. Stack.** 1992. Inoculum dynamics of *Gliocladium virens* associated with roots of cotton seedlings. Microb. Ecol. **23:**169–179.
44. **Park, Y. H., J. P. Stack, and C. M. Kenerley.** 1991. Production of gliotoxin by *Gliocladium virens* as a function of source and concentration of carbon and nitrogen. Mycol. Res. **95:**1242–1248.
45. **Pitson, S. M., R. J. Seviour, and B. M. Mcdougall.** 1993. Noncellulolytic fungal β-glucanases—their physiology and regulation. Enzyme Microb. Technol. **15:**178–192.
46. **Pozo, M. J., J. M. Baek, J. M. Garcia, and C. M. Kenerley.** 2004. Functional analysis of *tvsp1*, a serine protease-encoding gene in the biocontrol agent *Trichoderma virens*. Fungal Genet. Biol. **41:**336–348.
47. **Ravagnani, A., L. Gorfinkiel, T. Langdon, G. Diallinas, E. Adjadj, S. Demais, D. Gorton, H. N. Arst, and C. Scazzocchio.** 1997. Subtle hydrophobic interactions between the seventh residue of the zinc finger loop and the first base of an HGATAR sequence determine promoter-specific recognition by the *Aspergillus nidulans* GATA factor AreA. EMBO J. **16:**3974–3986.
48. **Saloheimo, A., N. Aro, M. Ilmen, and M. Penttila.** 2000. Isolation of the *ace1* gene encoding a Cys(2)-His(2) transcription factor involved in regulation of activity of the cellulase promoter *cbh1* of *Trichoderma reesei*. J. Biol. Chem. **275:**5817–5825.
49. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
50. **Sarrocco, S., L. Mikkelsen, M. Vergara, D. F. Jensen, M. Lubeck, and G. Vannacci.** 2006. Histopathological studies of sclerotia of phytopathogenic fungi parasitized by a GFP transformed *Trichoderma virens* antagonistic strain. Mycol. Res. **110:**179–187.
51. **Schep, G. P., M. G. Shepherd, and P. A. Sullivan.** 1984. Purification and properties of a β-1,6-glucanase from *Penicillium brefeldianum*. Biochem. J. **223:**707–714.
52. **Schirmbock, M., M. Lorito, Y. L. Wang, C. K. Hayes, I. Arisanatac, F. Scala, G. E. Harman, and C. P. Kubicek.** 1994. Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. Appl. Environ. Microbiol. **60:**4364–4370.
53. **Sophianopoulou, V., T. Suarez, G. Diallinas, and C. Scazzocchio.** 1993. Operator derepressed mutations in the proline utilization gene-cluster of *Aspergillus nidulans*. Mol. Gen. Genet. **236:**209–213.
54. **Steyaert, J. M., H. J. Ridgway, Y. Elad, and A. Stewart.** 2003. Genetic basis of mycoparasitism: a mechanism of biological control by species of *Trichoderma*. N. Z. J. Crop Hortic. Sci. **31:**281–291.
55. **Steyaert, J. M., A. Stewart, M. Jaspers, M. Carpenter, and H. J. Ridgway.** 2004. Co-expression of two genes, a chitinase (*chit42*) and proteinase (*prb1*), implicated in mycoparasitism by *Trichoderma hamatum*. Mycologia **96:**1245–1252.
56. **Thomas, L., R. Leduc, B. A. Thorne, S. P. Smeekens, D. F. Steiner, and G. Thomas.** 1991. Kex2-like endoprotease-Pc2 and endoprotease-Pc3 accurately cleave a model prohormone in mammalian cells—-evidence for a common core of neuroendocrine processing enzymes. Proc. Natl. Acad. Sci. USA **88:**5297–5301.
57. **Viterbo, A., and I. Chet.** 2006. *TasHyd1*, a new hydrophobin gene from the biocontrol agent *Trichoderma asperellum*, is involved in plant root colonization. Mol. Plant Pathol. **7:**249–258.
58. **Viterbo, A., M. Harel, and I. Chet.** 2004. Isolation of two aspartyl proteases from *Trichoderma asperellum* expressed during colonization of cucumber roots. FEMS Microbiol. Lett. **238:**151–158.
59. **Viterbo, A., O. Ramot, L. Chemin, and I. Chet.** 2002. Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. Antonie Leeuwenhoek **81:**549–556.
60. **Vogel, H. J.** 1956. A convenient growth medium for *Neurospora* (Medium N). Microbiol. Genet. Bull. **13:**42–43.
61. **Weaver, M., and C. Kenerley.** 2005. Density independent population dynamics by *Trichoderma virens* in soil and defined substrates. Biocontrol Sci. Technol. **15:**847–857.
62. **Wiest, A., D. Grzegorski, B. W. Xu, C. Goulard, S. Rebuffat, D. J. Ebbole, B. Bodo, and C. Kenerley.** 2002. Identification of peptaibols from *Trichoderma virens* and cloning of a peptaibol synthetase. J. Biol. Chem. **277:**20862–20868.
63. **Yamamoto, S., R. Kobayash, and S. Nagasaki.** 1974. Studies on fungal enzymes active in hydrolyzing yeast-cell wall. 4. Purification and properties of an endo β-1,6-glucanase from *Rhizopus chinensis* R-69. Agric. Biol. Chem. **38:**1493–1500.
64. **Zeilinger, S., B. Reithner, V. Scala, I. Peissl, M. Lorito, and R. L. Mach.** 2005. Signal transduction by Tga3, a novel G protein α subunit of *Trichoderma atroviride*. Appl. Environ. Microbiol. **71:**1591–1597.