Regulation of Morphogenesis and Biocontrol Properties in *Trichoderma virens* by a VELVET Protein, Ve1

Prasun K. Mukherjee1,2 and Charles M. Kenerley1*

Department of Plant Pathology and Microbiology, Texas A&M University, College Station, Texas 77843,1 and Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Center, Trombay, Mumbai 400085, India2

Received 1 October 2009/Accepted 2 February 2010

Mycoparasitic strains of *Trichoderma* are applied as commercial biofungicides for control of soilborne plant pathogens. Although the majority of commercial biofungicides are *Trichoderma* based, chemical pesticides, which are ecological and environmental hazards, still dominate the market. This is because biofungicides are not as effective or consistent as chemical fungicides. Efforts to improve these products have been limited by a lack of understanding of the genetic regulation of biocontrol activities. In this study, using gene knockout and complementation, we identified the VELVET protein Ve1 as a key regulator of biocontrol, as well as morpho- genetic traits, in *Trichoderma virens*, a commercial biocontrol agent. Mutants with mutations in ve1 were defective in secondary metabolism (antibiosis), mycoparasitism, and biocontrol efficacy. In nutrient-rich media they also lacked two types of spores important for survival and development of formulation products: conidia (on agar) and chlamydospores (in liquid shake cultures). These findings provide an opportunity for genetic enhancement of biocontrol and industrial strains of *Trichoderma*, since Ve1 is very highly conserved across three *Trichoderma* species.

*Trichoderma*-based formulation products account for about 60% of the biofungicide market (35). Despite the use of *Trichoderma*-based biofungicides as an alternative and additive to chemical fungicides, the applications of these preparations are limited because their efficacy is lower than that of fungicides. A lack of understanding of the regulation of biocontrol has limited progress in enhancing the competitiveness of these fungi through genetic manipulation of desired traits. The success of a biocontrol agent also depends on the ability of researchers to develop an effective formulation based on active propagules that survive under the conditions that occur in nature and are effective against the target pathogens. *Trichoderma* spp. produce two types of propagules, conidia during solid-state fermentation and chlamydospores during liquid fermentation. Both types are used in commercial formulations depending on the growth conditions (17, 35). Thus, understanding how the two sporulation pathways are controlled is critical for obtaining an improved, balanced formulation product. Identification of a global regulator of morphogenesis and biocontrol properties (such as antibiosis and mycoparasitism) would provide an opportunity to manipulate the morphogenetic and antagonistic traits, leading to wider commercial acceptance of *Trichoderma* spp. in the long run.

*Trichoderma virens* is a commercially formulated biocontrol agent that is effective against soilborne plant pathogens, such as *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Pythium* spp.; its major direct mode of action is antibiosis and mycoparasitism (20, 36). This species has also been used as a model system for studies of biocontrol mechanisms, and the genome has recently been sequenced (http://genome.jgi-psf.org/Trive1). The role of beta-glucanases, chitinases, and proteases in biocontrol has been reported previously (2, 8, 29). Some strains of *T. virens* (designated Q strains) produce copious amounts of the antibiotic gliotoxin that is involved in biocontrol (10, 12, 39). In an attempt to identify regulators of biocontrol properties, the role of a mitogen-activated protein kinase (MAPK) pathway was studied previously (22, 24). Deletion of the TmkA/Tvk1 MAPK gene resulted in derepressed conidiation and different biocontrol behavior for two strains of *T. virens*; Mukherjee et al. (24) noted the reduced ability of these mutants to parasitize the sclerotia of *S. rolfsii* and *R. solani*, while Mendoza-Mendoza et al. (22) found that deletion of this MAPK gene improved the biocontrol activity of *T. virens* against *R. solani* and *P. ultimum*. The production of secondary metabolites was not affected by deletion of this gene. To date, no gene that regulates the balance between conidiation or chlamydospore formation, secondary metabolism, and antagonistic or biocontrol properties has been identified in any *Trichoderma* sp.

The Ve1 VELVET protein has been shown to be a regulator of morphogenesis and secondary metabolism in some filamentous fungi (6). In *Aspergillus nidulans*, VeA physically interacts with VeIB and the regulator of secondary metabolism LaeA to form a complex that regulates secondary metabolism and sexual reproduction (3). Deletion of the VeA gene leads to an increase in asexual development (conidiation in the dark) and reduced biosynthesis of sterigmatocystin (the product of a polyketide synthetase [PKS]) and penicillin (the product of a nonribosomal peptide synthetase [NRPS]), while it reduces and delays sexual reproduction (15, 16). VeA is also required for the production of sclerotia and for aflatoxin biosynthesis in *Aspergillus parasiticus* (7). Deletion of the VeA gene in *Neu*...
sucrose (VMS) at the ambient temperature in the presence of light, unless arginine auxotroph GV10-4 have been described previously (1). Routinely, the A. nidulans economically important fungus. regulator of morphogenesis and antagonistic properties in this trol efficacy in this organism by using gene knockout and complementation. Here we report that in addition to a role in conidiation and secondary metabolism, Ve1 also regulates conidiophore aggregation, chlamydosporogenesis, mycoparasitism, and biocontrol efficacy in T. virens. Thus, we identified the first master regulator of morphogenesis and antagonistic properties in this economically important fungus.

MATERIALS AND METHODS

Fungal and bacterial strains and growth conditions. T. virens GV29-8 and its arginine auxotroph GV10-4 have been described previously (1). Routinely, the parental strains and transformants were grown in Vogel's minimal medium with sucrose (VMS) at the ambient temperature in the presence of light, unless otherwise stated. Escherichia coli strain TOP10 (Invitrogen) was used for cloning. All cultures were stored as glycerol stocks at –80°C to maintain genetic stability. Deletion of the vel1 gene. The vel1 gene was amplified using the veFor and veRev primers (all of the primer sequences used in this study are listed in Table S1 in the supplemental material) and cloned in the pGEM-T Easy vector (Promega). The BglII-EcoRV fragment encompassing the entire vel1 open reading frame (ORF) was replaced with the arg2/arginate cassette (BamHI-EcoRV fragment of the pMBB4 vector) (1). The resulting vector, vel1-Arg, was linearized with NotI and transformed into GV10-4 protoplasts as previously described (1). The transformants were purified by serial transfer on VMS and finally by single-sporo isolation. Mutant colonies were identified by Southern hybridization.

Complementation of the mutant with the wild-type vel1 gene. The entire vel1 gene with approximately 2-kb native promoter and 500-bp terminator sequences was amplified (with the Expand long-template PCR system, Roche) by using the VeCompSal and VeCompRI primers, digested with SalI and EcoRI, and ligated to predigested pBSS-G containing the Genetin resistance gene under control of the Cochliobolus heterostrophus gpd promoter and N. crassa beta-tubulin termin- nator sequences (31). We previously optimized transformation of T. virens with this cassette, and the transformants expressing this cassette were identical to the wild type (WT) with respect to colony morphology, growth, and biocontrol of Pythium ultimum (P. K. Mukherjee and C. M. Kenerley, unpublished data).

Protoplasts were generated from regenerated hyphal fragments of mutant ve3 and transformed with the complementation vector pVel-Gen. The protoplasts were plated on regeneration agar amended with 200 mg/liter G-418 (Genetin) and incubated in the presence of light. Sporulating colonies were selected after 7 days, tested to determine their stability, and purified by single-sporo isolation. A stable transformant with a single-copy integration (confirmed by Southern hybridization) was selected for further study.

Growth on agar and in liquid media. The mutants and the complemented strain were grown on VMS agar and incubated at the ambient temperature in the dark or light, as required. The colony diameter was measured at intervals, and details of colony morphology were examined with a light or stereo microscope. For growth in liquid culture, three mycelial disks were inoculated into 100 ml of medium and incubated at the ambient temperature with shaking at 125 rpm. To measure biomass production, the culture was harvested after 5 days, and the oven dry weight was determined. The effect of nutrients on chlamydospore production was determined with strains grown in either distilled water, VMS, or nutrient-rich molasses-yeast extract medium (30 g molasses per liter and 5 g yeast extract per liter).

Test for hydrophobicity. The hydrophobicity of colonies was tested by applying 15 µl of water or 0.5% aqueous aniline blue to fully grown colonies and observing the disappearance of the water or dye over an 8-h period. Gliotoxin production and regulation of secondary metabolism-related genes.

Gliotoxin production was monitored in Weindling medium, a substrate highly conducive for gliotoxin production (37). After 3 and 6 days, filtrates were extracted with an equal volume of chloroform, dried, and reconstituted in 0.01 volume methanol, and 30-µl portions were loaded on thin-layer chromatography (TLC) plates along with a gliotoxin standard (Sigma). The TLC plates were observed under short-wavelength UV and photographed. Expression of the gliP gene was studied by using reverse transcription (RT)-PCR and real-time PCR. We also studied the expression of other secondary metabolism-related genes (genes encoding three NRPs, two PKSs, O-methyl transferase B, and cytochrome P450; these genes were selected after an initial screening of a set of secondary metabolism-related genes based on high levels of expression in the wild type) in cultures grown for 3 days on VMS agar plates overlaid with cellophane membranes.

Myocoparasitism assays. The abilities of the WT, mutants, and the complemented strain to parasitize, lyse, and overgrow R. solani or P. ultimum strains were studied using a confrontation assay in which cultures co-inoculated onto VMS plates. The plates were incubated at 26°C, observed regularly for overgrowth, and also observed microscopically for mycoparasitic coiling and lysis. The viability of the Pythium culture was also assessed by plating it on VMS plates amended with 1 mg/liter benomyl (which selectively inhibited Trichoderma growth but allowed Pythium to grow). The abilities of the strains to respond to cell walls of a pathogen were assessed using a simulated myocoparasitism assay as described previously (22), except that a mycelial inoculum was used instead of a conidial inoculum. Briefly, three mycelial disks were inoculated into 50 ml VMS and incubated with shaking for 2 days. The growth was harvested, blended in a Waring blender for 30 s, and then transferred to 100 ml fresh VMS. After further incubation for 1 day, the cultures were washed and transferred either to VMS with 0.5% sucrose or to medium containing P. ultimum or R. solani cell walls (20) was assessed by RT-PCR and real-time PCR.

Biocontrol assays. Biocontrol assays were conducted as described previously (2, 22), with some modifications. The chlamydospore preparations were obtained by growing Trichoderma cultures in VMS instead of molasses-yeast extract medium. Cotton seeds (cultivar 112; Stoneville, Memphis, TN) were coated with chlamydospores of the appropriate strains and then planted in a sterile sand-soil mixture preinfested with R. solani or P. ultimum. Seeds planted in noninfested medium were used as positive controls. Healthy, surviving seedlings were counted after 7 days of incubation at 25°C in a growth chamber. Additionally, the severity of root infection in R. solani-infected plants was evaluated by using an arbitrary scale from 0 (no symptoms) to 5 (entire root system discolored and decayed); a maximum score of 6 was used for nongerminated or dead seeds. Each treatment was replicated three times with 10 seeds each, and the entire experiment was repeated twice.

DNA and RNA manipulations and expression studies. Restriction digestion, labeling, and PCR were performed using standard protocols (32). For RNA isolation, the tissues were snap-frozen in liquid nitrogen and ground, and RNA was extracted with TriReagent (MRC) using the manufacturer’s protocol. For RT-PCR, cDNA was synthesized from DNase-treated RNA using a high-capacity cDNA reverse transcription kit (Applied Biosys- tems), and cDNA from 100 ng RNA equivalents was PCR amplified using Taq polymerase (NEB) and 26 cycles. Real-time PCR was performed using 100 ng DNase-treated RNA and a Quantitect SYBR green RT-PCR kit (Qiagen) with an Applied Biosystems 7500 fast real-time PCR system for 40 cycles. The fold change in the mRNA was calculated using the ∆∆Ct method. The histone h3 gene was used as the housekeeping gene.

Statistical analysis. All of the experiments were performed using three replications and were repeated at least twice, with reproducible results. The figures were plotted using Microsoft Office Excel with standard error bars. Where appropriate, a statistical analysis was performed using StatView software.

RESULTS

T. virens vel1 gene. The gene encoding T. virens Ve1 (protein ID 80197) is a single-copy gene in the T. virens genome (http://genome.jgi-psf.org/Trive1/Trive1.home.html). The 1,771-bp ORF, interrupted by a single 88-bp intron, codes for a protein consisting of 560 amino acids. A phylogenetic tree (see Fig. S1 in the supplemental material) of Ve1 proteins from T. virens...
and other fungi revealed that *T. virens* Vel1 is most closely related to *Trichoderma reesei* Vel1 and has strong homology with VeA of *Fusarium verticillioides* (56% amino acid identity). Interestingly, in a phylogenetic analysis (http://www.phylogeny.fr/) performed with the VeA sequence, *Aspergillus* spp. and *Penicillium marneffei* formed a clade distinct from the other group of fungi analyzed (see Fig. S1A in the supplemental material). The Vel1 sequences of the three *Trichoderma* spp. are highly conserved (see Fig. S1B in the supplemental material).

**Generation of knockout strains and complementation of the loss-of-function mutants by the wild-type allele.** We obtained four stable mutants showing the expected Southern hybridization pattern (lack of the wild-type band and presence of a 5.6-kb band due to replacement of the *vel1* ORF with the *arg2* cassette [see Fig. S2 in the supplemental material]). When these mutants were grown on VMS agar, they lacked conidia, produced large numbers of chlamydospores, and displayed a wet mycelial phenotype (Fig. 1). To confirm that these phenotypes were indeed due to loss of the *veA* gene, we complemented a mutant with the wild-type allele. The complemented strain was able to sporulate and was resistant to G-418 (Geneticin), a selectable marker (see Fig. S3A and B in the supplemental material). The morphological defects in the mutants could not be restored by addition of osmoticum to the growth media (see Fig. S3C in the supplemental material). Genetic analysis of the complemented strain confirmed the presence of the *vel1* gene, as well as the Geneticin resistance cassette (see Fig. S3D and E in the supplemental material). On water agar, the WT produced conidiophores in large aggregates, compared to the more dispersed arrangement of conidiophores observed for the complemented strain (Fig. 2); the mutants produced large numbers of chlamydospores within 2 days after inoculation, whereas chlamydospore formation was delayed by 36 to 48 h in the wild type and the complemented strain. After prolonged incubation (more than 10 days) on water agar, a few conidiophores and conidia were observed for the mutants (data not shown). The level of expression of the *vel1* transcript in the complemented strain was lower than that in the wild type (see Fig. S4B in the supplemental material). There was a marginal (less than 2-fold) increase in expression of the *veA* transcript in the WT in the presence of light (see Fig. S4B and C in the supplemental material).

**Growth rate, biomass production, pigmentation, and hydrophobicity.** The mutants had a higher radial growth rate than the WT under continuous light conditions; however, the WT grew faster when the organisms were grown in the dark (see Fig. S5A and B in the supplemental material). The mutants
produced significantly greater biomass than the WT when the organisms were grown in liquid shake cultures. Under these conditions, the complemented strain produced the greatest biomass (see Fig. S5C in the supplemental material). In liquid shake cultures, the WT and complemented strains produced pigmented mycelia, as well as a diffusible yellow pigment in the filtrate, while the mutants did not produce the pigment (Fig. 3A and B). The WT colonies that formed in shake culture had smooth edges, whereas the mutant colonies had striations, and the outgrowth was more pronounced for the complemented strains (Fig. 3C). The colonies of the WT and the complemented strain were highly hydrophobic, while the colonies of the mutants were extremely hydrophilic (Fig. 4A). The transcript level data for a hydrophobin gene (tvh1) corroborated the physical properties of the colonies; this gene was expressed at a high level in the WT but was not present in the mutant (Fig. 4B and C).

**Mutants exhibit early chlamydospore differentiation under nutrient stress conditions.** Since we observed that the mutants produced large numbers of chlamydospores instead of conidia, we examined the time course of chlamydospore morphogenesis in various nutrient media (water, VMS, molasses-yeast extract medium). In general, under nutrient stress conditions the mutants exhibited early chlamydospore differentiation, while under nutrient-rich conditions chlamydospore development was delayed in the mutants. In water, the mutants produced chlamydospores within 2 days, while the WT formed chlamydospores after 4 days. In VMS, the WT formed chlamydospores in 5 days, while the mutants produced chlamydospores in 7 days (data not shown). In the nutrient-rich molasses-yeast extract medium, the mutants did not produce chlamydospores even after 12 days of incubation, even though the wild type and the complemented strains produced numerous chlamydospores (see Fig. S6 in the supplemental material).

**FIG. 2.** Production of conidia and chlamydospores by WT, mutant, and complemented (Comp) strains on 1% water agar. The cultures were incubated in the presence of light for 2 or 3 days (2d and 3d, respectively). Note that the WT produced conidiophores in large aggregates (indicated by arrows), compared to the small dispersed elements produced by the complemented strain. The mutants did not produce conidia but produced chlamydospores as early as 2 days after inoculation.

**FIG. 3.** Growth of wild-type (WT), mutant (ve3), and complemented (Comp) strains in shake cultures. (A) Growth after 8 days of incubation. Note the absence of pigmentation in the mycelia of the mutant. Cultures were transferred to plates for photography. (B) Culture filtrates after 8 days. Note the absence of the yellow pigment in the supernatant from cultures of the mutant. (C) Colony morphology after 4 days of incubation of a shake culture. Note the smooth edges of the developing WT colonies compared to the striated growth of the mutant and the complemented strain. (Insets) Enlargements of single colonies.
Mutants are defective in gliotoxin production and induction of other secondary metabolism-related genes. A TLC analysis of chloroform-extracted culture filtrates for organisms grown on a medium conducive to gliotoxin production (Weindling minimal medium) indicated that the mutants are defective in gliotoxin production. The lack of gliotoxin was correlated with a low level of expression of gliP, which encodes the NRPS responsible for gliotoxin synthesis in Aspergillus fumigatus (Fig. 5). The mutants did not produce gliotoxin in two other culture media (VMS and malt extract medium) over a 6-day period (see Fig. S7 in the supplemental material). In addition to gliP expression, the mutants were also defective in expression of a set of secondary metabolism-related genes, including genes encoding NRPSs (putative peptaibol synthetase np1, unknown NRPS np2, and putative ferrichrome synthetase np3), two PKSs (pk1 and pk2, orthologues of C. heterostrophus polyketide synthase 2 and the P. marneffei conidial pigment polyketide synthase PksP/Alb1), an O-methyl transferase, and cytochrome P450 (see Fig. S8 in the supplemental material).

Mutants are defective in mycoparasitism. In the confrontation assay in which the Trichoderma strains were paired with the plant pathogens R. solani and P. ultimum, the mutants did not overgrow and lyse the pathogen mycelia (Fig. 6A and B). In a simulated mycoparasitism assay, in response to R. solani and P. ultimum cell walls, the mycoparasitism-related gene nsp1/prb1 (encoding a serine protease) was highly induced in the WT and the complemented strain, but it was underexpressed in the mutants (Fig. 6C and D).

Mutants are ineffective for biocontrol of plant pathogens. In a growth chamber assay in which Trichoderma-coated seeds were sown in pathogen-infested soil, the mutants did not protect cotton seedlings against P. ultimum and R. solani infection.

DISCUSSION

Trichoderma spp. are economically useful biofungicides, plant growth promoters, sources of secondary metabolites for agricultural and pharmaceutical applications, and sources of industrial enzymes (11, 21). T. virens is a commercial biofungicide and also produces important secondary metabolites, such as gliotoxin, gliovirin, viridin, and viridil, which are antimicrobial (gliotoxin, gliovirin), have herbicidal properties (viridil), and have pharmaceutical or clinical significance (gliotoxin, gliovirin, viridin) (13, 14, 23, 26, 30, 38). Gliotoxin, the most abundant secondary metabolite synthesized by T. virens, is also a pathogenicity determinant in the human opportunistic pathogen A. fumigatus (34). Since it is an asexual fungus, T. virens produces only two types of propagules, conidia on solid substrates and chlamydospores in liquid submerged cultures; both types of propagules are used in commercial formulations depending on the type of fermentation used (viz., solid-state versus liquid fermentation) (35). Even though some genes (mostly genes involved in signal transduction) that modulate biocontrol properties and conidiation have been identified in Trichoderma spp. (22, 24), a single gene controlling these economically important traits has not been found. In this work, we report for the first time identification of a gene (vel1) that regulates secondary metabolism, conidiation, chlamydospore development, mycoparasitism, and biocontrol potential. Deletion of this gene eliminated conidiation in nutrient-rich media, such as molasses-yeast extract medium that is widely used for production of chlamydospores for biocontrol (27, 35). Furthermore, the mutants were not hydrophobic, did not produce...
mycelial and extracellular pigments and gliotoxin, and had defects in the regulation of many other secondary metabolism-related genes, including genes encoding three NRPSs, two PKSs, an O-methyl transferase, and cytochrome P450. The mutants were also defective in mycoparasitism and expression of a mycoparasitism-related gene, tvsp1/prb1, and did not exhibit biocontrol efficacy in growth chamber studies. Unlike the findings for *A. nidulans* or *N. crassa*, where

![Figure 6](http://aem.asm.org/content/2350/9/1034/F1.large.jpg)

**FIG. 6.** Antagonistic interactions between the WT, the *vel1* mutant, and the complemented strain in confrontation and simulated mycoparasitism assays. (A) Results of confrontation assay with *P. ultimum* (Pu) 6 days after coinoculation at the edges of VMS plates. (B) Results of confrontation assay with *R. solani* (Rs) 10 days after coinoculation of the organisms 4 cm apart. (C) Induction of serine protease (*tvsp1/prb1*) expression in a simulated mycoparasitism assay (RT-PCR). VM, sucrose used as the carbon source; Rs, *R. solani* cell walls used as the carbon source; Pu, *P. ultimum* cell walls used as the carbon source; W, wild type; 3, *vel1* mutant; C, complemented strain; sp, *tvsp1*; and h3, histone. (D) Confirmation of downregulation in the mutant by real-time PCR.

![Figure 7](http://aem.asm.org/content/2350/9/1034/F2.large.jpg)

**FIG. 7.** Biocontrol of *P. ultimum* and *R. solani* in cotton. (A) Percentage of healthy seedlings in *P. ultimum* - or *R. solani*-infested soil. (B) Extent of root rot in *R. solani*-infested soil. Pu, *P. ultimum*; Rs, *R. solani*; UC, uninoculated control; Cont, pathogen-inoculated control; WT, *T. virens* wild type; ve3, *vel1* mutant 3; ve5, *vel1* mutant 5; Comp, complemented strain. The values for bars labeled with the same letter are not significantly different (*P* = 0.01).
deletion of the *velvet* gene leads to increased conidiation, here we report that there was no conidiation in nutrient media. Deletion of *vel1* led to a defect in secondary metabolism in *T. virens*, as it has in other fungi, indicating that there is functional conservation with respect to secondary metabolism but not with respect to conidiation. This is the first report on the regulation of the important secondary metabolite glutoxin (antibiotic and mycotoxin) by Vel1.

Thus, in this paper we extend the role of this fungus-specific regulatory protein to chlamydosporogenesis, mycoparasitism (including regulation of a mycoparasitism-related enzyme, serine protease), and biocontrol. Chlamydospores, described as early as 1954, are important for survival of *Trichoderma* spp. in soil (5, 28). No information on the genetics of chlamydospore development in filamentous fungi is available, although limited data for *Candida* spp. indicate that chlamydospore development is genetically programmed (33). Since these propagules are also an important component of commercial media. Deletion of *velvet* led to a defect in secondary metabolism is genetically programmed (33). Since these propagules are also an important component of commercial media. Deletion of *velvet* led to a defect in secondary metab-

### ACKNOWLEDGMENTS

We thank David Laughlin for excellent technical assistance and Benjamin Horwitz for critical comments on the manuscript. We also thank Won-Bo Shim for providing the Genetcin resistance cassette. This research was supported by USDA competitive grant 2008- 35319-04470.

### REFERENCES


